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### Research Article

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## <sup>1</sup>H-NMR Detectable Fatty Acyl Chain Unsaturation in Excised Leiomyosarcoma Correlate with Grade and Mitotic Activity

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### Abstract

We report on the use of <sup>1</sup>H-NMR two-dimensional total correlated spectroscopy (2D TOCSY) at 600 MHz for an ex vivo analysis of fatty acyl chain lipid in normal smooth muscle and a series of primary retroperitoneal leiomyosarcomas. These TOCSY spectra were used to identify and quantitate the methylene protons situated between unsaturated site protons (D) to those bordered by only one unsaturated site proton (C). The D/C cross-peak volume ratios determined for oleic (18:1), linoleic (18:2), linolenic (18:3), and arachidonic (20:4) acids were 0.0, 1.3, 2.7, and 4.0, respectively, suggesting that this ratio can be a measure of the degree of unsaturation for fatty acyl chains of lipids. The D/C cross-peak volume ratio was found to be proportional to the mean mitotic activity ( $r = 0.94$ ) in nine smooth muscle tissues. These results suggest, that for leiomyosarcoma, the degree of fatty acyl unsaturation may be an important determinant of the metastatic potential of these tumors. Furthermore, application of TOCSY for the ex vivo study of smooth muscle tumors would potentially serve as a pathologist-independent and quantitative method for assessment of leiomyosarcoma grade and mitotic activity thereby rendering a more accurate staging of patients. (*J. Clin. Invest.* 1996. 98:244–250.) Key words: sarcoma • membrane lipids • tumor markers, biochemical • nuclear magnetic resonance • fatty acids, unsaturated

### Introduction

Malignancy grading of soft tissue sarcoma and of leiomyosarcoma in particular is of prognostic importance. Different grading systems have been used taking into account sarcoma cellularity, degree of cellular differentiation, mitotic activity, pleomorphism, and necrosis. Several recent studies have shown significant discordance among pathologists in grading soft tissue sarcoma for as many as 30–40% of cases (1, 2). These

studies emphasize that grading of sarcoma is often pathologist-dependent and requires significant experience and expertise. An ex vivo pathologist-independent and quantitative technique to assess sarcoma grade would thus be highly desirable.

Selection of a judicious combination of surgery, radiation, and chemotherapy for treatment of leiomyosarcoma remains a problem. Two kinds of clinical problems are frequently encountered. In one situation, where the primary sarcoma is treated by surgical resection followed by adjuvant chemotherapy, it has not yet been possible to demonstrate the efficacy of the chemotherapy or to predict those patients that will go on to develop metastatic disease. In the second case, where large sarcomas are treated with chemotherapy before surgery, there does not as yet exist an effective method for predicting response to chemotherapy. In general, the sole primary indicator of response to chemotherapy used by the physician is decrease in tumor size. Change in sarcoma size can be evaluated only late in the course of chemotherapy, i.e., after two or three cycles; this, however, often entails significant patient morbidity. A quantitative noninvasive NMR measurement that directly correlates with mitotic activity would allow for early assessment of change in sarcoma growth rate before and after therapy. Toxicity in those patients unlikely to respond could thereby be minimized and chemotherapy could be intensified for those who might respond. This biochemical information together with clinical data on patient response would be used to identify those patients who, on the one hand, are at high risk for relapse and who, on the other hand, have sarcomas that are sensitive to chemotherapy.

Alterations in cell membrane phospholipid synthesis and composition, membrane fluidity and order, and lipid-dependent pharmacology are common during tumorigenesis and may play a key role in determining the metastatic behavior of tumor cells (3–6). It is well established that lipid structural order in membranes is largely determined by cholesterol and sphingomyelin content and by the degree of saturation of the phospholipid fatty acyl chains (7, 8). Most of the techniques used to study cellular membrane lipid involve destruction or chemical perturbation of the membrane under study. <sup>1</sup>H-NMR spectroscopy can provide unique and important structural information about plasma membrane composition and function in cells and tissues with the additional advantage of being non-destructive to the system under study (9–12). Correlation spectroscopy (COSY)<sup>1</sup> experiments, a standard method for establishing proton coupling networks, have been used to detect

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1. Abbreviations used in this paper: COSY, correlation spectroscopy; TOCSY, total correlated spectroscopy.

and identify amino acids, phospholipids, plasma membrane triglycerides and cell surface carbohydrates in cancer cells and tissues (13–15). 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. It still remains to be determined if these measured changes in lipid and carbohydrate metabolites can be used as indicators of tumor proliferation and metastatic potential.

In this paper we investigate the changes in the degree of fatty acyl unsaturation associated with leiomyosarcoma grade and mean mitotic activity using two dimensional  $^1\text{H}$ -NMR total correlated spectroscopy (TOCSY). Homonuclear polarization transfer by isotropic mixing (also known as TOCSY) (16, 17) has become the method of choice for two-dimensional correlation spectroscopy. In the 2D TOCSY experiment a multiple-pulse, coherent averaging sequence is used that has the net effect of permitting the nuclear spins (protons) to share the same energy levels. This in effect removes any resonance differences between spins, and makes all spins have the same energy separations. The resultant energy match in the presence of couplings (which are not removed by the multiple-pulse train) causes the spins to evolve as if they were strongly coupled. Now there is an efficient flow of magnetization energy throughout a coupled spin (proton) network and additional cross peaks are observed to more distant bonded protons in a contiguous spin system. The resulting 2D TOCSY spectrum displays intensity as a function of two frequencies  $F_1$  (reflects the environment of the magnetization during the evolution period) and  $F_2$  (reflects the environment of the magnetization during the detection period). Thus, at the  $F_2$  value for any given proton there are cross-peaks at a variety of different  $F_1$  values. These are at  $F_1$  chemical shifts for protons within the mutually coupled spins. For example, the olefinic protons in linoleic acid (see Fig. 4 A) show correlations to the diallylic meth-

ylene protons (cross-peak D), the allylic methylene protons (cross-peak C) and as well to the methylene protons 4 bonds away (cross-peak BC). Thus, a chemical shift correlation data matrix is produced in which ideally each proton shows a correlation cross-peak to each of the other protons in a contiguous spin system, regardless of the exact coupling topology. In terms of the spectral appearance, there are a few very significant advantages to TOCSY experiments, (1) the cross peaks are in phase and absorptive (only positive peaks are present and no self-cancellation occurs), therefore broad linewidths do not reduce the signal-to-noise as they would for anti-phase resonances, (2) many more correlations are observed, (3) the spectrum shows the partitioning of the spin system into its basic chemical components.

We demonstrate that the TOCSY method can be used to determine the degree of unsaturation of fatty acyl chains for oleic (18:1), linoleic (18:2), linolenic (18:3), and arachidonic (20:4) fatty acids. Leiomyosarcoma grade and mean mitotic activity were found to correlate with the cross-peak volume ratio of the fatty acyl chain lipid methylene protons situated between unsaturated site protons (D) to those bordered by only one unsaturated site proton (C). These data suggest that TOCSY measurements of sarcoma tissue are sensitive to the degree of unsaturation of the NMR visible fatty acyl chain lipid. The TOCSY technique may potentially serve as a quantitative and pathologist-independent adjunct to conventional histologic assessment of sarcoma grade and mean mitotic activity.

## Methods

*Tissue samples for NMR measurements.* Multiple one-gram tissue samples were acquired from patients with primary leiomyosarcomas of the abdomen/retroperitoneum undergoing surgical resection. All tissue samples were obtained from homogeneous and viable portions of the resected tumors by the pathologist. Normal smooth muscle tissue

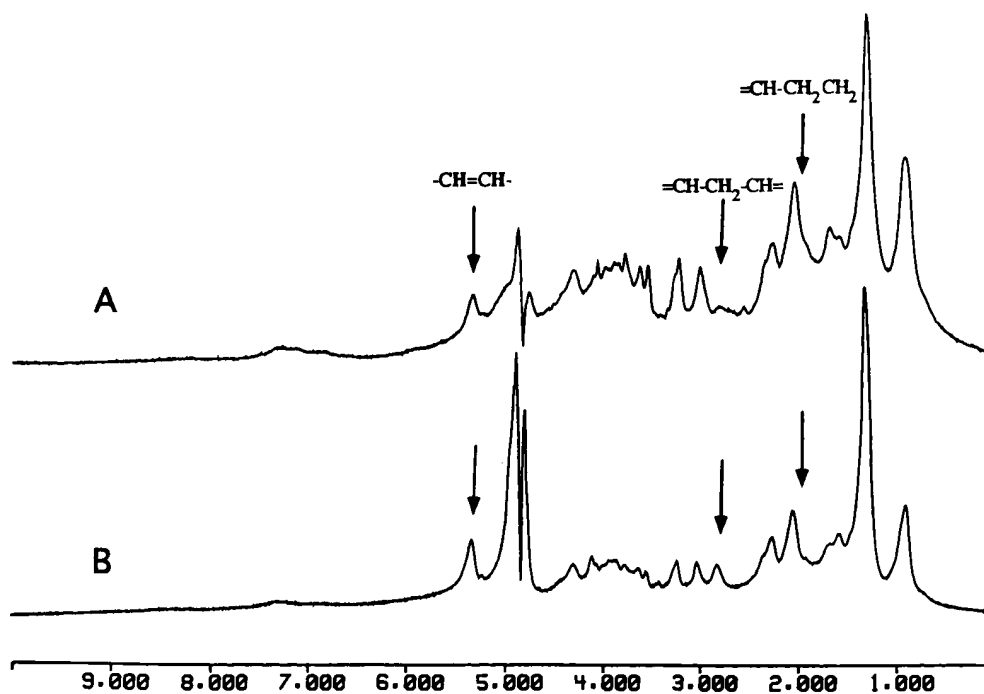


Figure 1. Ex vivo 1D  $^1\text{H}$ -NMR spectra at 600 MHz and 20°C of: (A) low grade leiomyosarcoma with a mean mitotic activity of 1.5 mitoses/10 hpf and (B) a high grade leiomyosarcoma with a mean mitotic activity of 12 mitoses/10 hpf. NMR acquisition parameters are given in Methods. These spectra were normalized to yield the same peak height for the bulk methylene resonance (1.2 ppm).

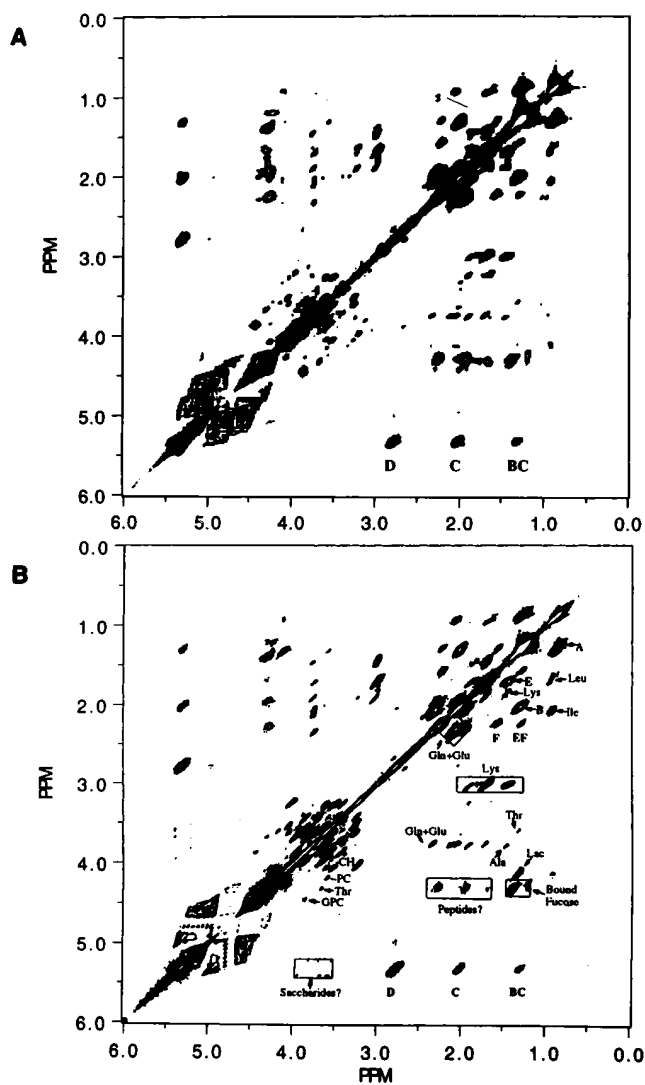


Figure 2. Ex vivo symmetrized TOCSY spectra at 600 MHz and 20°C of: (A) low grade leiomyosarcoma with a mean mitotic activity of 1.5 mitoses/10 hpf and (B) a high grade leiomyosarcoma with a mean mitotic activity of 12 mitoses/10 hpf. Total measuring time was 5 h for each spectrum. NMR acquisition parameters are given in Methods.

was obtained from esophageal specimens with careful dissection to remove all nonmuscle tissue. Tissue samples were placed in a  $-80^{\circ}\text{C}$  freezer no longer than 10 min from the time of excision. Histologic material in each sample was evaluated for tissue of origin of the dominant tumor cell type. All histologic material was reviewed by a single pathologist at the Brigham and Women's Hospital (J.M. Corson) and classified according to histologic type, grade, mitotic activity and surgical margin. Grade was determined by evaluating cellularity, pleomorphism, necrosis, and mitotic activity. The degree of malignancy was scored as low, intermediate or high grade based on a total evaluation of the above histologic features, with a major emphasis placed on the mean mitotic activity (18). Mean mitotic counts of  $0 < 2$ ,  $2 - 5$ ,  $> 5$  per 10 high power fields (hpf) served as a guide to assignment of low, intermediate and high grades, respectively. These were variably modified by the degree of cellularity and pleomorphism and the extent of necrosis; the mean mitotic count, however, remained the primary determinant of grade. The mitotic activity counts were performed with a high power field size of  $0.120\text{ mm}^2$  for all mitotic counts measured in our study. At least 50–100 high power fields were counted from the

most cellular areas of the sarcoma and used to determine mean and maximum mitotic activity.

Before 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  $20^{\circ}\text{C}$ . The piece was then incubated in deuterated phosphate-buffered saline (PBS/ $\text{D}_2\text{O}$ ) for 30 min, then rinsed once in fresh PBS/ $\text{D}_2\text{O}$ . A glass wool plug of 1.5 cm in height was placed at the bottom of the NMR tube so as to position the tissue sample in the center of the rf coil. The tissue was then placed in the NMR tube in PBS/ $\text{D}_2\text{O}$ .

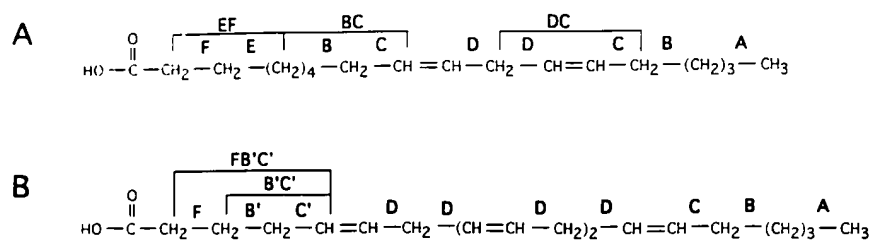
**Fatty acid standards.** 99% pure oleic, linoleic, linolenic, and arachidonic acids were purchased from Sigma and were diluted in deuterated chloroform prior to NMR analysis.

**NMR measurements.** NMR measurements were performed on a 600 MHz spectrometer designed, constructed and sited at the Francis Bitter Magnet Laboratory equipped with a Nalorac triple resonance 5-mm probe. The probe temperature was maintained at  $20^{\circ}\text{C}$  using a home-built variable temperature unit. One-dimensional (1D)  $^1\text{H}$ -NMR spectra were digitized into 8 K data points over a spectral sweep width of 7000 Hz. A  $90^{\circ}$  pulse ( $10\ \mu\text{s}$ ) was used and 128 transients were coadded before Fourier transformation. Low-power continuous wave irradiation for 1.5 s was used for solvent suppression along with a relaxation delay of 0.3 s. Resonances in the 1D and TOCSY spectra were referenced to aqueous 3-(trimethylsilyl) propanesulfonate (TSP).

TOCSY spectra of intact tissue and fatty acid standards were digitized into 1K data points along the  $t_2$  dimension using a sweep width of 7000 Hz. 256  $t_1$  increments were collected over a sweep width of 7000 Hz. Low power continuous wave irradiation for 1.5 s was used for water suppression for spectra of intact tissue along with a relaxation delay of 0.3 s and an acquisition time of 0.15 s. Spin-lattice relaxation times ( $T_1$ ) of the diallylic resonance (2.8 ppm) were estimated in high and low grade leiomyosarcoma using the inversion recovery method. A  $T_1$  of 0.8 s was found for the diallylic resonance for both the high and low grade leiomyosarcomas. The isotropic mixing sequence MLEV-17 was applied for 72 ms since this provided complete long range transfer of magnetization for visualization of the entire spin system network for the fatty acyl chains. For intact tissue, using longer mixing times (90 and 120 ms) added no additional cross-peaks; shorter mixing times (20 and 50 ms) resulted in fewer total cross-peaks but did not change the total number of fatty acyl chain cross-peaks. The total acquisition time for each TOCSY experiment was 5 h.

The TOCSY spectra were processed as follows. The data were linear predicted forward to 512 points in the  $t_1$  dimension and zero-filled to 1 K points. The linear prediction algorithm used was that described by Barkhuysen et al. (19). Exponential line-broadening was applied along both dimensions prior to Fourier transformation. A line-broadening of 3 Hz for the first transform and 8 Hz for the second transform was used. All cross-peak volume measurements were determined from unsymmetrized spectra using the RNMR software package developed at the Francis Bitter Magnet Laboratory. Cross-peak volume ratios are given as the average  $\pm$ SD of at least three data sets taken from samples of the same leiomyosarcoma which contained the highest cellularity as shown by histological examination.

Resonance assignments were based on previously published chemical shift values and spin-spin coupling patterns (20) and TOCSY spectra of phosphocholine, glycerophosphocholine, choline, phosphoethanolamine, *N*-acetylgalactosamine, galactose, triolein, and fatty acids recorded by us at 600 MHz. The cross-peaks assigned to C and D in our TOCSY spectra of linoleic, linolenic and arachidonic acid in chloroform all possess the same chemical shifts. Furthermore, chloroform extracts of the leiomyosarcoma tissues show fatty acyl chain assignments for the C and D cross-peaks identical to those made with the free fatty acids in chloroform as well as having the same assignments for intact leiomyosarcoma tissue (data not shown). Esterified fatty acids such as triglycerides do not have significant changes in their chemical shift assignments of their acyl chain protons compared to free fatty acids except for a minor chemical shift change (0.05 ppm) in the  $\beta$ -methylene protons of the acyl chain (21).



Chemical shifts of scalar coupled fatty acyl chain protons in smooth muscle tissues<sup>a</sup>

Cross-peak symbol	Cross-peak protons	Chemical shift (ppm)
A	<b>CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>3</sub></b>	1.33-0.90
B	<b>CH-CH<sub>2</sub>-CH<sub>2</sub></b>	2.07-1.33
C	<b>CH-CH<sub>2</sub>-CH<sub>2</sub></b>	5.38-2.07
D	<b>CH-CH<sub>2</sub>-CH</b>	5.38-2.79
E	<b>OC-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub></b>	1.65-1.33
F	<b>OC-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub></b>	2.36-1.65
G	<b>O-CH<sub>2</sub>-CH-O</b>	4.16-5.29
B'C'	<b>OC-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH</b>	1.67-5.38
FB'C'	<b>OC-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-CH</b>	2.36-5.38

<sup>a</sup>The protons in bold participate in the scalar coupling.

Figure 3. Structures and scalar connectivity assignments for linoleic (A) and arachidonic (B) acids. Table of chemical shifts for the scalar connectivities are also presented.

## Results and Discussion

Fig. 1 shows representative 1D <sup>1</sup>H-NMR spectra of intact tissue from a low grade leiomyosarcoma (A) with a mean mitotic activity of 1.5 mitoses/10 hpf and a high grade leiomyosarcoma (B) with a mean mitotic activity of 7.4 mitoses/10 hpf. The chemical shifts of the olefinic, diallylic, and allylic methylene protons are indicated by arrows. The diallylic resonance is significantly larger in spectra of the high grade leiomyosarcoma compared with the low grade leiomyosarcoma. The allylic methylene resonance is not resolved in 1D spectra from either tissue. Fig. 2 shows TOCSY spectra of intact tissue from a low grade leiomyosarcoma (A) with a mean mitotic activity of 1.5 mitoses/10 hpf and a high grade leiomyosarcoma (B) with a mean mitotic activity of 12 mitoses/10 hpf. The majority of cross-peaks exist in the 0-6 ppm region. These cross-peaks correspond to both small metabolites and macromolecules; most have been assigned (see Fig. 1) and correspond to free amino acids, triglycerides and lipid (cross-peaks A-F), phospholipids, bound fucose and lactate.

Fig. 3 shows the structures and spectral assignments for linoleic and arachidonic acids. The corresponding TOCSY spectra for linoleic acid and arachidonic acid are shown in Fig. 4, A and B, respectively. The acyl chain assignments are based on the TOCSY of the following fatty acid standards: oleic, linoleic, linolenic, and arachidonic. Cross-peaks A through F arise from the acyl chain. Cross-peaks C and D are methylene proton couplings in the acyl chain to the unsaturated site protons. Cross-peak C arises from spin-spin coupling between olefinic (-CH=CH-) and allylic methylene protons (-CH<sub>2</sub>-CH<sub>2</sub>-CH=) and thus appears only if the methylene protons is adjacent to only one unsaturated site proton. Cross-peak D arises from spin-spin coupling between olefinic (-CH=

CH-) and diallylic methylene protons (=CH-CH<sub>2</sub>-CH=) and appears if there are at least two unsaturated sites and the methylene protons are located between the unsaturated site protons. The ratio of cross-peaks D/C is thus a measure of the degree of unsaturation. An increase in the number of unsaturated site protons in a fatty acyl chain increases the number of methylene protons situated between two unsaturated site protons compared to the number of methylene protons bordered by only a single unsaturated site proton. Table I shows the D/C cross-peak volume ratio measured from TOCSY spectra for a series of fatty acids containing a variable number of unsaturated site protons. Oleic acid (18:1) has only one double bond and thus has no diallylic protons and therefore has no measurable D cross-peak. Linoleic (18:2), linolenic (18:3), and arachidonic (20:4) all have two or greater double bonds resulting in a progressive increase in the D/C ratio as a function of the number of double bonds. Thus, the degree of fatty acyl saturation directly correlates with the D/C cross-peak volume ratio for these model fatty acids. Cross-peak E is replaced by B' in arachidonic acid due to the presence of only three methylene groups between the first unsaturated carbon and the carboxyl group. Cross-peaks B'C and FB'C are unique to arachidonic acid. Quantitation of the A and B cross-peaks in spectra of intact tissue is problematic since they reside in a region close to the diagonal and are seldom baseline resolved (see Fig. 2A).

Table II shows the D/C cross-peak ratio as a function of tissue type, mitotic activity and grade. The D/C ratios changed only by 2.7±2.6% (mean±SD, n = 3) for two consecutive TOCSY spectra of the same leiomyosarcoma sample. Thus, there was no significant NMR-detectable degradation of tissue lipid over a 10-h total measurement time. The D/C ratio for the four patients with high grade leiomyosarcomas (2.6±0.4, n = 13) was significantly higher compared with the low grade

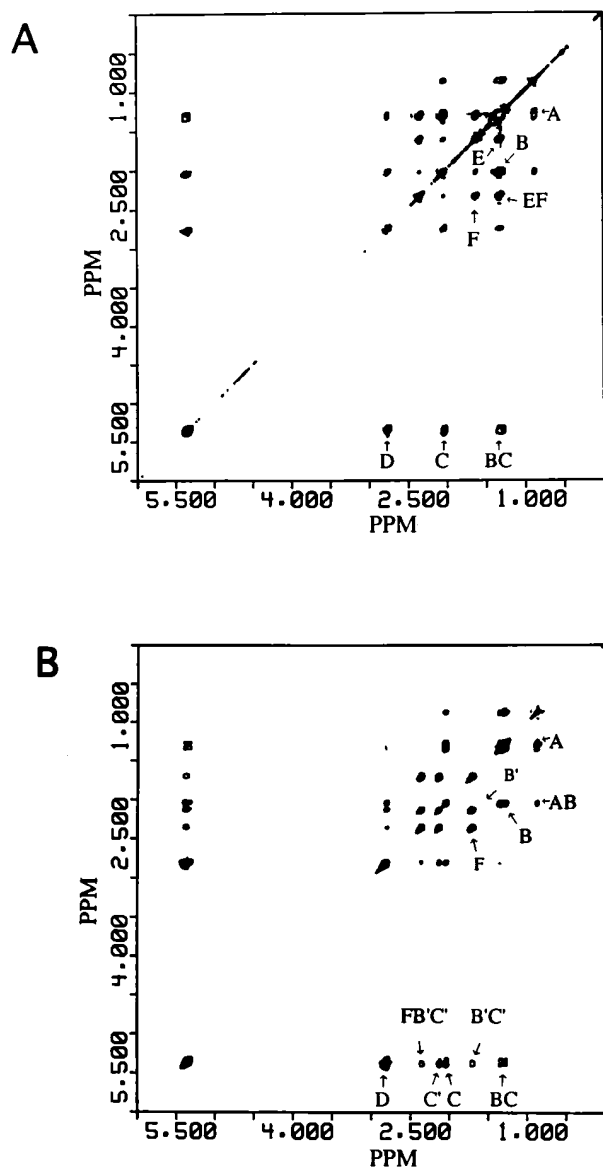


Figure 4. Symmetrized 2D  $^1\text{H-NMR}$  TOCSY spectra of linoleic acid (A) and arachidonic acid (B) in deuterated chloroform measured at 600 MHz.

leiomyosarcomas ( $1.3 \pm 0.2, n = 6$ ) ( $P = 0.001$ ). In addition, the D/C ratio for low grade leiomyosarcoma ( $1.3 \pm 0.2, n = 6$ ) was significantly higher compared with normal smooth muscle ( $0.5 \pm 0.1, n = 3$ ) ( $P = 0.001$ ). Fig. 3 shows the average D/C cross-peak ratio as a function of the mean mitotic activity of the smooth muscle tissue and tumor. For the nine smooth muscle tissues analyzed the D/C cross-peak ratio was found to be proportional to the mean mitotic activity using a weighted least squares linear regression with standard deviation as the weight ( $r = 0.94$ ) (Fig. 5). Our results suggest that high grade leiomyosarcomas contain a higher degree of fatty acyl unsaturation than low and intermediate grade leiomyosarcomas. High resolution NMR spectroscopy, however, can only detect signals from molecular species which possess sufficient mobility. For this reason, our results suggest that grade and mitotic ac-

Table I. D/C Cross-Peak Volume Ratios for Fatty Acid Standards

Fatty acid	D/C ratio
Oleic (18:1)	0.0
Linoleic (18:2)	1.3
Linolenic (18:3)	2.7
Arachidonic (20:4)	4.0

tivity of leiomyosarcoma correlates with the “NMR visible” fraction of fatty acyl chain lipid.

A change in the degree of unsaturation of fatty acyl chains of phospholipids would be expected to alter the physical properties of cell membranes. Electron spin resonance studies of L1210 murine leukemia membranes demonstrated that an increase in polyunsaturated fatty acid content resulted in a lower order parameter ( $S$ ), a quantitative measure of membrane fluidity (3, 22). These studies indicate that an increase in the degree of unsaturation of fatty acids resulted in a more fluid lipid membrane phase. Thus, the apparent increased degree of fatty acyl unsaturation observed in the high grade leiomyosarcomas compared with low and intermediate grade leiomyosarcomas may result in increased cell membrane fluidity for the high grade tumors and thus serve as an important determinant of metastatic potential.

Doxorubicin is the most active chemotherapeutic agent for the treatment of leiomyosarcoma. The mechanism of doxorubicin cytotoxicity is generally thought to occur by interfering with DNA. However, doxorubicin has been shown to interact with acidic phospholipids, affecting both membrane order and function. An increase in the degree of polyunsaturation of membrane fatty acids has been shown to increase accumulation of doxorubicin in leukemia cells (23). In addition, cell lines resistant to doxorubicin have been found to have phospholipid composition and membrane fluidity different from those of sensitive cells (24). The demonstration that modification of lipid unsaturation can affect the uptake of drugs that enter the cell by diffusion (such as doxorubicin) may be an important predictor of response to chemotherapy. Recently, studies have suggested a direct correlation between the degree

Table II. D/C Cross-Peak Volume Ratios for Smooth Muscle Tissues

Tissue type	Mean mitotic activity	Grade	D/C ratio ( $n$ )*
Normal smooth muscle	0.0	NA	$0.5 \pm 0.2$ ( $n = 3$ )
Leiomyosarcoma	0.3	low	$1.2 \pm 0.3$ ( $n = 3$ )
Leiomyosarcoma	1.5	low	$1.3 \pm 0.1$ ( $n = 3$ )
Leiomyosarcoma	4.6	intermediate	$2.0 \pm 0.5$ ( $n = 3$ )
Leiomyosarcoma	5.0	intermediate	$2.2 \pm 0.2$ ( $n = 3$ )
Leiomyosarcoma	6.2	high	$2.2 \pm 0.2$ ( $n = 3$ )
Leiomyosarcoma	6.8	high	$3.0 \pm 0.4$ ( $n = 3$ )
Leiomyosarcoma	7.4	high	$2.7 \pm 0.2$ ( $n = 3$ )
Leiomyosarcoma	12.0	high	$2.7 \pm 0.4$ ( $n = 4$ )

\* $n$  samples analyzed from the same leiomyosarcoma or smooth muscle sample.

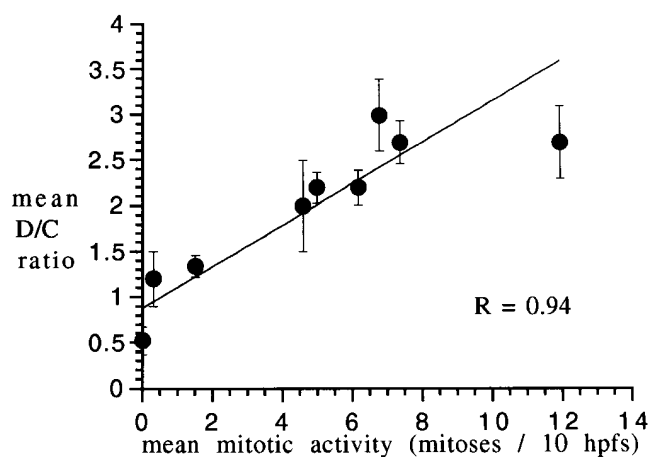


Figure 5. D/C cross-peak volume ratio as a function of leiomyosarcoma mean mitotic activity (mean mitoses/10 hpf). Each point represents the mean D/C ratio for at least three separate samples derived from the same tissue specimen.

of polyunsaturation of membrane lipids in L1210 leukemia cells and sensitivity to doxorubicin chemotherapy as measured in a soft-agar clonogenic assay (25). The increase in sensitivity of cells to doxorubicin was found to be directly related to greater intracellular accumulation of the drug and the number of double bonds in the fatty acid used to enrich the cells (23, 26). Paradoxically, high grade leiomyosarcomas tend to be more responsive to doxorubicin-based chemotherapy than low grade leiomyosarcomas. The increase in the degree of polyunsaturated fatty acids seen in the high grade leiomyosarcoma might allow for an increase in drug uptake into the high grade tumors compared with low grade leiomyosarcomas. Future studies correlating intracellular levels of doxorubicin with leiomyosarcoma grade and degree of fatty acid unsaturation will be required to test this postulate.

We have identified a number of amino acid spin systems (Ala, Glu, Gln, Arg, Lys, Pro, Thr, Val, Leu, Ile) based on spin-spin coupling patterns and chemical shift values. These assignments are indicated in Fig. 2. The amino acids appear to exist as isolated molecules based on the chemical shifts, especially of the alpha carbon protons (27). The broad cross-peaks labeled "peptides?" (see Fig. 2) at  $\omega_1 = 4.3\text{--}4.5$  ppm and  $\omega_2 = 1.5\text{--}2.4$  ppm are detected in all leiomyosarcoma spectra. The large linewidths of these cross-peaks are consistent with large, aggregated, or surface bound peptides. Three different forms of bound fucose (FucI, FucII, and FucIII) are detected in the TOCSY spectra for some of the leiomyosarcomas. The fucosylation pattern was not significantly different between high and low grade leiomyosarcomas.

In summary, cross-peaks assigned to amino acids, peptides, phospholipids, triglycerides, fucose, and other saccharides are easily identified in the TOCSY spectra. We have assigned cross-peaks corresponding to the fatty acyl chains of tissue lipids. The cross-peak ratio, D/C, is a measure of the degree of unsaturation of the fatty acyl chains because TOCSY spectra of oleic, linoleic, linolenic, and arachidonic acids yield D/C cross-peak ratios of 0.0, 1.3, 2.7, and 4.0, respectively. For nine smooth muscle tissues, the D/C cross-peak ratio was found to correlate with the mean mitotic activity ( $r = 0.94$ ). These results demonstrate that we can use 2D  $^1\text{H-NMR}$  TOCSY spec-

tral profiles to determine the degree of unsaturation of the mobile fraction of fatty acyl chain lipids in smooth muscle tumors and that the extent of unsaturation correlates with mean mitotic activity and thus grade of smooth muscle tumors. Thus, the 2D TOCSY technique may serve as an ex vivo pathologist-independent method for determining sarcoma grade and prognosis. In addition, changes in the degree of fatty acyl unsaturation are known to change protein motional characteristics as well as affect activity of membrane enzymes, altering many cell functions. Thus, assessment of the degree of fatty acyl unsaturation may be predictive for uptake of chemotherapeutic drugs such as doxorubicin.

Furthermore, ex vivo 2D  $^1\text{H-NMR}$  TOCSY measurements of leiomyosarcoma tissue fatty acyl unsaturation would potentially allow for a more quantitative and functional assessment of sarcoma grade and mitotic activity thereby rendering a more accurate staging of patients. We are currently developing selective one dimensional proton NMR methods to determine the integral ratio of diallylic to allylic methylene protons. This will permit a substantial decrease in total measurement time with some increase in spectral resolution. Furthermore, these selective 1D methods could be potentially applied for use in vivo at lower field strengths and thus allow for the non-invasive assessment of sarcoma grade and mitotic activity.

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