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NMR AND GAS CHROMATOGRAPHY STUDIES OF LYOPHILIZED HUMAN BRAIN TUMORS

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Abstract: ^{1}H HR MAS (high resolution magic angle spinning) NMR is a promising tool in the *in vitro* characterisation of brain tumors. Brain tissue samples were collected from patients with intracranial tumors during surgery, frozen and lyophilized. The analysis of solid samples was performed by high-speed rotation (33 kHz) ^{1}H MAS NMR. The most intense resonances arise from lipids (methylene mid-chain and methyl carbons of fatty acids). ^{1}H MAS spectra obtained from the same histological type of brain tumors are similar; spectra of glioblastomas were different from those of meningiomas. ^{1}H and ^{13}C NMR solution spectra of lipid extracts confirmed the presence of aliphatic chains fatty acids and cholesterol as major constituents. Biochemical information on liberated fatty acid composition was obtained by gas-chromatography (GC). The glioma content of the linoleic acid (18:2n6) was found to be greater, whereas the level of docosahexaenoic acid (22:6n3) was significantly reduced.

Keywords: ^{1}H MAS NMR, solid state NMR, fatty acids composition, brain tumors, glioblastoma, meningioma, docosahexaenoic acid

Magnetic resonance imaging (MRI) techniques are widely used in the *in vivo* localization of brain tumors (1). Magnetic resonance spectroscopy (MRS) is expected to characterize them by establishing biochemical criteria for differentiation. However, the interpretation of *in vivo* NMR data is difficult and requires parallel studies *in vitro*. Actually, the diagnosis of brain tumors relies mainly on histopathological criteria. Nevertheless, NMR techniques may be useful in detection of metabolic alternations of tumor specimens (2, 3). Low molecular weight compounds which are present in fairly high concentration are MRS-visible, however, most macromolecules cannot be accessed because of their limited mobility. Qualitative and quantitative information on metabolites that are soluble in water can be obtained by analysis of ^{1}H NMR spectra of perchloric acid extracts (4-6). However, interesting biochemical information on insoluble constituents of brain tissue is lost. The lipids have to be extracted from tissue sample by chloroform/methanol mixture, and analyzed by gas-liquid chromatography or solution NMR.

NMR spectra of rigid solids exhibit a broad contour due to the dipole-dipole and chemical shift anisotropy interactions, these contributions can be averaged by sample spinning at the ''magic angle'' of 54˚44'. Unfortunately, the ^{1}H-^{1}H couplings are frequently of 40 – 50 kHz, and actually it is difficult to spin the sample fast enough to remove them. However, rapid lipid vesicle rotation and molecular motions reduce the line width. Magic angle spinning (MAS) has been used to eliminate residual dipolar interactions and to obtain high resolution ^{1}H NMR spectra of lipid dispersions and myelin membrane (7).

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^{1}H MAS NMR spectra of unprocessed, intact brain tissues were measured by Cheng et al. (8, 9). High spectral resolution was achieved by spinning with a speed of 2.5 kHz (the rotated sample was kept at 2^{o}C to prevent tissue degradation), and the concentrations of 11 metabolites could be determined. The comparison of *in vivo* ^{1}H MRS of human brain tumors with ^{1}H HR-MAS NMR of intact biopsy samples *in vitro* was made by Barton et al. (10). MAS gave greatly improved line-shape in comparison to the spectroscopy *in vivo*. Spectra derived from ^{1}H HR MAS NMR were used to distinguish three neural cell types: cortical astrocytes, cerebellar neurons and O-2A progenitors, and the large lipid content of neuronal cells was evidenced (11).

The aim of our study was to characterize brain tumors in solid phase (lyophilized) using high speed ^{1}H MAS NMR. ^{1}H NMR spectrum obtained from a whole piece of tissue is more likely to reflect tumor composition since all metabolites and macromolecules are maintained. Water-soluble metabolites in perchloric acid extracts obtained from the same collection of samples have been assessed previously by high resolution ^{1}H NMR [Czernicki et al. (4)]. In order to collect information on the water insoluble compounds, lipids extraction was performed. The extracts were analyzed by ^{1}H and ^{13}C NMR in solution and also by gas chromatography.

EXPERIMENTAL

Clinical material

Brain tissue samples: tumors (17) and normal tissue (3) adjacent to pathology were collected from 17 male patients (aged 43-67) with intracranial tumors. The samples extirpated at surgery (fresh tissue weight range 67-1895 mg) were frozen immediately in liquid nitrogen and stored at -80^{o}C. A part of each specimen was subjected to routine histopathological examination, the rests were subsequently lyophilized. The dry powders were stored at -20^{o}C until analyzed by ^{1}H MAS NMR and prior to lipids extraction. According to histopathological criteria, the specimens were classified as: normal brain tissue (**1N – 3N**), gliomas (glioblastoma multiforme: **4GM – 11GM**), meningiomas (**12M – 16M**) and other types (haemangioblastoma (**17MG**), craniopharyngiomas (**18CP, 19CP**) and carcinoma microcellulare (**20MC**).

Chemicals and reagents

Hexane (chromatography-grade), sodium sulfate, chloroform and methanol (analytical grade) were obtained from POCh (Poland). Boron trifluoride-methanol solutions (14% BF_3) and GC standard of fatty acids were purchased from Sigma. Deuterated solvents ($CDCl_3$) for NMR were purchased from Glaser (Basel).

Lipid extraction

The extraction from lyophilized samples was performed by Dual-Phase Extraction (DPE) method (12) in which water-soluble metabolites are recovered in the methanol-water phase and cellular lipids in the chloroform phase. The lipids were redissolved in $CDCl_3$ and transferred to NMR tubes for analysis. One sample of normal brain tissue was subjected to standard lipid extraction with chloroform, the unsoluble residue was dried in a stream of dry nitrogen and analyzed by ^{1}H MAS NMR.

NMR measurements

^{1}H and ^{13}C NMR spectra of liquid lipid extracts were recorded for $CDCl_3$ solution on a Bruker DSX-400 WB AVANCE spectrometer (at 400,13 and 100.13 MHz, respectively) using standard pulse sequences from Bruker library. Chemical shifts are reported in ppm relative to internal tetramethylsilane (TMS). Solid state ^{1}H MAS NMR spectra were recorded on the same instrument at 400.13 MHz with a probehead enabling high speed rotation; the samples were spun at 33 kHz in a 2.5 mm ZrO_2 rotor (a repetition time of 6 s, a spectral width of 10 kHz, 90-deg pulse length of 2.6 μs, were used for accumulation of 64 scans). Data were zero-filled and 1 Hz line broadening applied prior to Fourier transformation. Phase and baseline corrections were done automatically. Chemical shifts were referenced indirectly to TMS.

Gas chromatography

Aliquots of lipid extract from each sample contain a mixture of fatty acids. Their composition was determined by gas chromatography (GC) (13, 14). The analysis was performed with a Shimadzu 15A gas chromatograph equipped with a SGE 2561 M03 column (Supelco) and a flame ionization detector (FID). The acids were converted to methyl esters with 14% BF_3, in methanol, transferred to 1 mL of hexane and dried with Na_2SO_4; 5 mL of hexane solution was then moved to evaporation tube and taken to dryness with a stream of nitrogen at 37^{o}C. Dry residue was dissolved in 50 mL of hexane and analyzed with GC. Separation parameters: column temperature 140 – 220^{o}C, increasing 3^{o}C per min, final 15 min at 220^{o}C; carrier gas – helium (2 cm^{3} per min). The resultant peaks were identified by comparing the retention times with those of known fatty acid methyl ester standards. The GC method was validated according to Seppanen-Laakso T. et

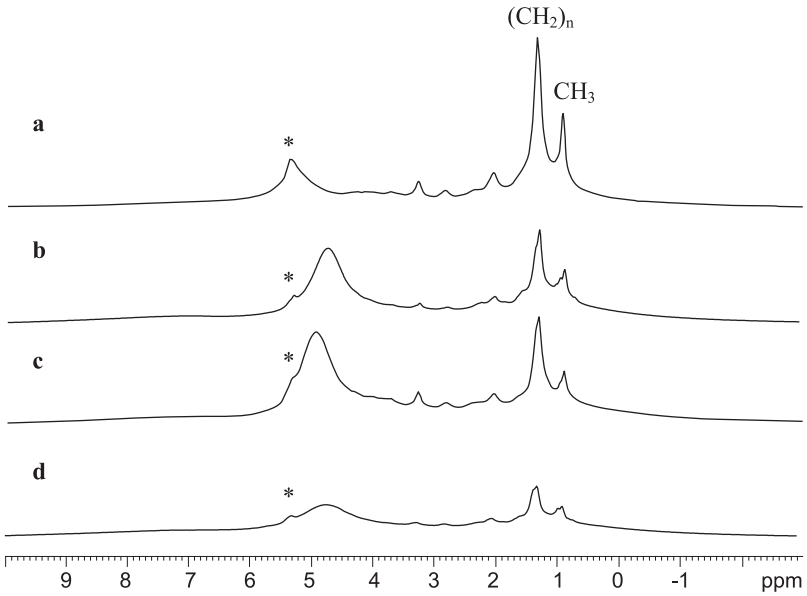

Figure 1. ^{1}H-MAS NMR spectra of **a**) „normal" brain tissue (**1N**), **b**) glioblastoma multiforme (**7GM**), **c**) meningioma (**12M**), **d**) carcinoma microcellulare (**20MC**). The signals of C**H**=C**H** at 5.3 ppm are marked with an asterisk, broad resonances at 4.5 – 4.8 ppm arise from water

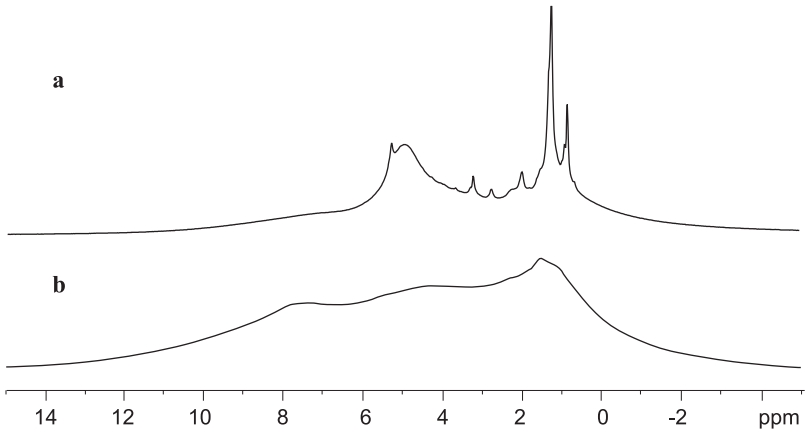

Figure 2. ^{1}H MAS NMR spectra of lyophilized brain tissue (**2N**), before (**a**) and after (**b**) the lipid extraction

al. (15). Few samples studied by GC (three of normal tissue and three glioblastomas) do not allow for statistical analysis. Such analysis would require a collection of samples from the patients of similar age, same sex and the same histopathological diagnosis.

RESULTS AND DISCUSSION

The samples of intracranial tumors were first examined by histopathology, and on that basis the specimens were classified as gliomas, meningiomas or other types. Three samples were qualified as normal brain tissue, however, obtained from the region adjacent to pathology (glioblastoma multiforme).

Solid state HR ^{1}H MAS NMR

The lyophilized brain tissues, beige to brown powders, were packed into NMR rotors and subjected to solid state ^{1}H MAS NMR studies. The ^{1}H MAS NMR spectra of all specimens were measured at rotation speed of 33 kHz; the spectra of four selected samples, are shown in Figure 1a-d, as an illustration.

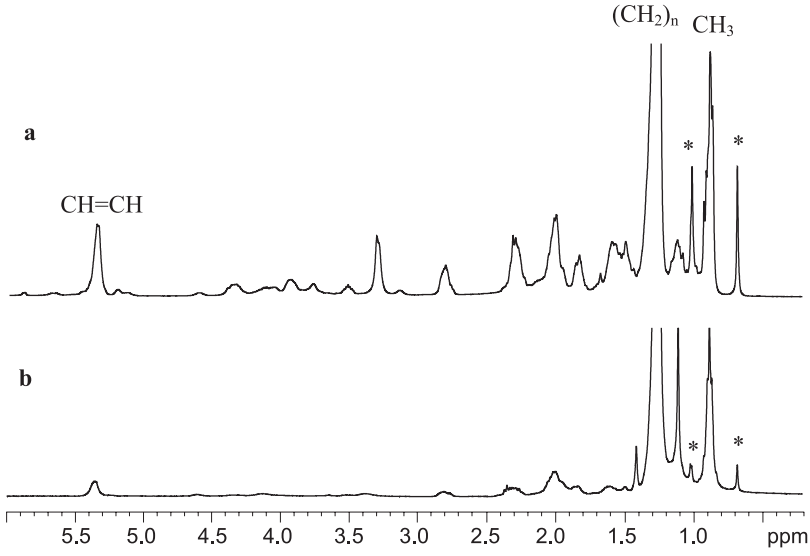

Figure 3. ^{1}H NMR spectra of lipid extracts from lyophilized brain tumors, **a**) glioblastoma multiforme (**5GM**), **b**) carcinoma microcellulare (**20MC**). The signals of cholesterol methyl groups are marked with an asterisk

During the MAS experiment, increasing temperature and centrifugal forces (cased by the high frequency spinning) can have an influence on a sample. At spinning of 10 kHz the temperature increases by 5-10 degrees, higher spinning frequencies result in a further increase of sample temperature. However, repeated measurements of the same sample (without opening of the rotor), do not produce different NMR spectra. The lyophilization of samples seemed to prevent tissue degradation during MAS NMR measurements.

The ^{1}H MAS spectra exhibit mainly the signals of lipids which can be assigned as: d 0.9 – C**H**$_3$, d 1.3 – (C**H**$_2$)$_n$, d 2.0 – allylic methylene =CH-C**H**$_2$, d 5.3 C**H**=C**H**, d 3.25 N(C**H**$_3$)$_3$. Separate resonances indicated that the brain tissue lipids are fairly mobile, in spite of the dehydration of the tissue sample.

Intense, broad resonances observed at 4.5-4.8 ppm (Fig. 1) inform that the lyophilized samples still contain significant amount of water. Chemical shift changes of H_2O signal may reflect susceptibility effects suggesting different water localization. Although lyophilization was performed in standard conditions, the content of water remaining in the samples cannot be linked to the progress of disease without further detailed experiments, such as the measurements of relaxation times. Water content of a normal brain is ca. 65%, and changes up to 10-20% may occur due to disease. The increase of the free water content and varying cellular environment of tumor cells are reflected in the relaxation rates. In gliomas, the changes of T_2 relaxation time of the metabolites were reported (16); T_2 of tissue water was significantly extended, as compared to normal subject.

The signals of lipid methylene and methyl groups are less clearly seen in the spectra of tumors (Fig. 1b, c, d) than in the spectrum of "normal" tissue. It is worth to note that histopathologically "normal" or "healthy" brain tissue was obtained from an ill patient, and may suffer the disturbed metabolism. The spectrum of the lowest resolution was observed for high-grade carcinoma microcellulare. This tissue does not show accumulation of mobile lipids but remarkable broadening and reduction in most signals, including lipids. This brain sample was unique because metastases constitute only few percent of brain tumors, and carcinoma microcellulare extremely rarely metastases into central nervous system.

Comparing ^{1}H MAS NMR spectra of all lyophilized samples of tumors, some characteristic features have been noticed: the spectra of glioblastomas are similar, also those of meningiomas resemble each other; however, no further details on molecular level can be differentiated.

In order to confirm that the well resolved intense peaks come mainly from lipids, the ^{1}H MAS

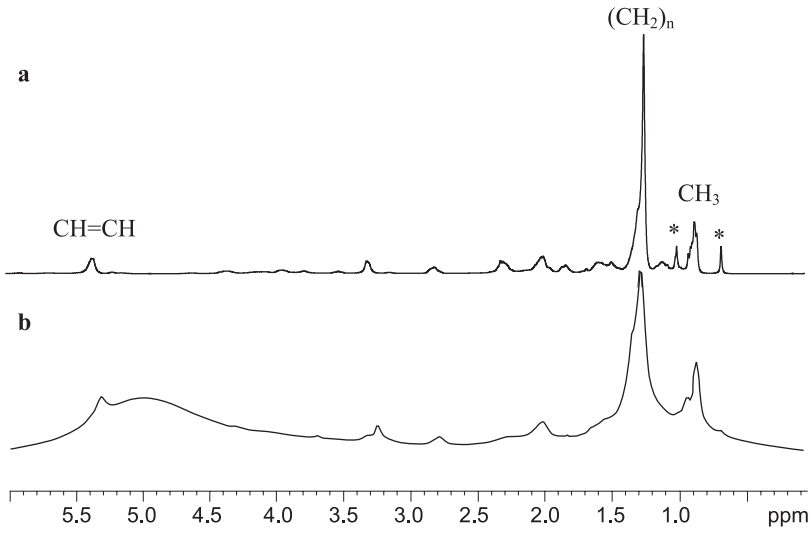

Figure 4. **a**) ^{1}H NMR spectrum (in $CDCl_3$) of lipid extract from meningnioma (**12M**), **b**) HR ^{1}H MAS NMR spectrum (at 33 kHz) of lyophilized, solid tumor tissue (**12 M**). The signals of cholesterol methyl groups are marked with an asterisk

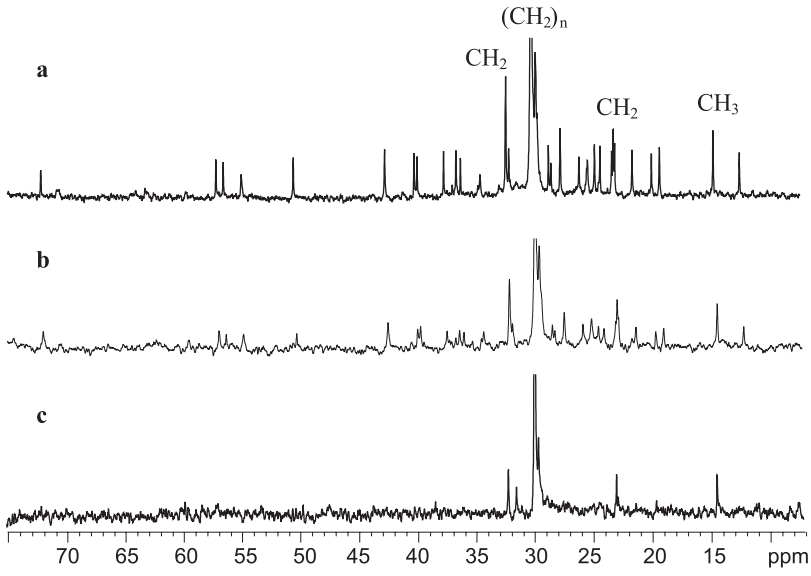

Figure 5. ^{13}C NMR spectra of lipid extracts of brain tissues: **a**) glioblastoma multiforme (**7GM**), **b**) meningioma (**13M**), **c**) carcinoma microcellulare (**20MC**). The main methylene and methyl resonances of lipids (**a**) are exclusively present in the spectrum (**c**). All other resonances observed in (**a**) and (**b**) arise from cholesterol (i.e. 25 resonances in the range 10-75 ppm, of the all 27 carbons of this molecule)

spectrum was recorded for the dried solid residue obtained after lipids extraction. The ^{1}H MAS NMR spectra of normal brain tissue before and after lipid extraction are shown in Figure 2a, b, for comparison. Broad contour, covering whole spectral region demonstrates that the mobile components have been removed and the remaining ones (proteins, saccharides) are rigid, and probably amorphous.

The signals of lipids arise predominantly from fatty acyl moieties which are relatively mobile and probably not organized in a phospholipids bilayer structure. Their association with cellular necrosis in

Table 1. Fatty acid levels (%) in normal brain samples **1N-3N** and tumor (glioblastoma) **4GM-6GM**, as determined by GC.

Fatty acid		1N	2N	3N	Ref.*	4GM	5GM	6GM	Ref.**
Palmitic	16:0	25.8	26.1	27.7	21,5	35.2	23.6	34.3	26.3
Stearic	18:0	25.4	31.6	33.0	23,9	20.4	22.4	15.5	14.7
Palmitooleic	16:1n7	2.9	1.8	1.4	-	3.9	2.3	3.4	4.0
Oleic	18:1n9	28.8	26.2	24.3	18,3	26.5	28.9	25.7	24.0
Linoleic	18:2n6	2.4	1.3	2.5	0,72	3.2	14.9	13.0	4.4
Arachidonic.	20:4n6	7.0	5.4	5.2	10,1	7.0	7.3	6.5	4.4
Docosahexaenoic	22:6n3	7.7	7.5	5.8	13,6	3.7	0.03	1.5	4.4

*(24), calculated from regression equation, since the levels of brain fatty acids are dependent on age of the subject; ** (25), total lipids (%), for glioma samples

high grade glial tumors may be related to membrane breakdown. In 1994 Kuesel et al. (17, 18) found large amounts of mobile lipids in high grade astrocytomas. In the case of glioblastoma multiforme, mobile lipids were also observed in the absence of apparent necrosis. Mobile lipids were observed (19) *in vivo* using ^{1}H MRS. Meolic heterogeneity of brain tumors were detectable *ex vivo* by ^{1}H MRS (20, 21). The spectra of subsequent stages of metastatic brain tumors showed either unambiguous lipid signals or lactates. Unfortunately, lactate signal (δ 1.33 ppm) is superimposed on the $(CH_2)_n$ lipid signal; without special pulse sequences (2D) it is not possible to distinguish lactate from lipids in the ^{1}H HR MAS spectra of solid samples.

Solution NMR of tissue extracts

More biochemical information can be gained from NMR spectra measured for solutions. Tissue extracts containing water-soluble compounds of intracranial tumors were previously characterized using ^{1}H NMR by Czernicki et al. (4). The signals normalized to that of total creatine provided good discrimination between gliomas and meningiomas. Both types of tumors were characterized by the high concentration of lactate, remarkably higher in the studied meningiomas than in gliomas. The dominated signal (doublet at 1.31/1.33 ppm) in the spectra came from the lactate. However, the quantification of metabolites was difficult because extirpated tumor samples contain variable proportion of viable and necrotic tissues.

Tissue extracts containing water-insoluble compounds of the tumors were analyzed by us using both ^{1}H and ^{13}C NMR spectroscopy. The extracted lipids (oils) were dissolved in $CDCl_3$. Figure 3 shows ^{1}H NMR spectra of lipid extracts from two tumor samples: glioblastoma multiforme and carcinoma microcellulare. The dominant signal is an unresolved resonance at 1.25-1.30 ppm from fatty acid methylene groups, followed by that of terminal methyl groups at 0.89 ppm. Most of the signals are broad because they consist of superimposed resonances of similar (mainly methylene) protons. ^{1}H NMR spectra confirm the presence of saturated (δ 0.89, 1.25-130, 1.65, 2.35 ppm) and unsaturated (δ 2.05, 2.8, 5.35 ppm) fatty acids and cholesterol (sharp singlets at δ 0.70 and 1.02 ppm from angular methyl group carbons C18 and C19, respectively). The differences in the intensities of lines (which may be connected to the tumor type or the progress of disease) can be noticed, however, the quantification is impossible due to the signal overlap.

The HR ^{1}H MAS of the brain tissue and ^{1}H NMR spectra of the lipid extract from the same sample are illustrated in Figure 4. Both solid state and liquid state NMR spectra show the dominating resonances from lipids and supply similar information on tissue constituents. ^{1}H MAS NMR contributes to our understanding of brain tissue composition because it allows to detectable major constituents of lyophilized material, without preparation procedures. The advantage of solid-state technique is its non-destructive character; i.e. the sample removed from rotor can be further used for extraction and analysis by solution NMR.

^{13}C NMR solution spectra of lipid extracts are more informative than ^{1}H ones. The spectra recorded for three extracts from various tumor samples are illustrated in Figure 5a-c. Major detectable compounds are fatty acids and cholesterol. The cholesterol chemical shifts observed in these spectra corre-

spond to literature data (6). The dominant signal in the region 29.5-30.0 ppm arises from mid-chain carbon atoms; easily recognized shifts are associated with carbons from saturated chain: ω **C**H_3 – 14.5 ppm, ω1 **C**H_2 -22.8 ppm, ω2 – 32.2 ppm, α (proximal to **C**OO) 34.8 ppm, β 25.7 ppm, γ 30.2 ppm and **C**O at 174 ppm. The presence of unsaturated fatty acids is indicated by small signals at 128-130 ppm. Unequivocal assignment of particular phospholipids, as well as quantification of the signals is not an easy task. However, changes in the phospholipids and cholesterol content are detectable in the ^{13}C NMR spectra of glioblastoma and meningioma (Fig. 5a, b). No cholesterol signals are observed in the ^{13}C spectrum of carcinoma microcellulare (Fig. 5c). Interestingly, the ^{13}C NMR spectra of normal brain tissue extracts confirm high content of cholesterol in the brain tissue.

The brain is highly enriched in cholesterol and important neuronal processes require its presence. A number of neurological diseases result in defective cholesterol metabolism and altered interaction with membrane lipids and proteins. However, much less is known about its interaction with polyunsaturated phospholipids (22). The relative affinity of cholesterol for saturated *versus* polyunsaturated acyl chains has been proposed as a mechanism for lateral separation into cholesterol-rich and poor microdomains.

The intact brain tissue undergoing MRS measurements even at low temperature will still be subjected to neurochemical degradation, unlike tissue extraction. The oxidized fatty acids were observed by Willker et al. (23), in ^{1}H (800 MHz) and ^{13}C NMR spectra of blood plasma lipids from lyophilized samples stored for several weeks. No signals of keto or hydroxyl groups from the oxidized lipid species were detected by us in ^{13}C NMR spectra of lipid extracts of brain tumors, although such compounds may appear as storage artifact. Metabolic reactions are terminated at the point when the tissue is frozen in liquid nitrogen and subsequently lyophilized (extracted).

Gas chromatography studies of fatty acids

GC was used to perform quantitative analysis of fatty acids for tissue samples of glioma tumors and compare this with fatty acid composition of normal brain tissue. Glioblastoma multiforme (GM) was selected because it is the most common and aggressive tumor with short survival rate. The fatty acid levels in terms of total lipid extract for glioma samples (**4GM- 6GM**) and normal brain samples (**1N – 3N**) are collected in Table 1. The literature data for normal human brain and for gliomas are included, for comparison. The fatty acid composition of human gliomas differs from that found in normal brain tissue. The most striking change in the fatty acid composition observed (Table 1) is negligible amount of DHA in the tumor sample (**5GM**). The docosahexaenoic acid is the final product of long sequence of PUFA (polyunsaturated fatty acids) synthesis pathway steps, therefore the most vulnerable towards any genetic/enzymatic defects. The deficiency of DHA may be due to the inherited peroxisomal disorders or extensive genome rearrangement in the neoplastic cells present in the tumor.

Neural tissue fatty acid composition depends on the brain area, age, sex and the diet. Fatty acid composition in normal human brain changes significantly during periods of rapid brain growth, and also later through the late adulthood. Therefore, the data were treated as bilinear relationship (24), with changes occurring after the age of 18 years. In the older cohort, the levels of 18:2n6 increased significantly with age, while several of the polyunsaturated and elongated n6 fatty acids (particularly 20:4n6) decreased with age.

Brain tissue samples obtained from 13 glioma patients and from 3 nonmalignant patients were analyzed by Martin et al. (25), using gas chromatography, following lipid extraction. The percentage of PUFA was essentially identical in the normal brain and glioma samples. However, the glioma content of the linoleic acid was found to be significantly greater, whereas the levels of DHA were significantly reduced. These results are in agreement with our data.

CONCLUSIONS

^{1}H MAS NMR spectra of lyophilized brain tissues are almost "liquid like" and show well resolved dominating signals of mobile lipids. The spectra of glioblastomas are similar; those of meningiomas are broader but also resemble each other. The advantages of solid-state technique: i) it is fast and non-destructive, ii) MAS NMR spectra supply information on tissue constituents – mobile lipids, iii) the sample removed from rotor can be further used for extraction and analysis of metabolites. ^{1}H and ^{13}C NMR solution spectra of lipid extracts enabled the identification of phospholipids with saturated and unsaturated fatty acids and cholesterol. Quantitative analysis of fatty acids extracted from tumor samples was performed by gas chromatography. The reduced content of docosahexaenoic acid was found in gliomas.

Acknowledgments

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