Cell Type-specific Fingerprinting of Meningioma and Meningeal Cells by Proton Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

We compared the properties of six human meningiomas with normal rat meningeal cells using cell culture techniques, high resolution in vitro 1H-NMR (nuclear magnetic resonance) spectroscopy, and chromatographic analysis. Cell cultures were immunocytochemically characterized at all stages with specific antibodies. Quantitative and qualitative metabolite assessments in cell extracts were obtained from 1H-NMR spectra and chromatographic analysis. Human meningioma cells expressed a characteristic spectrum of metabolites including free amino acids, compounds related to membrane phospholipid metabolism, energy metabolites, and other intermediary products. These spectral characteristics, although different in some respects, were strikingly similar to the ones of rat meningeal cells. Particularly, several metabolites that allow discrimination between meningeal cells and other cell types of the central nervous system were preserved in meningiomas. These similarities suggest that the regulation of intracellular levels of such metabolites is so intrinsic to the identity of cell type as to be conserved across species and through transformation. Additionally, human meningioma cultures expressed some spectroscopic characteristics that enabled them to be clearly distinguished from primary rat meningeal cultures. Thus, human meningiomas may be both specifically recognizable by 1H-NMR spectroscopy and also distinguishable from normal rat meningeal tissue. Our results raise the eventual possibility of using NMR in the noninvasive diagnosis of brain tumors in vivo.

INTRODUCTION

The evolution of tumor classification has been closely correlated with the development of methods for identifying the normal cells from which tumors arise. For example, as the cellular specificity of labeling with various antibodies has become more clearly defined, the use of histological criteria for tumor classification has been gradually supplemented and, in some instances, replaced by the use of immunocytochemical analysis. In addition, the increase in our understanding of the cellular lineages involved in normal development has led to the recognition of categories of tumors that were previously not appreciated.

One of the tools for cell type recognition that has lately proved of potential interest is 1H-NMR spectroscopy. NMR analysis of extracts obtained from cultured cells provides a reliable method to assess the metabolites present in these cells (1). A major advantage of NMR spectroscopy is that it is a noninvasive technique that can be applied in vivo, enabling information to be obtained about the metabolic composition of living tissue. Cerebral metabolites such as creatine and phosphocreatine, Cho and NAA can be detected routinely by 1H-NMR spectroscopy in vivo in animals and humans (2, 3). Additional signals in normal brain, including those of glutamate, inositol, and glucose, can be detected in animal studies or in humans with the latest generation of clinical NMR scanners (4). Glutamine, alanine, lactate, and GABA are present in lower amounts in normal brain and are difficult to detect due to sensitivity and resolution problems but can be detected with the aid of spectral editing techniques or in pathological states when their concentration is elevated (5, 6). NMR spectra of extracts obtained at high magnetic field strengths offer much improved sensitivity and resolution compared to spectra recorded in vivo and enable a wide range of hydrogen-containing metabolites to be identified and quantified. Thus, such extracts can be used to detect fine biochemical differences between various cell lines or types of cells.

In examining purified populations of different cell types derived from a variety of cellular lineages of the central nervous system, we (7) have found that it is possible to distinguish unambiguously cerebellar granule neurons, meningeal cells, cortical astrocytes, oligodendrocyte-type-2 astrocyte progenitor cells, and oligodendrocytes by analysis of their 1H-NMR spectra. Each cell population studied expressed a characteristic distribution and amount of metabolites, including free amino acids and derivatives, compounds involved in membrane biosynthesis and catabolism, and compounds related to energy metabolism. Thus, different cell types could be recognized by the application of 1H-NMR spectroscopy.

An important question that follows from our observations that different cell types express characteristic 1H-NMR spectra relates to the applicability of this technique to tumor diagnosis, i.e., whether related tumor cells express similar spectra. To investigate this possibility, we compared 1H-NMR spectra from six human meningioma cell lines. We found that these spectra were not only similar to each other but also shared characteristics with spectra obtained from primary rat meningeal cells. Thus, our results suggest that it might eventually be possible to use NMR for noninvasive diagnosis of meningiomas and that cell type-specific metabolites are so integral to the identity of a cell type that they are conserved across species and through transformation.

MATERIALS AND METHODS

Preparation and Analysis of Cell Cultures

All cells were cultured in DMEM containing 1 g/liter glucose (GIBCO-BRL, Paisley, United Kingdom), supplemented with 10% heat-inactivated FCS (Imperial Laboratories), 2 mM glutamine (Sigma Chemical Co., Poole, United Kingdom), and 25 µg/ml gentamicin (Flow Laboratories, Rickmansworth, United Kingdom; DMEM-FCS). Cells were grown in NUNC tissue culture flasks (GIBCO-BRL) coated with 13 µg/ml poly-L-lysine (Sigma) and maintained in a 37°C incubator with humidified atmosphere containing 7.5% CO2. The immunocytochemical characterization of all cell cultures was performed at each passage by plating cells in parallel onto poly-L-lysine-coated glass coverslips and growing them in the same conditions as above. Antibodies and staining methods described in the literature (8, 9) were used with minor modifications. The specificity of immunocytochemical labeling was tested for all antibodies on mixed cultures of cerebral cortices.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: NMR, nuclear magnetic resonance; Cho, choline-containing compounds; NAA, N-acetyl-aspartate; GABA, y-aminobutyric acid; GFAP, glial fibrillary acidic protein; Gab C, galactocerebroside; PCA, perchorlic acid; TSP, 3-trimethylsilyl-3-sodium propionate; 2D-COSY, homonuclear proton two-dimensional correlation spectroscopy; PFG, pulsed field gradients; NAAG, N-acetyl-aspartyl-glutamate; PC, phosphoryl choline; GPC, glycerophosphoryl choline; GFA, o-phthaldialdehyde; BCA, bicinchoninic acid; PK, pyruvate kinase.
Meningeal Cells. Six human meningeal cell lines, of which one was derived from a recurrent meningeal, were cultured. One of the cell lines originated from a spinal meningocele. The composition of cultures was evaluated by immunolabeling cells on coverslips with antibodies at 48–72 h after plating onto coverslips (initial density of 500 cells/coverslip). Cells were tested for the presence of cytoplasmic intermediate filaments, such as glial fibrillary acidic protein (protein specific to astrocytes in mature brain; Refs. 10–12) and vimentin (11, 13), and also for fibronectin (12). Additionally, contamination by other cell types of the nervous system was assessed by testing with monoclonal antibody A2B5 (a marker characteristic of neurones and cells of the oligodendrocyte–type-2 astrocyte lineage (14); hybridoma supernatant, diluted 1:2), or anti-GaC (a specific surface marker for oligodendrocytes; Ref. 15; hybridoma supernatant, diluted 1:10).

Primary Meningeal Cells. Cell cultures were prepared as described previously (9). Leptomeninges of 1-day-old Wistar rats were removed and dissociated with collagenase and trypsin and then plated onto 175-cm² flasks and grown in DMEM-FCS. The cells formed a confluent monolayer within 5–6 days after plating, when they were passaged and replated at 1:4 dilution into 175-cm² flasks. Cells were plated in parallel onto coverslips (density of 3000 cells/coverslip). When meningeal cells formed a confluent monolayer, they were harvested from culture flasks. The meningeal cultures were assessed for purity by immunocytochemical labeling on coverslips with anti-Ran-2 (a monoclonal antibody which recognizes an antigen present among others on the surface of astrocytes and leptomeningeal cells; Ref. 16). All antibodies used in the characterization of human meningeal cells were also used for rat meningeal cells.

Cell Harvesting, Extraction, and Preparation of Samples

All cells were continuously cultured until the amount necessary for high-resolution NMR spectroscopy was obtained (typically 10⁴–10⁶ cells for a sample). They were harvested at 95–100% confluence and always 24 h after a final medium change. Cells were detached from culture flasks with a 3-min rinse with Ca²⁺/Mg²⁺-free DMEM (CMF-DMEM) containing 0.54 mm EDTA (Sigma), followed by 5–5 min incubation with a solution of 300 units/ml trypsin (Sigma; bovine pancreas, type III) in CMF-DMEM. One ml soybean trypsin inhibitor-DNase (5200 units/ml soybean trypsin inhibitor, 74 units/ml bovine pancreas DNase I, and 3 mg/ml BSA; Sigma) was added per 10 ml cell suspension to prevent the cells from clumping and to stop the trypsinization process. Cell suspensions were centrifuged at 500 X g for 5 min, followed by a 30-s spin at 700 X g. Cells were washed three times with 30 ml PBS, and the pellet finally was extracted with 2 ml ice-cold PCA (12% v/v; Merck, Ltd.). PCA extracts were sonicated (2 times 30 s, 4°C; Soniprep 150, M/S Scientific Instruments, Crawley, United Kingdom) and then centrifuged at 3000 X g at 4°C for 20 min. The pellets were kept at −20°C for protein quantification, and the supernatants were further treated. At least two PCA extracts were prepared for each human meningeal cell line, and four extracts were obtained from primary cultures of rat meningeal cells (replicate samples).

The pH of the supernatant was adjusted to slightly alkaline (pH > 7.5) and then mixed with 5 mg Chelex-100 (Bio-Rad Laboratories, Richmond, CA) per 0.2 ml sample in order to remove multivalent ion contaminants. A subsequent adjustment of pH to 8.5–8.9 with 3 M KOH was followed by removal of the precipitated KC104 by centrifugation at 3000 X g for 5 min. The sample was lyophilized and redissolved in 0.5–0.7 ml D₂O (Goss Scientific Instruments, Ltd., lngatestone, United Kingdom). As internal concentration and chemical shift standard TSP (Goss Scientific Instruments, Ltd.) was added (10 μl of 10 mM TSP in D₂O). Finally, the pH of the sample was adjusted to 8.9 with DCl or NaOD (Aldrich, Gillingham, United Kingdom).

1H-NMR Spectroscopy

All spectra were obtained at 26°C–30°C on a Varian Unity-plus NMR spectrometer (Varian Associates, Inc., NMR Instruments, Palo Alto, CA) operating at a proton frequency of 500 MHz. Single-pulse spectra (approaching full relaxation) were acquired with 45° pulses applied every 5 s. The sample spinning rate was 20 Hz, spectral width was 5000 Hz, and data size was 32K points. A second radiofrequency pulse was applied at the frequency of the water peak in the delay between acquisitions in order to suppress the residual water (HOD) signal. For a satisfactory signal:noise ratio, 512 or 1024 scans were accumulated and Fourier transformed. A line broadening of 0.2–0.3 Hz was applied for spectral processing.

2D-COSY was also used for identification of coupled resonances. Absolute-value and, where possible, phase-sensitive conventional COSY and double quantum filtered COSY spectra were acquired, either using standard phase cycling or with the application of PFG (20 G/cm; duration 2 ms, rise and fall time of 100 μs) to select coherence pathways (17). Typically, 192 transients were collected for each of 256 T₁ increments (repetition time, 1.2 s; spectral width, 5000 Hz). Water suppression was achieved by presaturation of the HOD peak between acquisition periods as in the one-dimensional spectra. Data were collected into 2K data points in F2, and 1K in F1, which was then zero-filled to 2K. Sine-bell and Gaussian weighting functions were applied during the double Fourier transform, and the COSY plots were symmetrized after initial analysis.

Metabolite Identification and Quantification. Metabolite peaks found in the one-dimensional spectra were identified by: (a) their chemical shift and coupling pattern as described in the literature (1, 18); (b) comparison with spectra of metabolites in known concentrations obtained at the same pH and spectroscopic conditions; and (c) two-dimensional spectroscopic methods.

The following metabolites were identified from their strongest and best resolved resonances: β-hydroxybutyrate -γCH3 1.19 ppm (doublet); threonine -γCH3 1.30 ppm (doublet); lactate -CH3 1.34 ppm (doublet); alanine -CH3 1.47 ppm (doublet); acetate -CH3 1.92 ppm (singlet); NAA -NCOCH3 2.02 ppm (singlet); NAAG -NCOCH3 2.05 ppm (singlet); glutamate -γCH2 2.34 ppm (triplet); succinate -CH2 2.41 ppm (singlet); glutamine -γCH2 2.44 ppm (triplet); aspartate -CH2 2.56 and 2.75 ppm (double doublet); creatine -NCH3 3.04 ppm (singlet); taurine -SCH2 3.08 ppm (triplet) and -NCH2 3.42 ppm (triplet); choline -N(CH3)2 3.21 ppm (singlet); PC -N(CH3)2 3.22 ppm (singlet); GPC -N(CH3)2 3.23 ppm (singlet); glycine -CH2 3.56 ppm (singlet); and inositol (H2) 4.05 ppm (triplet). Metabolite amounts were calculated from their intensities in 1H-NMR spectra by reference to the internal standard TSP after baseline correction. The intensity of a given signal in the proton spectrum is proportional to the amount of compound and number of protons contributing to that signal.

HPLC Analysis of Metabolites

Quantitative determination of amino acids, NAA, and NAAG was performed by HPLC to complement the spectroscopic data. Amino acids were analyzed by derivatization with OPA (19), with the concentrations being calculated from external standards. A LiChroGraph HPLC system (Merck-Hitachi, Darmstadt, Germany) equipped with a LiChrosorb 100HPL 18–22 separation column (5 μm; 4 mm internal diameter, 25-cm length) was used. The OPA derivatives were detected with a UV/VIS fluorimeter (LiChroGraph F1050), characterized by wavelengths of 340 nm for excitation and 450 nm for emission. Aliquots taken from NMR samples were diluted 5–200-fold with ultrapure water, and a 150–250-μl sample was mixed with 350 μl reagent containing 0.8 mg/ml OPA (Sigma). The compounds were allowed to react for 3 min and then 150 μl mixture was injected onto the column. Elution was achieved at 40°C with a three-step linear gradient of methanol in phosphate buffer (50 mM in ultrapure water, pH 6) starting at 20% methanol, increasing to 35% methanol in 5 min, then decreasing to 30% methanol in 30 min, and finishing at 35 min with 20% methanol.

NAA and NAAG were detected with an HPLC System Gold (Beckman Instruments, San Ramon, CA) equipped with a UV detector (wavelength, 210 nm), and their concentrations were calculated from external standards. NMR samples were diluted 1:10 with 5 mM H2SO4, and 50-μl aliquots were injected onto the column. NAA was analyzed by a method described previously (7) using the same conditions. Detection for NAAG was carried out according to Koller et al. (20) using an Anachem SAX (46 mm internal diameter, 25-cm length) column fitted with a 1-cm guard column.

Protein Quantification

Protein quantification was performed on the final PCA precipitate by complexing with BCA (21). The pellet was dissolved in 1 ml of 1 mM KOH by incubation at 37°C for 30 min. BCA reagent (BCA kit, Pierce, Rockford, CA) was further added and the resulting solution was detected after incubation (wavelength, 256 nm; UV/VIS Spectrophotometer Ultraspecll), and protein was calculated by reference to BSA standards (BCA kit, Pierce).
were prepared for each antibody, or combination of antibodies, for each cell line. Tests were carried out in the following combinations: A2B5 and aGalC; aFN; aGFAP; aVim. Figures are given for the start and for the end passage number, where these results differ for the two passage numbers.

Vimentin was expressed by all six meningioma cell lines in all passages, the pror
glycoprotein with adhesive and opsonic properties (12), was widely
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Metabolites Identified in 1H-NMR Spectra and Comparison

with HPLC Results. Representative high-resolution 1H-NMR
spectra obtained from extracts of acid-soluble metabolites from
cultured rat meningeal and human meningioma cells are shown in
Fig. 1. These high-field regions of spectra (0.5—4.5 ppm) contain
signals from a variety of metabolites and present qualitative simi-
larities and also quantitative and qualitative differences. Metabo-
lites detected and quantified include amino acids and related comp-
ounds; substances involved in membrane biosynthesis such as choline, Cho, and inositol; and intermediary metabolites such as succinate and lactate. Metabolite contents (nmol/mg protein) of the cultures examined are given in Tables 2 and 3. In Table 4, selected metabolite ratios are presented for rat meningeal and human meningioma cells. These ratios were chosen because of their rele-
vance either to NMR in vivo (involving creatine, Cho, and inositol) or to previous work (7) identifying metabolite ratios which could
distinguish between preparations of rat meningeal cells and other primary cultures from rat brain (involving Cho, glutamate, aspar-
tate, and creatine). Not all identifiable compounds in the 1H-NMR
spectra could be accurately quantified because of incomplete res-
olution in certain zones of the spectra (e.g., isoleucine, leucine, and valine with signals between 0.9—1.05 ppm). In addition, signals
from some of the identifiable compounds were often too low for accu-
rate quantification.

Within each category of cells (rat normal and human tumor),
spectra were qualitatively identical (i.e., all identifiable peaks were present in all spectra from that category), with the exception of
creatinine, which was not detectable in spectra from one-half of the
meningioma cell lines (see below). Quantitatively, the spectra from
the meningioma cell lines displayed more variability between differ-
ent cell lines (Table 2), but excellent reproducibility was achieved in
replicate samples (i.e., preparations obtained from one cell line).
There was very good reproducibility in replicate preparations from
normal rat meninges. Good agreement was obtained between quanti-
fication from NMR spectra and by HPLC analysis in all samples. The
taurine content of human meningioma cell lines could not be reliably
quantified in the NMR spectra, although it was present at levels
detectable by HPLC. The greatest variability in absolute metabolite
amounts was displayed by glutamate in human meningiomas, but the
data for glutamate from NMR spectra and by HPLC analysis were in
good agreement for individual cell lines.

The identification of coupled metabolites in single-pulse spectra
was confirmed and supplemented by identification of crosspeaks in
2D-COSY spectra. Fig. 2 displays a 2D-PFG-COSY spectrum from one
of the meningioma cell lines.

<table>
<thead>
<tr>
<th>Human meningioma cell line</th>
<th>Start</th>
<th>End</th>
<th>A2B5 (%)</th>
<th>aGalC (%)</th>
<th>aFN (%)</th>
<th>aGFAP (%)</th>
<th>aVim (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IN1067</td>
<td>P6</td>
<td>P9</td>
<td>0</td>
<td>0</td>
<td>29.0 ± 8.7 (P6)</td>
<td>82.0 ± 8.9 (P9)</td>
<td>0</td>
</tr>
<tr>
<td>IN1069</td>
<td>P3</td>
<td>P6</td>
<td>0</td>
<td>0</td>
<td>26.7 ± 3.4 (P3)</td>
<td>58.6 ± 2.6 (P6)</td>
<td>0</td>
</tr>
<tr>
<td>IN1081 (recurrent)</td>
<td>P3</td>
<td>P6</td>
<td>0</td>
<td>0</td>
<td>26.0 ± 4.2 (P3)</td>
<td>78.3 ± 3.3 (P6)</td>
<td>0</td>
</tr>
<tr>
<td>IN1114</td>
<td>P5</td>
<td>P8</td>
<td>0</td>
<td>0</td>
<td>43.1 ± 5.1 (P5)</td>
<td>80.1 ± 8.5 (P8)</td>
<td>0</td>
</tr>
<tr>
<td>IN1123</td>
<td>P3</td>
<td>P7</td>
<td>0</td>
<td>0</td>
<td>32.2 ± 6.4 (P3)</td>
<td>67.0 ± 5.6 (P7)</td>
<td>0.5 ± 0.5 (P3)</td>
</tr>
<tr>
<td>IN1239 (spinal)</td>
<td>P3</td>
<td>P7</td>
<td>0</td>
<td>0</td>
<td>31.5 ± 6.4 (P3)</td>
<td>79.5 ± 14.9 (P7)</td>
<td>0</td>
</tr>
</tbody>
</table>

* aGalC, anti-galactocerebroside; aFN, anti-fibronectin; aGFAP, anti-gliarial fibrillary acidic protein; aVim, anti-vimentin.
Table 2. Comparative composition of metabolites quantified from 1H-NMR spectra of rat meningeal and human meningioma cells

Metabolite concentrations (nmol/mg protein) are expressed as mean ± SD. Spectra obtained from replicate preparations for each of the independent observations for human meningioma cell lines and rat meningeal cells, respectively, were analyzed by reference to TSP. P obtained from two sample Student’s t test are given without correction for the number of comparisons. The results for normal cells were considered statistically different from the results for tumor cells if \( P < 0.05/13 \) (5% level) or \( P < 0.01/13 \) (1% level).

<table>
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<tr>
<th>Metabolites</th>
<th>Rat meningeal cells (n = 4)</th>
<th>Human meningioma cell lines (n = 6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>17.8 ± 3.8</td>
<td>22.5 ± 7.1</td>
<td>0.264</td>
</tr>
<tr>
<td>Arginine</td>
<td>2.0 ± 0.9</td>
<td>3.1 ± 2.3</td>
<td>0.026</td>
</tr>
<tr>
<td>Asparagine</td>
<td>1.4 ± 1.0</td>
<td>4.9 ± 1.9</td>
<td>0.018</td>
</tr>
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<td>Aspartate</td>
<td>20.3 ± 0.4</td>
<td>27.0 ± 14.6</td>
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<td>GABA</td>
<td>5.9 ± 0.8</td>
<td>6.6 ± 0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Glutamate</td>
<td>43.6 ± 5.6</td>
<td>72.9 ± 23.6</td>
<td>0.145</td>
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<tr>
<td>Glutamine</td>
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<td>305.5 ± 47.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hypotaurine</td>
<td>19.4 ± 2.9</td>
<td>5.0 ± 3.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NAA</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>NAAG</td>
<td>30.0 ± 1.5</td>
<td>4.5 ± 3.9</td>
<td>0.474</td>
</tr>
<tr>
<td>Serine</td>
<td>18.0 ± 1.9</td>
<td>28.6 ± 5.9</td>
<td>0.018</td>
</tr>
<tr>
<td>Taurine</td>
<td>38.4 ± 4.2</td>
<td>18.0 ± 2.8</td>
<td>&lt;0.001</td>
</tr>
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<td>Tyrosine</td>
<td>2.3 ± 0.6</td>
<td>10.3 ± 2.1</td>
<td>&lt;0.001</td>
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The amount of NAA was too low to be detected by HPLC investigations in five of the six meningioma cell lines we examined, but one meningioma cell line (IN1239) contained a very low amount of NAA (2.9 ± 1.7 nmol/mg protein).

Table 3. Comparative composition of metabolites determined by HPLC analyses in rat meningeal and human meningioma cells

Metabolite concentrations (nmol/mg protein) are expressed as mean ± SD of the independent observations for rat meningeal cell preparations and for human meningioma cell lines. P obtained from two sample Student’s t test are given without correction for the number of comparisons. The results for normal cells were considered statistically different from the results for tumor cells if \( P < 0.05/13 \) (5% level) or \( P < 0.01/13 \) (1% level).

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Common Features of the Spectra of Rat Meningeal and Human Meningioma Cell Extracts. In the light of existing data on metabolites detectable by 1H-NMR in whole brain extracts and cell preparations from either rat or human brain (5, 7, 23), we note that spectra from both rat meningeal cells and human meningioma cell lines were characterized by either very low or not detectable signals for NAA (2.02 ppm, singlet), NAAG (2.05 ppm, singlet), and the neuroactive amino acid GABA (3.0 ppm, triplet). NAA was not detectable by NMR but was detectable by chromatographic analysis. Complementary determinations by HPLC showed statistically
significant differences at the 1% level in the concentration of amino acids such as GABA, hypotaurine, and tyrosine in extracts of rat meningial cells compared to human meningioma cells.

Spectra of preparations from rat meninges and human meningioma cell lines had common features in the 1.0—1.5 ppm region, displaying the doublet signals of β-hydroxybutyrate, threonine, lactate, and alanine. The concentrations of none of these metabolites were significantly different between rat normal and human tumor cells. Relative to 1H-NMR spectra in vivo, lactate signals were elevated in the spectra obtained from cultured cells, probably due to anaerobic glycolysis taking place in cells during the extraction process. Lactate content was highly variable in the cell preparations (from 25 to 230 nmol/mg protein), depending on the rapidity of cell extraction and on the dimension of the cell pellet. The signals for alanine were relatively high in spectra from preparations of both categories of cells compared to the typical levels of alanine detected by spectroscopy in vivo in normal human brain (5, 23).

In the methylene region of the spectra (2—3 ppm), common signals for both types of cells included those of glutamine and succinate. No statistically significant differences were obtained in the concentrations of these metabolites between the preparations of rat meningial and human meningioma cells. There was a large variation in the glutamine concentration of tumor cells, ranging from 29.3 nmol/mg protein for one cell line up to 105.0 nmol/mg protein for another, but the averaged glutamine content in human meningiomas was not statistically significantly different from the value for rat meningial cells.

The methine region of the spectrum (3.2—4.0 ppm) had some common general characteristics for both types of cells examined, including a relatively high signal for glycine and relatively low signal for inositol. No significant difference in the inositol content determined from the 1H-NMR spectra was obtained between normal and transformed cells. We did not have a reliable independent assessment for glycine and threonine content in samples because their separation on the HPLC column was poor. Allowing for correction of the Ps for the number of comparisons, the difference in threonine and glycine contents of rat meningial and human meningioma cells was not significant at the 5% level.

**Table 4 Metabolite ratios in rat meningeal and human meningioma cells**

<table>
<thead>
<tr>
<th>Metabolite ratios</th>
<th>Rat meningeal cells (n = 4)</th>
<th>Human meningioma cell lines (n = 6)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cho/creatinine</td>
<td>1.12 ± 0.18</td>
<td>5.5 ± 2.9</td>
<td>0.019</td>
</tr>
<tr>
<td>creatine/inositol</td>
<td>3.2 ± 0.9</td>
<td>0.63 ± 0.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>aspartate/creatinine</td>
<td>1.2 ± 0.3</td>
<td>5.6 ± 3.3</td>
<td>0.024</td>
</tr>
<tr>
<td>Cho/aspartate</td>
<td>0.7 ± 0.1</td>
<td>1.3 ± 0.7</td>
<td>0.120</td>
</tr>
<tr>
<td>Cho/glutamate</td>
<td>0.12 ± 0.02</td>
<td>0.1 ± 0.05</td>
<td>0.217</td>
</tr>
<tr>
<td>Cho/glycine</td>
<td>1.2 ± 0.4</td>
<td>1.7 ± 1.6</td>
<td>0.277</td>
</tr>
<tr>
<td>glycine/creatine</td>
<td>0.78 ± 0.23</td>
<td>5.9 ± 4.6</td>
<td>0.063</td>
</tr>
</tbody>
</table>

Differential Characteristics in Spectra of Normal Rat Meninges and Human Meningiomas. There were also differences in the 1H-NMR spectra obtained from cell extracts of cultured rat meninges and human meningiomas. For example, a prominent compound in preparations of meningial cells obtained from rat was taurine, a metabolite which was either not detected by NMR in meningioma extracts, or which gave very low signals in spectra. Quantification of taurine in spectra obtained from extracts of human transformed cells was not possible due to overlap of the creatine signal at 3.04 ppm with one of the taurine peaks (3.08 ppm, triplet) and because of the complexity of the 1H spectrum where the second taurine signal lies (3.42 ppm, triplet). Taurine was among the most abundant free amino acid detected by HPLC (Table 3) in rat meningial cells and was present in amounts significantly different (P < 0.001) from those existent in human meningiomas.

Among other signals in the high field region, the singlets at 3.04 ppm and 3.94 ppm, which are assigned to creatine, were more prom-
inent in $^1$H-NMR spectra from normal rat meninges than in spectra from human meningioma cell lines. The creatine peak in vitro represents the sum of creatine and phosphocreatine present in the living cells, since hydrolysis of phosphocreatine to creatine occurs during the cell harvesting and extraction process. Spectra from preparations of three meningioma cell lines were characterized by the absence of creatine peaks; in two other cell lines, the creatine levels were below the lowest amount in any of the meningioma preparations; in one cell line, the creatine level appeared relatively "normal" (16.9 nmol/mg protein). However, overall there was a significant difference ($P = 0.002$) in the creatine content of rat normal and human transformed cells. The creatine:inositol metabolite ratio was also significantly different at the 1% level in meningioma compared with meningeal cells (Tables 2 and 4). The signals for glutamate were relatively higher in spectra of human tumor cells than in those of normal rat cells. This was also reflected, by HPLC, in the significantly elevated ($P < 0.001$) content of glutamate in human meningioma lines (305.5 ± 47.4 nmol/mg protein) versus normal rat meningeal cells (105.4 ± 2.6 nmol/mg protein), and related to this, in a significantly different ($P < 0.001$) glutamate:alanine ratio.

Peaks from Cho were prominent in spectra of both normal and tumor cells. The signals in the 3.2–3.3 ppm region are assigned to choline itself (3.21 ppm, singlet), PC (3.23 ppm, singlet), and GPC (3.24 ppm, singlet); these metabolites were identifiable in the spectra of both categories of cells. Other metabolites, including carnitine, ethanolamine, and phosphoethanolamine might also have contributed to signals in this region. A significant difference at the 5% level ($P = 0.004$) was detected in total Cho (sum of the three peaks) in rat meningial compared to human meningioma cell extracts (Table 2).

The relative heights of individual peaks contributing to the Cho signal in the two categories of cells were also different. There was a significant increase ($P < 0.001$) in the amount of PC in human meningiomas compared to rat meninges. A shift was observed in the PC:GPC ratio from below one in rat meningeal cells to greater than one in the human meningioma cell lines. PC is synthesized in vivo in the first step of phospholipid biosynthesis, whereas GPC is a phosphodiester breakdown product of phospholipids. The predominance or otherwise of one kind of phosphoesters in the composition of the Cho peak in these spectra may indicate differences in phospholipid metabolism in meningioma cells of human origin versus meningial cells from rat.

**DISCUSSION**

We used cell culture techniques and investigations by $^1$H-NMR spectroscopy complemented with HPLC analysis in order to compare metabolite profiles of normal rat meningeal cells and human meningioma.

**Meningioma Cell Lines in the Light of Their Immunocytochemical Characteristics and Cell Types of the Meninges.** All cell types of meningeal tissues covering the brain and spinal cord originate in the primitive meninx derived from the layer of mesenchymal tissue that comes to surround the neural tube in the early development of the embryo (24). Meningiomas, as primary tumors of the meninges composed of or differentiating towards arachnoidal cells (25), can involve dura mater, leptomeninges, or both and account for about 15% of all human brain tumors. Tumors of the meninges display a high phenotypical variability in cell products, shape, pattern, and stroma, but equally, human meningiomas of various origins have similarities in their immunocytochemical characteristics, histological features, metabolism, behavior, etc. (11, 13, 25). The results we obtained were in agreement with these latter observations of commonalities among meningiomas.

Our findings related to fibronectin and vimentin expression of meningioma cell lines were in concordance with previous studies on human intracranial meningiomas. It has been shown (12) that neoplastic cells retain the intermediate filaments of their tissue of origin and that virtually all meningeal neoplasms have a uniform distribution of fibronectin filaments. Additionally, coexpression of vimentin and desmosomal proteins and the association of this type of intermediate filaments to desmosomal plaque proteins are a combination unique to meningioma cells (13). The negative GFAP labeling obtained in the meningioma cells was in agreement with their cell of origin and their usual localization outside brain tissue (10, 24).

**Interpretation of HPLC Results and NMR Findings.** Although there were quantitative variations for certain metabolites between the human meningioma cell lines, qualitatively identical $^1$H-NMR spectra were obtained from all six cell lines we examined. This fact suggests a preservation of the characteristic metabolic profile within the type of tumor, irrespective of its origin.

Spectra from preparations of rat meningial cells displayed obvious similarities to those we have reported previously from extracts of primary cultures of rat meningial cells (7). These results confirm the reproducibility of cell culture techniques, metabolite extraction procedures, and NMR spectroscopy investigations. Although cell culture procedures and methods of extract preparation could be a source of variability (1,26), in studies such as ours, these potential problems can be readily overcome if factors including composition of the culture medium, degree of cell culture confluence, time of harvesting, and method of extraction are carefully controlled.

We have shown previously that rat meningial cells can be distinguished from other primary cultures of central nervous system rat cells (neurones, astrocytes, oligodendrocyte-type-2-astrocyte progenitors, and oligodendrocytes) on the basis of $^1$H-NMR spectra of extracts (7). The distinguishing features were the presence of high amounts of succinate and β-hydroxybutyrate, the absence of NAA and hypotaurine, and relatively low amounts (compared to oligodendrocytes) of creatine. These characteristics were shared by all the human meningiomas we examined, with no significant differences in the succinate and β-hydroxybutyrate concentrations between rat meningial cells and human meningioma cell lines, and an even lower creatine content in the meningioma preparations. In addition, the metabolite ratios Cho:aspartate and Cho:glutamate, which were used previously to discriminate rat meningial cells from rat astrocytes, do not differ significantly between rat meningial and human meningioma cells (Table 4). The aspartate:creatine ratio, which was more than 10-fold higher in rat meningial cells compared to cortical rat astrocytes, was even higher in human meningioma cell lines, reflecting their lower creatine content.

Although the key features described above clearly relate human meningioma cells to their tissue of origin even when considered across these widely separated species, there were also a number of characteristics which discriminated the transformed from normal cells. These statistically significant features of the human meningioma metabolite profiles were reductions in creatine and taurine and increases in Cho (attributable mainly to increased PC) and glutamate. Taurine is known to be much lower in human brain than in rat brain (5, 27); therefore, it is possible that the reduced taurine in the meningioma cell lines reflected a species difference rather than an effect of transformation. Although it is not yet known whether the dissimilarities in other signals reflect differences across species or differences between normal and transformed cells, arguments can be raised that at least some of these changes may be associated with transformation.

A feature of human meningioma cell lines that we examined was...
their high content of glutamate, consistently 2.5- to 3-fold higher than rat meningeal cells. As previous studies (24) have demonstrated that human meningiomas in culture express a relatively low uptake of glutamate (lower even than normal human fibroblast cultures), the question arises as to how the high glutamate content in meningioma cells might be achieved. A variety of observations raise the possibility that such an elevation would be a predicted consequence, both of enzymatic alterations characteristic of brain tumors and of the high alanine content of primary meningeal cells (7). We propose the following hypothesis to explain the high glutamate levels observed in our human meningioma cultures.

One of the enzymes involved in the glycolytic pathway in eukaryotic cells is PK, which catalyses the transfer of phosphate from phosphoenolpyruvate to ADP, yielding free pyruvate and ATP. PK is "turned off" when ATP, or other fuels including alanine, are present in high concentration in the cell, and it "turns on" when there is a buildup of the preceding glycolytic intermediates, especially phosphoenolpyruvate (28). It has been shown (24) that this enzyme is present both in normal brain and brain tumors mostly as the muscle type (M), in the form of two subgroups: M1, which is not inhibited by alanine in its action; and M2, which is inhibited by alanine. The M1 form is present in normal brain, while the M2 form is predominant in tumors. These observations, considered in the light of the results of our analyses of amino acids, especially alanine and glutamate, in rat normal and human tumor meningeal cells, raise the possibility that there might be a relationship between the relatively high alanine content of both meninges and meningiomas and PK in these cells. It might be that in normal meningeal cells, PK (the M1 form which is not inhibited by alanine) will be active, while in meningioma cells, the M2 form of PK which is inhibited by alanine is "turned off." Such an inhibition of PK would lead to a lack of production of pyruvate in the glycolytic process and accumulation of phosphoenolpyruvate. Alternatively, pyruvate could be generated in the tumor cells via the transamination reaction from alanine and α-ketoglutarate, yielding glutamate in addition to pyruvate. The presence of this reaction would account for the buildup of glutamate in meningioma cells without uptake from the culture medium. This reversible transamination process could also replenish alanine. Our results showing that human meningiomas display a constant glutamate:alanine ratio, which is also significantly different from the ratio for rat meningeal cells, are consistent with this suggestion. Subsequently, when phosphoenolpyruvate has accumulated in meningioma cells in high amounts, it will "turn on" the PK (M2 form), and glycolysis will follow its normal path. This hypothesis is also consistent with observations that normal allosteric factors regulating the glycolytic rate to that of the tricarboxylic acid cycle are inactive or altered in transformed cells (28).

Our results showed that the PC peak was predominant amongst signals contributing to Cho in spectra from all six human meningioma cell lines we examined, as opposed to a predominant GPC peak in the 3.2 ppm region of the 1H-NMR spectra obtained from rat meningeal cells. This finding was consistent with the idea that increased levels of phosphomonoesters (e.g., PC and phosphoethanolamine) may be associated with intensified cell membrane synthesis and increased rate of cell replication and have been, therefore, proposed, as an indicator of cell proliferation (29).

Our findings showing raised amounts of Cho in preparations from human meningiomas compared to extracts from rat meningeal cells were also in agreement with the outcome of studies performed on human meningiomas in vivo and on human biopsy extracts. Clinical studies by 1H-NMR spectroscopy on humans have detected an increase of Cho levels in meningiomas compared to normal contralateral brain (30), but the differences in distribution of individual peaks contributing to the Cho signal (i.e., phosphomonoesters and phosphodiesters) cannot be detected due to the decreased spectral resolution achievable in NMR spectroscopy in vivo. 31P-NMR spectroscopy has showed elevated phosphomonoester signals in untreated meningiomas of human patients in vivo (31, 32), as well as in spectra from PCA extracts of resected human meningiomas (32).

In the comparison of NMR spectra from extracts to those obtained in vivo, it is important to appreciate that metabolite levels may differ because certain metabolite pools may be "NMR-invisible" in vivo. There is little evidence to suggest that most of the major metabolites detected by 1H-NMR spectroscopy are to any extent "invisible," in contrast to the situation for 31P-NMR spectra in vivo in which ADP and inorganic phosphate certainly contain NMR-invisible pools (33). An exception to this is glutamate, which is about 80% NMR-visible in rat brain (34) in vivo or in guinea pig brain slices (35). It has been suggested that the invisible pool is neurotransmitter glutamate (36), in which case interpretation of our data from nonneuronal preparations would be unaffected.

The 1H-NMR profiles we obtained from cell populations derived from human meningiomas were very similar to the 1H-NMR spectra that have been reported (30) for extracts of meningiomas obtained from human biopsy samples. These similarities included relatively high signals for alanine and Cho, a reduced signal for creatine, and low or not detectable signals for neuroactive amino acids and related compounds such as GABA, NAA, and NAAG. The low or not detectable levels of such compounds (which are present in analyses of human central neural tissue) were consistent with the embryonic derivation of the meninges and the location of meninges and meningiomas outside of the central nervous system.

Metabolic regulation in cultured cells may diverge compared to the situation in vivo. Furthermore, such changes may be different in normal cells and in transformed cells. However, all of the features that we observed in spectra from preparations of cultured meningioma cells in vitro (see above) were in agreement with data reported (23,30) by noninvasive NMR spectroscopy on meningiomas in vivo. Such a consistency lends support to the ideas that: (a) the metabolic pattern in vivo is generally preserved by cells in culture when culture conditions are carefully controlled; and (b) that meningioma cell lines may offer a suitable model for studying at least some of the properties of their parental tumors.

In conclusion, our investigations showed that human meningioma cell lines derived from excised primary meningiomas expressed similar characteristic metabolic profiles, even when isolated from different patients. Furthermore, both rat meninges and human meningioma cell lines shared a number of features, which makes them distinguishable from other cells of the central nervous system. It is interesting to note the conservation of metabolites such as β-hydroxybutyrate, alanine, glutamine, succinate, inositol, threonine, and maybe glycine across species and across transformation from normal to tumor tissue. Additionally, we identified certain characteristics such as Cho, PC/ GPC, creatine, glutamate, and taurine which enabled the spectra from the human transformed cells to be distinguished from those of normal rat meningeal cells. If these differences prove to be due to metabolic alterations in transformed cells rather than due to species differences, they might enable neoplastic and normal tissues to be distinguished noninvasively by 1H-NMR spectroscopy. Together, our findings raise the possibility of identifying meningiomas in vivo by performing noninvasive 1H-NMR spectroscopy on patients.

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