The Relationship between Nuclear Magnetic Resonance-Visible Lipids, Lipid Droplets, and Cell Proliferation in Cultured C6 Cells

Ignasi Barba, Miquel E. Cabañas, and Carles Arús

Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, 08193 Cerdanyola del Vallès, Spain

ABSTRACT

There is an ongoing controversy about the subcellular origin of the fatty acyl chains that give rise to the NMR visible mobile lipids (MLs) resonance at -1.24 ppm in the H spectra of cells and solid tumors. Some groups have been supporting the hypothesis that triglycerides originating MLs are isotropically tumbling in small membrane microdomains, whereas other authors back the proposal that they are inside cytosolic or extracellular (necrotic areas) lipid droplets. Furthermore, MLs are frequently present in in vivo spectra recorded from human brain tumors, but the meaning of this detection is not fully clear.

We have addressed the possible contribution of intracellular droplets to the ML pattern recorded from human brain tumors in vivo by studying cultured C6 rat glioma cells as a model system for astrocytic tumors. We show here that cultured C6 cells display ML resonances in high field (9.4 T) 1H NMR spectra recorded at 136 ms echo time when grown at saturation density conditions, but no MLs are visible for log-phase cells. Fluorescence microscopy analysis of cells stained with the lipophylic dye Nile red shows intracellular spherical yellow-gold droplets containing neutral lipids; cells at saturation density present lipid droplets of diameters about 1.6 µm in most cells (85%), whereas they are almost absent in log-phase cells (only 6% of the cells contain them). Furthermore, log-phase cells can be induced to display MLs and accumulate Nile red-positive droplets by culturing them for 24 h at pH 6.2. This acid pH effect can be fully reversed by 24 h of standard media incubation. Lipid droplet volume calculated from fluorescence microscopy preparations in an average cell is different for both culture conditions (2.2 times higher volume for saturation density than for pH-stressed cells). This difference in lipid droplet volume is reflected by a different ML peak height at 1.24 ppm (about 2 times higher for saturation density than for pH-stressed cells). Flow cytometry analysis shows that both culture conditions result in a slowing down of the proliferation rate of the cells.

The fact that MLs are found to originate in lipid droplets inside cells that are growth compromised but still viable suggests that changes in the proliferative state of tumor cells, in the absence of necrosis, may be detected non invasively by in vivo NMR spectroscopy.

INTRODUCTION

Nuclear magnetic resonance spectroscopy is a noninvasive methodology that is increasingly applied to the diagnosis and prognosis of human brain tumors (1–8). In some tumors, the spectral features that differ most between normal brain parenchyma and tumoral masses are the resonances arising from fatty acyl chains of lipids, which we will call MLs.3 It is important to understand the origin of these MLs, so that the full potential of NMR can be exploited for diagnosis, prognosis, therapy planning, and follow-up.

There has been some controversy as to the subcellular origin of the compounds giving rise to ML resonances. In vitro studies on human brain tumor postsurgical biopsies (9) have related the observation of MLs to the extent of necrosis present in the biopsy sample as measured by routine histopathology staining (H&E). Furthermore, studies on an animal model of human brain glioblastoma, a rat brain glioma induced by stereotactic injection of C6 cells, showed that MLs originated in vivo from large (diameter >2.6 µm) triglyceride-containing lipid droplets found in necrotic areas (10–12). Such accumulation of lipid droplets has also been observed by electron microscopy in necrotic areas of human brain tumor biopsies (13). Nonetheless, Kuesel et al. (14) reported tumor spectral patterns displaying MLs in biopsies where no apparent necrosis was present. This could be interpreted as caused by very small necrotic foci not detected by the staining procedure or alternatively could suggest a nonnecrotic origin of the MLs. This nonnecrotic origin of MLs in certain cases would agree with a number of studies carried out on various cell systems (10, 15–19) for which MLs have been observed from otherwise viable cells. A study using transformed murine fibroblast cells showed that cell culture conditions, confluence, serum deprivation, and acidic extracellular pH (pHe 6.1) could all induce the appearance of MLs in the spectral pattern recorded, although the subcellular origin of the ML resonances was not addressed in this work (20). An increase in MLs upon treatment of a human malignant breast cell line (DU 4475) with a cytotoxic compound was also reported (21), although the subcellular origin of the ML resonance was not clarified. Furthermore, work on a human leukemia cell line (K562wt) and its Adriamycin-resistant derived cell line (K562adr) showed differences in ML content between both cell lines and changes induced upon passage of those cell lines in the presence or absence of Adriamycin (22). The same authors also showed Nile red-positive, lipid droplet-containing cells in the cell lines studied, although they concluded that no correlation could be found between MLs and lipid droplet detection in their cellular system (23). Finally, Knijn et al. (24) have shown that H-ras transformation of NIH-3T3 mouse embryo fibroblasts, which makes the cell line tumorigenic in mice, decreases the ML pattern observed for the parental cell line (nontumorigenic). These results would suggest that transformation would decrease MLs. On the other hand, Delikatny et al. (25) suggested that MLs could correlate with tumoral aggressiveness. In summary, the origin and especially the meaning of ML detection in tumoral samples are still not fully understood.

We think that it is going to be important to clarify not only the experimental conditions at which MLs in cells and in tumors appear but also the tissue or subcellular location to fully understand their relevance to the cellular biology of the tumor. For example, do MLs in tumor cells originate from isotropically tumbling triglycerides in small microdomains (25–28 nm in diameter) embedded in the plasma membrane as was proposed some time ago by Mountford et al. (16, 17), or do they originate from large (>500 nm) intracellular/extracellular lipid droplets, as suggested by others (10, 19)? What is the biochemical origin of the fatty acyl chains in MLs? Do they originate from a halt in cell proliferation and a temporary detour of the phospholipid biosynthetic pathway at the level of diacylglycerol toward triacylglycerol (anabolic origin), or do they originate from fatty acids released after membrane phospholipid degradation upon cell death processes, necrosis, and/or apoptosis (catabolic origin)? Do both
origins coexist, and, if so, can we differentiate MLs coming from the different origins?

In this report, we describe our attempt at answering some of the questions outlined above with a well-characterized cell line, the C6 cells. This cell line was initially obtained from a chemically induced rat brain glioma (26, 27) and can be used as a model for human brain glial tumors, the most abundant type of primary brain tumor.

For this purpose, we have characterized the growth curve of C6 cells under various culture conditions and followed ML pattern changes in their NMR spectra, the appearance and volume of intracellular lipid droplets by optic fluorescence microscopy and cell proliferation status by cytofluorometry. We will show in this work that the increase in ML intensity correlates with cell proliferation arrest and that ML changes closely follow intracellular lipid droplet volume change. The relevance of our results to in vivo studies of human brain tumors will be discussed.

MATERIALS AND METHODS

Cell Culture Technique. C6 cells (European Collection of Animal Cell Cultures, Salisbury, United Kingdom) were grown in 150-cm² plastic flasks (TPP, Barcelona, Spain) using DMEM-F12 medium (Sigma, Madrid, Spain) containing 10% (v/v) FCS (Life Technologies, Inc., Barcelona, Spain) and antibiotics, 1000 units of penicillin and 10 µg/ml streptomycin (Sigma). Standard medium pH was adjusted between pH 7.2 and 7.4 with 1 N NaOH.

Cells were maintained at 57°C in a sterile air atmosphere containing 5% CO₂ in a NAPCO incubator (NAPCO, Chicago, IL). Cells were plated at the same initial concentration as described in the growth curve (see below); the medium was exchanged routinely every 2 days.

The growth curve was characterized by measuring the number of cells during 9 days at 24-h intervals. Cells were seeded in plastic round Petri dishes (6 cm in diameter; TPP) at an initial concentration of 1.5 × 10⁵ cells/cm². Cell number and viability, as denoted by trypan blue exclusion, were counted on a Neubauer chamber four times for each of the three samples assayed for every time point. Trypan blue exclusion was measured as follows. Cell suspension (about 10³ cells) was mixed with trypan blue at a final 0.125% w/v concentration. Cells were maintained at 37°C in a sterile air atmosphere containing 5% CO₂ by the standard medium. Measurements on acid pH-recovered cell cultures were carried out after 24 h of standard media incubation.

Low pH Experiments. Low pH (≤6.2) experiments were performed by exchanging the standard medium by a pH 6.2 adjusted medium during 24 h before the NMR experiment. The only difference between the standard pH and the acid pH media was on the sodium bicarbonate (Sigma) content initially present in the media (1.2 g/l for the standard pH medium or 0.5 g/l for the acid pH medium). pH recovery experiments were carried out by exchanging the acid pH media by the standard medium. Measurements on acid pH-recovered cell cultures were carried out after 24 h of standard media incubation.

PCA Extraction. PCA extraction was carried out essentially as described for other tissues (28). Briefly, the cell pellet used for NMR (see below) was frozen at liquid nitrogen temperature and powdered by percussion with a mortar and pestle. Five ml of ice-cold 0.5 M PCA were added to the ground frozen at liquid nitrogen temperature and powdered by percussion with a cell cultures were carried out after 24 h of standard media incubation.

Statistical Analysis. Statistical comparison of peak height ratios and cell cycle fractions was carried out using a two-tailed Student’s t test. Significance level was set at P < 0.05.

Nile Red Staining. Nile red staining was done essentially by following the protocol described by Greenspan et al. (33). About 10⁵ cells were directly stained with 1 ml of 0.1 µg/ml final concentration, of the fluorescent stain Nile red (Sigma) in PBS (prepared by dilution of a stock solution which was 0.1 mg/ml in acetone) for 5 min. The solution of Nile red in PBS was prepared just before its use. Excess of Nile red was washed away once with 1 ml of PBS. In some cases, cells were grown on microscopy cover-slides and stained without detaching for better observation of cellular morphology. Sample observation was carried out as soon as possible after its preparation, always in <1 h. Samples were kept in the dark until being observed.

NMR Spectroscopy. Cells were harvested by trypsinization. For this, the culture flask was washed once with PBS, then 0.5 ml of trypsin-EDTA stock solution was added (0.5 g of trypsin and 0.2 g of EDTA in 1 liter of HBSS (Sigma), the minimum amount of solution needed to wet the whole culture flask surface. The cells were centrifuged for 3 min at 425 × g at 4°C and washed once with PBS-3H₂O at pH* 7.2 or 6.2 (pH* is the pH meter reading uncorrected for the deuterium isotope effect), depending on the pH of the medium in which the cells had been cultured. This was done to facilitate water resonance suppression. A cell pellet of about 50 × 10⁶ cells was resuspended in 500 µl of PBS-3H₂O. This volume of cells was enough to fill the sensitive volume of the observation coil in the 5-mm probe.

NMR spectra were recorded on a Bruker AM-400-WB spectrometer (Bruker Spectrospin, Wissembourg, France) at 35 ± 1°C. In a previous experiment (results not shown), it was observed that the recording temperature had a reversible effect on the apparent intensity of the lipid peak at 1.24 ppm, being more intense at 35°C than at 21°C. Accordingly, all subsequent experiments were carried out at 35°C to mimic more closely the experimental conditions that are found in tumors in vivo.

A coaxial glass capillary (internal diameter, 0.8 mm; external diameter, 1.1 mm) filled with 10 mM 2,2,3,3-tetradeo-tetramethyl-silylpropionate, sodium salt; Sigma) at pH* 7.8 in 3H₂O was used as an external chemical shift reference at 0.00 ppm.

The pulse sequence used to record cell pellet spectra combined water presaturation (1 s, 2 mW) with a spin-echo sequence (30), where the first pulse had been replaced by a "jump and return" (31): Presaturation → 90° − τ → TE/2 → 180° − TE/2 → Acquisition τ was set at 184 ms, which gave an excitation maximum at 1.24 ppm, the echo time (TE) was set at 136 ms, and the total repetition time was 2.8 s. Data were acquired over a 5000 Hz sweep width and digitized with 4096 data points and were the result of the accumulation of 128 scans. Total acquisition time was ~6 min.

The whole experiment, cell preparation and NMR spectroscopy, lasted 60–75 min after the addition of PBS to the cell culture flasks before trypsinization. Cell viability, as measured by trypan blue exclusion, was always above 95% after trypsinization and above 80% after NMR spectroscopy.

Spectra of PCA extracts were acquired with a pulse-and-acquire sequence (90° pulse), with presaturation of the water signal during 1 s at 2 mW; the total delay between pulses was set to 10 s to allow for full relaxation of the metabolites of interest (32). Spectra were the result of the accumulation of 128 scans with 16,386 data points. 2,2,3,3-Tetradeo-tetramethyl-silylpropionate, sodium salt was added to the lyophilized material as an internal standard at a final concentration of 1 mM. In one case, the same pulse sequence and acquisition parameters used for the cell pellet spectra acquisition were applied to the PCA extract of saturation density cells, with the only change being that 16,386 data points were used to digitalize the free induction decay.

Cell pellet spectra were processed using the Bruker Software Win-NMR (Bruker). A line broadening of 1 Hz was applied to the free induction decay prior to Fourier Transform; only zero order phase correction was applied to avoid baseline roll-up. Cell pellet spectra were characterized quantitatively by measuring the height of different peaks, e.g., corresponding to lipids at 0.93 and 1.24 ppm, lactate at 1.34 ppm, and creatine at 3.05 and 3.95 ppm.

PCA spectra were processed in the same mode as cell pellet spectra except that only 0.2 Hz line broadening was applied. Resonance areas were measured by deconvolution of the resonances of interest with the Win-NMR software (Bruker).

Sample observation was carried out as soon as possible after its preparation, always in <1 h. Samples were kept in the dark until being observed.
Nile red-stained cells were studied with a Leica DMRB fluorescence microscope (Leica, Barcelona, Spain) equipped with a Hamamatsu C-5310 color chilled CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Droplet size measurements were done with Q500MC software (Leica) on yellow-gold fluorescence images. Red fluorescence was observed after excitation at 515–560 nm at an emission wavelength of 580 nm using a fluorescein filter, whereas yellow-gold fluorescence was observed at 580 nm, using a rhodamine filter, after excitation at 510 nm (33). Images were recorded at different integration times, between 0.2 and 2 s, depending on the fluorescence intensity of the subcellular structures being stained. The smallest pixel size usable to digitalize the image was 172 × 172 nm due to the digital resolution of the CCD camera. This fact sets a lower limit (172 nm of diameter) to the resolution attainable in the measurement of the size of the subcellular structures being observed.

Flow Cytometry. After the NMR measurements, cells were treated for flow cytometry analysis. They were washed twice in PBS and then stained with 2 ml of a solution containing 50 μg/ml propidium iodide (Sigma), 0.2% Triton X-100, and 0.2 mg/ml RNase A (Sigma) for 30 min in the dark prior to filtration through an 80 μm mesh filter. Between 10 × 10^6 and 12 × 10^6 cells were measured using an EPICS Profile II (Coulter Electronics, Hialeah, FL) flow cytometer. The percentages of cells in the different phases were calculated with the software provided by the manufacturer, considering diploid cycle and correcting for cell clusters.

RESULTS

C6 Cells Cultured under Standard Conditions

Growth Curve. Fig. 1 shows the growth curve for the C6 cells. The cells reproducibly reached saturation density (with significant slowing down of the growth rate) 1 week after seeding under our experimental conditions; this was between 48 and 72 h after the culture flask surface was completely covered (confluence). The doubling time was 18 h in the log phase of the growth curve (4 days after seeding).

NMR Spectroscopy. The 1H NMR spectral pattern of C6 cells changes in a reproducible way with the time point of the growth curve (Fig. 2). At saturation density, clear peaks can be seen at 0.93 ppm (methylene groups of fatty acyl chains; Lac, lactate; Cr, creatine, and TMA, trimethylamine group containing compounds) and at 1.24 ppm (Fig. 3). At saturation density, 1 week after seeding. Tentative assignments for the major contributors to the peaks as follows: Lip1, methyl group of fatty acyl chains; Lip2, methylene groups of fatty acyl chains; Lac, lactate; Cr, creatine, and TMA, trimethylamine group containing compounds. Arrows mark the spectral points used for the peak height ratio measurements shown in Table 1. Figures were scaled to display similar heights for the creatine peak at 3.05 ppm.

VALUES ARE PRESENTED AS MEAN RATIO ± SD.

The ratio between creatine at 3.05 ppm and TMA at 3.18 ppm does not change significantly between the two phases of the growth curve studied by NMR.

The creatine concentration in cells at both time points of the growth curve was measured in spectra from PCA extracts (Fig. 3). We found 8.9 ± 0.4 nmol/mg of protein (n = 2) for log-phase cells and 10.5 ± 2.3 nmol/mg of protein (n = 4) for saturation density cells. These values were not statistically different. Accordingly, ratios calculated in Table 1 using the creatine peaks height as a reference should reflect relative changes of the concentration and NMR visibility of the other compounds being observed.

The spin echo spectra of PCA extracts (Fig. 3B) also showed that there were no positive peaks at 1.24 or 0.93 ppm, in agreement with previous literature that has shown compounds responsible for MLs to be PCA insoluble. Furthermore, the creatine peaks appear clearly resolved from neighboring resonances, which are diminished in apparent intensity by phase modulation due to J-coupling effects. This validates its use as internal quantitation reference signals in the intact cell spectra (Table 1).

Nile Red Staining. Nile red staining of saturation density cells (Fig. 4) clearly showed distinct spherical yellow-gold droplets over a faint background of pale yellow-reddish staining of the cell plasma and subcellular membranes.

These droplets (Table 2) were seen in 85% of the saturation density cells, whereas only 6% of the log-phase cells contained them (Fig. 4,
Cell Cycle Measurements. Cell cycle measurements showed clear differences between exponentially growing cells and saturation density cells (see Table 3). Saturation density C6 cells are slowing down differences between exponentially growing cells and saturation density cell doubles. Furthermore, this lipid droplet appearance can be reversed, as seen in the spectral pattern, by returning to standard media conditions.

Flow Cytometry. Low pH-treated log-phase cells approach the situation observed for saturation density cells; the percentage of G0-G1 significantly increases, whereas the one for cells at the S phase decreases. These effects are fully reversed by 24 h of culture in standard media (Table 3).

DISCUSSION

MLs and Growth Curve. Clear ML resonances at 1.24 and 0.93 ppm were present in the spin-echo spectra of C6 cells harvested at the saturation density phase of the growth curve, 48–72 h after reaching confluence. According to Moreton et al. (34), C6 cells at this time point would be approaching the quiescent (G0) stage with dramatically reduced incorporation of [3H]thymidine and basically no cell number increase after it. On the other hand, MLs were reproducibly absent from C6 cell spectra when the cells were harvested in the log phase of the growth curve.

The increase in the 1.24-ppm peak upon saturation density using the creatine peak at 3.05 ppm as quantitation reference (total creatine does not change between log phase and saturation density) is on the order of 27 times. This ratio is possibly underestimated because of the closeness of the 1.24 ppm resonance height to the noise level in the log-phase cell spectra, and to the fact (Fig. 3A) that other PCA-soluble metabolites may have minor contributions to the 1.24-ppm peak.

We agree in this respect with the work of previous authors who observed similar results for different cell lines. Callies et al. (19) mentioned in their work on AgX63.653, a murine spleen myeloma cell line, that ML-type signals were observed in a postconfluent state (the total number of cells was already decreasing) but were very small in log-phase cells when a spin-echo experiment (total echo time, 136 ms) was acquired. Delikatny et al. (20) also showed by pulse-and-acquire and two-dimensional NMR experiments on transformed murine fibroblast L cells that MLs were higher at confluence than at log phase. No spin-echo experiments were reported, and accordingly, it is difficult to predict the NMR visibility of their detected ML at long
Fig. 4. Fluorescence micrographs of C6 cells stained with 0.1 μg/ml of Nile red, final concentration, viewed at yellow-gold fluorescence (see text for details). A, C, and E are from log-phase cells; few droplets are present (see arrow in C as an example). B, D, and F are from saturation density cells; in the case of F, the average droplet diameter \( (n = 5) \) is 2.94 ± 0.22 μm; the droplet diameter measurement in D \( (n = 50) \) is 1.77 ± 0.62 μm. G is from 24-h, low pH-treated cells whereas H is from cells recovered for 24 h from the low pH treatment. A, B, and E are from cells stained while attached to the growth surface; all of the others correspond to cells harvested by trypsinization prior to staining.
echo times (136 ms). Other studies on stabilized mouse embryo NIH-3T3 fibroblasts and their transformed line transfected by the human ras (H-ras) oncogene have shown MLs appearing in pulse- and-acquire spectra of cells harvested in the late log phase of growth (24). The ML signals were more intense for the untransformed than for the transformed fibroblasts, although no full follow-up of their growth curve was described by the authors.

In summary, our data on C6 cells and data from other groups working with different cell lines seem to agree with a general trend. MLs are detected or reach a maxima for tumoral cell lines at, or past, the confluent state. In the particular case of C6 cells, this could be interpreted as suggesting that no MLs visible in an astrocytic tumor would be compatible with a high proliferation rate, and then, aggressiveness of the tumor.

**ML Pattern and Lipid Droplets.** Lipid droplets in tumor cells have been studied by electron microscopy (13, 18, 26, 35–37) and optical microscopy with stains specific for neutral lipids (37–39). We have used the method of Greenspan et al. (33) because it gives us a hands a quicker and more complete view of the changes taking place in the cell population, being more amenable to faster quantitation than, for example, electron microscopy. With this method, we can deduce from Table 2 that lipid droplets are seen in most (85%) saturation density cells, whereas they are practically absent (6% only) in log-phase cells.

The calculated lipid droplet volume increase (119 times) can fully explain the ML relative height increase (27 times). The difference could be reconciled if we consider the possible underestimation of the ML relative peak height increase as mentioned above. Nonetheless, other possibilities could be taken into account. For example, we could consider that not all of the lipid droplet content may be equally NMR visible; the fatty acyl chains of triglycerides in the periphery of the droplet could have a restricted mobility and, accordingly, lower NMR visibility. Finally, the presence of variable proportions of cholesterol esters in addition to triacylglycerols in the droplet core cannot be discarded from our data. If this would be the case, the volume of the droplet occupied by the cholesterol skeleton would not contribute to the 1.24-ppm peak height.

The accumulation of droplets in C6 cells at saturation density would agree with previous work by Hirakawa et al. (40), who studied established rat embryo fibroblasts transfected with H-ras and a temperature-sensitive SV-40 large T antigen. They showed that growth arrest induced by culture at a restrictive temperature (39°C) resulted in the massive accumulation of neutral lipids, mostly triglycerides and cholesterol esters, in the cellular cytosol in the form of “cytoplasmic vacuoles,” which stained positive for Oil Red O (a lipophylic dye).

Nile red staining has also been used by others to study the correlation between the detection of Nile red-positive subcellular structures and ML pattern. Mackinnon et al. (18) detected Nile red-positive lipid droplets and MLs in malignant Chinese hamster ovary cell lines but concluded that the spectral pattern change in MLs between cell lines was larger than the variation in the number of Nile red-positive droplets or in the percentage of cytosol cross-sectional area occupied by droplets as deduced from electron microscopy micrographs. A qualitatively similar conclusion was reached by Le Moyec et al. (23) on a human leukemia cell line study, Nile red staining and ML pattern did not correlate. We are unable to explain the discrepancy between our results and those of Mackinnon et al. (18) because no original Nile red plates were shown in their work. In the case of Le Moyec et al. (23), we believe that the discrepancy could be caused by the counting as Nile red-positive cells those in which Nile red was staining subcellular structures like endoplasmic reticulum membranes but not droplets (see for example Fig. 4C in the present work). This background effect is caused by the tails of the red emission entering the yellow zone of the fluorescence spectra and, accordingly, should not be used as a sign of droplet presence. Besides, a recent communication by Ferretti et al. (36) would agree, for NIH-3T3 fibroblasts, in a close relationship between ML signals and the presence of intracytoplasmic lipid bodies, as detected by either Nile red or electron microscopy.

In the particular case of C6 cells, it seems clear that growth-arrested saturation density cells accumulate neutral lipids in the form of large

---

**Table 2: Lipid droplet-related parameters in C6 cells at different stages of their standard growth curve, under acidic stress and upon recovery**

<table>
<thead>
<tr>
<th></th>
<th>Log phase</th>
<th>Saturation</th>
<th>Acid pH</th>
<th>Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage of cells with droplets (%)</td>
<td>6 (n = 120)</td>
<td>85 (n = 143)</td>
<td>70 (n = 118)</td>
<td>9 (n = 112)</td>
</tr>
<tr>
<td>Droplet diameter (μm)</td>
<td>1.03 ± 0.4 (n = 12)</td>
<td>1.56 ± 0.40 (n = 138)</td>
<td>1.00 ± 0.5 (n = 72)</td>
<td>0.86 ± 0.4 (n = 15)</td>
</tr>
<tr>
<td>Droplets per cell</td>
<td>1.45 ± 1.86</td>
<td>3.6 ± 2.1</td>
<td>7.4 ± 4.4</td>
<td>2.1 ± 1.2</td>
</tr>
<tr>
<td>Volume of LD in average μm³ cell</td>
<td>0.05</td>
<td>5.98</td>
<td>2.67</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Lipid droplet volume was calculated from the average droplet diameter, assuming spherical droplets, considering the percentage of cells containing droplets and an ideal cell containing the average number of droplets per cell.

---

Fig. 5. NMR spectra of C6 cells under acidic stress. Spectral parameters are the same as in Fig. 2. A. log-phase cells after 24 h of acidic pH stress (pH 6.2). B. log-phase cells after 24 h recovering from the acidic pH stress; spectra were scaled to have similar creatine peak heights.
incorporation being diverted from newly synthesized phospholipids in the C6 glioma cell line, that growth arrest results in glycerol disposition. Indeed, the proliferation rate of C6 glioma cells was reduced to 20% of saturation density cells. Nevertheless, other possible origins have been proposed. Delikatny et al. (20) measured by two-dimensional NMR an increase of GPC upon culture of their transformed murine fibroblasts at pH 6.1, and they proposed that free fatty acids produced when membrane phosphatidylcholine was being hydrolyzed to GPC could be reesterified in the form of triacylglycerols that would then be detected as MLs. Galons et al. (46) have also detected comparable increases in GPC by 31P NMR from three types of mammalian cells perfused in a bioreactor and challenged with pHs of 6.4. The reported pH difference ΔpH (pHl — pHs) was close to 0.5 pH units. In our particular case, we have also measured 1H NMR pH gradients in perfused C6 cells exposed to acidic pHs of 0.5 pH unit (pHl, 6.0; pHs, 6.5; Ref. 47). The increase in the 3.26-ppm peak height (Fig. 5) in our pH-stressed C6 cells would agree with a possible GPC concentration increase, and accordingly, the catabolic origin of the fatty acids in the lipid droplets could also be considered in our case.

It is then open to question what causes cell cycle arrest, pHl or pHs. This may be an important point, because an acid pHl and/or pHs (see Ref. 48 for the subject of where the acid pH measurement in tumors really comes from) can be caused in vivo by regions of accelerated glucolitic metabolism in the absence of proper removal of the excess protons produced (49). This could be the case of hypoxic regions in tumors. Indeed, histochemical studies of solid tumors (Ehrlich carcinoma in mice) using the Nile red stain as a probe have demonstrated the existence of such thin layers of hypoxic cells sandwiched between necrotic and normoxic areas (within 130 μm of a capillary). These layers of hypoxic cells (sometimes only 1–2 cells deep) accumulated Nile red-positive droplets inside the cytoplasm (37).

In summary, ML detection from defined areas of astrocytic tumors in vivo could also point to hypoxic areas in which cell proliferation has been arrested by acidic extracellular/intracellular pH conditions.

Conclusions. Growth arrest caused by saturation density or acid pHl induces the reproducible detection of ML in C6 cells at long echo time (136 ms). The ML peak height intensity measured by NMR in the two different conditions mentioned correlates with the detection of Nile red-positive cyttoplasmic droplets measured by optical microscopy. Furthermore, higher average ML intensity (two times higher at saturation density versus acid pHl-stressed cells) closely correlates with higher lipid droplet volume inside the cells (2.2 times higher at saturation density versus acid pHl-stressed cells). On the other hand, actively proliferating cells do not show either ML or lipid droplets. These findings, taken together, suggest that the detection of MLs in vivo NMR spectra of astrocytic tumors may be able to provide information not only about the presence of necrosis but also about the proliferative state of the cells in the sampled voxel.

ACKNOWLEDGMENTS

We thank Dr. Chantal Rémy (INSERM U418) for initial help in C6 cell culture, Dr. Núria Garçon at Balager Center for help in flow cytometry analysis, Dr. Salvador Bartolomé (LAF-UAB) for help in cell image acquisition, Daniel Edo for help in cell culture, and Drs. A. R. Tate and C. Gasparovic for English language correction and useful suggestions.

REFERENCES


The Relationship between Nuclear Magnetic Resonance-Visible Lipids, Lipid Droplets, and Cell Proliferation in Cultured C6 Cells

Ignasi Barba, Miquel E. Cabañas and Carles Arús


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/59/8/1861

Cited articles This article cites 40 articles, 9 of which you can access for free at: http://cancerres.aacrjournals.org/content/59/8/1861.full.html#ref-list-1

Citing articles This article has been cited by 14 HighWire-hosted articles. Access the articles at: /content/59/8/1861.full.html#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.