In vitro Study of Astrocytic Tumour Metabolism by Proton Magnetic Resonance Spectroscopy

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Abstract. In vivo magnetic resonance spectroscopy (MRS) studies of glial brain tumours reported that higher grade of astrocytoma is associated with increased level of choline-containing compounds (Cho) and decreased levels of N-acetylaspartate (NAA) and creatine and phosphocreatine (Cr). In this work, we studied the metabolism of glioma tumours by in vitro proton magnetic resonance spectroscopy (¹H-MRS). ¹H-MR spectra were recorded in vitro from perchloric acid extracts of astrocytoma (WHO II) and glioblastoma multiforme (WHO IV) samples. We observed differences between astrocytoma and glioblastoma multiforme in the levels of Cho, alanine, lactate, NAA, and glutamate/glutamine. In astrocytoma samples, we found higher MR signal of NAA and lower signal of Cho and alanine. MR spectra of glioblastoma samples reported significantly higher levels of lactate and glutamate/glutamine. In contrast, levels of Cr were the same in both tumour types. We also determined NAA/Cr and Cho/Cr ratios in the tumour samples. The NAA/Cr ratio was higher in astrocytomas than in glioblastomas multiforme. Conversely, the Cho/Cr ratio was higher in glioblastoma multiforme. The results indicate that MRS is a promising method for distinguishing pathologies in human brain and for pre-surgical grading of brain tumours.

Key words: Proton magnetic resonance spectroscopy — Brain metabolites — Astrocytoma — Glioblastoma

Introduction

Neoplastic transformations of brain cells entail numerous biochemical changes including modification of cellular energy metabolism, protein synthesis, and expres-
sion of receptors and antigens. Inside the tumour region, there may even be necrotic and cystic tissues, and in the case of highly infiltrative tumours, such as gliomas, there may be also contributions from normal brain tissue (Paulus and Pfeiffer 1989). Tumour growth also leads to heterogeneity in blood flow (Del Sole et al. 2001), changes in metabolic demands and disruption of transport mechanisms in cell membranes. In accordance with the World Health Organization, an astrocytoma (WHO II) is characterized by a high degree of cellular differentiation, slow growth and diffuse infiltration of neighbouring brain structures. This lesion typically affects young adults and has intrinsic tendency to malignantly progress to anaplastic astrocytoma (WHO III) and, ultimately, glioblastoma (WHO IV) (Kleihues et al. 2000a). Glioblastoma multiforme is the most malignant astrocytic tumour, composed of poorly differentiated neoplastic astrocytes. Histopathological features include cellular polymorphism, nuclear atypia, brisk mitotic activity, vascular thrombosis, microvascular proliferation, and necrosis (Kleihues et al. 2000b).

Shimizu et al. (1996) demonstrated that higher grades of brain tumours were associated with higher content of choline-containing compounds (Cho) and lower N-acetylaspartate (NAA) levels. In vivo proton magnetic resonance spectroscopy ($^1$H-MRS) studies indicate that the differences in Cho/Cr and NAA/Cr ratios can help determine the presence or absence of neoplasm and grade of a solid brain tumour (Herminghaus et al. 1998; Möller-Hartmann et al. 1998; Fountas et al. 2000; Tamiya et al. 2000). Determination of total Cho using in vivo $^1$H-MRS provides a method of assessment of proliferative activity of neuroepithelial brain tumours (Herminghaus et al. 2002).

The aim of this work was to observe differences in low-molecular metabolites in astrocytoma and glioblastoma multiforme tumours using in vitro $^1$H-MRS, and to compare our findings with in vivo studies from literature.

Materials and Methods

We evaluated 19 specimens obtained from two patients affected by brain astrocytoma (WHO II) and from four patients affected by glioblastoma multiforme (WHO IV). Brain tumour samples were divided into the two groups based on their histological characteristics.

For the MRS study, the samples were frozen in liquid N$_2$ immediately after peroperation abscission of brain tumours, and extracted with perchloric acid (PCA) (Lachema Brno, Czech Republic) according to Klunk et al. (1994). Briefly, 3-aminopropylphosphonic acid (3-APP) (20 mmol/l, Sigma, USA) as an internal standard and 70% PCA were added to each sample in amount of 1/4 of tumour sample weight. The homogenized tissue was centrifuged (43,000 $\times$ g, 15 min at 4 $^\circ$C, Beckman, USA). In the supernatants, pH was adjusted to 6.5–7.5 by addition of KOH (Lachema Brno, Czech Republic) or HCl (Lachema Brno, Czech Republic). Afterwards, the PCA extracts were passed through potassium Chelex column (Bio-Rad, USA) and lyophilised (Jouan LP 3, France). After lyophilisation, 30–40 mg of powder was dissolved in 1 ml of 99.9% D$_2$O and pH was adjusted to 5.0–5.2.
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1H-MR spectra were measured by a Varian VXR 300 MHz (7.5 T) spectrometer (USA). The brain extracts were measured in the following conditions: t, 27°C; spectral width, 3000 Hz; time of scans, 2.5 s; angle of rotation, 45° (10 µs); time of presaturation, 2 s; number of accumulations, 200.

The recorded spectra were evaluated by the MestRe-C version 2.3 computer program (http://www.mestrec.com). Relative levels of low-molecular metabolites were obtained using integration of areas under individual peaks after phase and baseline correction of the spectra. Relative metabolite ratios were defined as percentages of signal intensity to intensity of a signal, corresponding to the CH₂-group of the internal standard (3-APP). The resonances of interest were assigned as follows: NAA at 2.02 ppm, Cho (including choline, phosphocholine and glycerophosphocholine) at 3.15–3.23 ppm, glutamate and glutamine (Glu/Gln) at 3.76 ppm, lactate (Lac) at 1.32 ppm, and alanine (Ala) at 1.48 ppm. The peaks of Cr were integrated at 3.93 ppm, but not at 3.03 ppm because the internal standard had resonance between 3.03 and 3.13 ppm. Integration of Cr peak at 3.03 ppm was made for determination of the NAA/Cr and Cho/Cr ratios, because in in vivo 1H-MRS, only this peak together with NAA and Cho are good distinguishable in tomographs with 1.5 T of magnetic induction, which are common used in hospitals. In these spectra, the signal of Cr at 3.93 ppm is very hard to recognise from the noise.

Results are represented as relative proportions (%) of metabolite levels to the internal standard (3-APP) and expressed as mean ± standard deviation (SD). For the statistical evaluation, we used an One-way analysis of variance test. Differences between types of tumours with \( p < 0.05 \) are considered statistically significant.

Results

Using in vitro MRS, 19 samples from 6 patients with cerebral gliomas were investigated. Brain tumours were divided into two groups according to histopathological classification of the WHO. There were two patients with astrocytoma (WHO II), with seven determined samples, and four patients with glioblastoma multiforme (WHO IV), with twelve determined samples. Representative 1H-MRS spectra of low-molecular metabolites of astrocytoma samples and glioblastoma multiforme samples are shown in Figures 1 and 2, respectively.

Relative levels of low molecular metabolites were obtained using integration of areas under individual peaks. Relative metabolite ratios were defined as percentage of signal intensity with respect to a signal corresponding to the CH₂-group of the internal standard (3-APP).

In PCA extracts of astrocytoma samples, we found a higher signal of NAA (80 ± 10) than in the samples of glioblastoma multiforme (59 ± 9). The level of Cr was similar in both groups of brain tumour samples. There were no differences associated with malignity grading in these metabolites.

Larger peak intensities of Cho were observed in samples of glioblastoma multiforme (169 ± 19).
Figure 1. $^1$H-MRS spectra of astrocytoma (WHO II) samples. Lac, lactate; Ala, alanine; Ace, acetate; NAA, N-acetylaspartate; St, internal standard (3-APP); Glu/Gln, glutamate/glutamine; Cr, creatine; Cho, choline-containing compounds; Tau, taurine; Ino/Gly, inositol/glycine; Ino, inositol.

A statistically significant difference was found in Lac levels ($p < 0.01$). High amount of Lac in both types of tumour samples may be attributed to a short period of time between their surgical removal and freezing. Anyway, there was a significantly higher intensity in the glioblastoma (302 ± 40) than in the astrocytoma samples (161 ± 12). Ala was elevated in the samples of glioblastoma multiforme (14 ± 3). The amount of Glu and/or Gln in the glioblastoma multiforme samples was also larger (167 ± 18) than in astrocytoma, with statistical significance $p < 0.01$ (Figure 3).

Signal intensities of glycine (Gly) and inositol (Ino) were not evaluated, because the 3.6 to 3.9 ppm region also features a group of resonances characteristic of mannitol. Mannitol and other anti-oedematous hyperosmolar drugs are administered to patients during craniotomy for cerebral decompression.

We also determined the NAA/Cr and Cho/Cr ratios in the tumour samples (Figure 4). The NAA/Cr ratio was higher in astrocytoma (0.5 ± 0.1) than in glioblastoma multiforme (0.4 ± 0.1) samples. Conversely, the Cho/Cr ratio was larger in glioblastoma multiforme (1.4 ± 0.2).
Figure 2. $^1$H-MRS spectra of glioblastoma multiforme (WHO IV) samples. Lac, lactate; Ala, alanine; NAA, N-acetylaspartate; St, internal standard (3-APP); Glu/Gln, glutamate/glutamine; Cr, creatine; Cho, choline-containing compounds; Tau, taurine; Ino/Gly, inositol/glycine; Ino, inositol.

Figure 3. Comparison of low-molecular metabolite levels in astrocytoma (WHO II) and glioblastoma multiforme (WHO IV). Numbers are relative proportions (%) of metabolites with respect to the internal standard, expressed as mean ± SD. ** statistically significant difference between tumour types; p < 0.01.
Discussion

MRS provides useful information for looking at intracellular pathology and tissue specificity of human brain.

In our study, PCA extracts of astrocytoma samples provided a higher signal of NAA than samples of glioblastoma multiforme. This is in agreement with the fact that glioblastoma multiforme is associated with loss of neurons. Tumours with high-grade malignity are more cytologically atypical and more mitotically active than grade II gliomas (Burger et al. 2002). Substitution or destruction of normal neuronal cells by tumour cells is expected to be more severe in grade III and IV tumours, resulting in more pronounced decrease in NAA intensity (Hsu et al. 2004). In MRSoft tumours, the presence of NAA within the spectrum is generally believed to indicate the presence of viable neurons within an infiltrative tumour such as a glioma (Howe and Opstad 2003).

The levels of Cr were found to be similar in both groups of tumour samples, although previous in vivo studies of human brain tumours reported a lower amount of Cr in higher grade astrocytomas (Kinoshita et al. 1993; Negedank et al. 1996; Majós et al. 2002).

Increase in Cho indicates increased cell membrane turnover (Kugel et al. 1992; Ott et al. 1993). In this study of PCA extracts, we found a lower level of Cho in astrocytoma than in glioblastoma multiforme samples. A correlation of the Cho peak with mitotic and proliferative activity and degree of tumour malignancy has also been reported (Negedank et al. 1996; Shimizu et al. 1996; Herminghaus et al. 1998; Möller-Hartmann et al. 1998). Anaplastic astrocytomas and glioblastomas multiforme are characterized by elevation of choline in accordance with tumor grading (Ott et al. 1993; Poptani et al. 1995; Möller-Hartmann et al. 1998). The
Cho peak can be also ascribed to reactive gliosis (Krouwer et al. 1998). The increase in total Cho was associated with tumour progression (Tadeshi et al. 1997; Wald et al. 1997) and for patients with gliomas (grade II to IV gliomas), a more than 45% increase in total Cho indicated progression, whereas a less than 35% increase, or even a decrease, was found in patients with stable disease (Tadeshi et al. 1997).

High amount of Lac in proton spectra of both types of tumour samples may be attributed to the short period of time between their surgical removal and freezing. The significantly higher Lac levels in glioblastoma multiforme may relate to rapid growth of the tumours (Möller-Hartmann et al. 1998). Both circumstances lead to anaerobic glycolysis, which produces Lac. The significance of occurrence or intensity of the Lac peak for glioma grading is controversial. Lac was identified more frequently in grade III and IV (~50%) than in grade II (10%) gliomas (Hsu et al. 2004).

Ala was elevated in samples of glioblastoma multiforme. This elevation with increase of Cho, Gly, and Ino reflects the degree of malignancy (Kinoshi et al. 1993; Sabatier et al. 2001). The amount of Glu/Gln was also significantly higher in glioblastoma multiforme. The higher level of Glu/Gln in glioblastoma possibly reflects altered energy metabolism involving partial oxidation of Glu rather than glycolysis (Manton et al. 1995), the end product being Ala, which is also elevated. Malignant tumours are characterized by increased glucose consumption, but the mechanism(s) leading to hypermetabolism have not been fully elucidated (Levivier et al. 2002).

We also determined the NAA/Cr and Cho/Cr ratios in the observed PCA extracts of tumour samples. The NAA/Cr ratio was higher in astrocytoma than in glioblastomas multiforme samples. Conversely, the Cho/Cr ratio was higher in the glioblastoma multiforme samples. This is in a good agreement with literature data, reporting that an increase in Cho/Cr and decrease in NAA/Cr ratios reflects the grade of solid brain tumours (Herminghaus et al. 1998; Möller-Hartmann et al. 1998; Fountas et al. 2000; Tamiya et al. 2000). The Cho/Cr ratio has also been used in serial studies following treatment. Hsu et al. (2004) showed that, among other metabolite ratios, the (Cho+Cr)/NAA ratio was the most significant predictor for discrimination of different grades of gliomas. This ratio was in significant correlation with the WHO glioma grading.

In conclusion, our results obtained from PCA extracts of glioma samples correspond to in vivo findings from literature. Limitations of this study are in that we did not have any controls to compare glioma samples with healthy brain tissue, and that the number of patients was low. We would like to continue this study and compare also other type of tumours.

In vitro 1H-MRS provides valuable information about biochemical composition and metabolism of the different types of brain tumours. This information can be helpful in the diagnostics of tumours using in vivo MRS. In comparison with other examination methods, the in vivo MRS is a non-invasive diagnostic method, does not burden organisms, offers information in real time, and has a high resolution and chemical specificity.
References


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