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In Vitro Proton Magnetic Resonance Spectroscopy of Four Human Prostate Cancer Cell Lines


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ABSTRACT: There is accumulating evidence that some biochemical pathways observable by magnetic resonance spectroscopy, e.g., citrate acid and phospholipid metabolism, are altered in human prostate cancer. Four well-established human prostate cancer cell lines were therefore studied with magnetic resonance spectroscopy to compare differences in metabolic content with tumor biological behavior. Herein we demonstrate that, although each cell line has its own metabolic profile, relative creatine and citrate levels can be used to discriminate the androgen-dependent LNCaP cell line from the androgen-independent DU-145, TSU, and PC-3 cell lines.

INTRODUCTION

The recent development and clinical introduction of transrectal probes not only improved the quality of magnetic resonance imaging (MRI) of the human prostate but also made this organ accessible to magnetic resonance spectroscopy (MRS) [1-4]. Transrectal MRI and MRS, basically employing the same instrumental setup, are potential powerful noninvasive methods for monitoring the diseased prostate, first, to identify with MRI the suspected lesion, and secondly, to monitor with MRS the metabolism of this lesion. Changes in various metabolic pathways associated with several cancers have been reported [5-7]. For prostate cancer, metabolic derangements might provide markers that improve diagnosis and allow more accurate prediction of its clinical behavior. In vitro and in vivo studies employing $^{31}$P, $^1$H, and $^{13}$C MRS have shown that differences in the metabolic content of human prostate tissue samples correlated with tumor grade [3,4,8-14]. Moreover, for the Dunning R-3327 rat tumor model, it has been demonstrated that the relative levels of phosphocreatine, glycerophosphorylglycerol, glycerophosphoethanolamine, and glycerophosphocholine, obtained by $^1$H and $^{31}$P MRS, can be used to differentiate sublines by differentiation grade, androgen sensitivity, and metastatic capacity [15,16]. However, interpretations of differences in MRS spectra between human prostate tissues with different biological behavior are hampered by tissue heterogeneity and the lack of a complete understanding of biochemical pathways. MRS examinations of well-defined human model systems are therefore needed to allow correlations between metabolic patterns and biological behavior. For this reason, studies of human prostate (cancer) cell strains are of value. Yacoe et al. [17] investigated cell strains derived from human prostates to investigate whether $^1$H MRS could reliably distinguish normal prostate epithelium from prostate cancer. In the present study we attempted to identify differences in metabolites which correlated with different biological behavior, e.g., androgen responsiveness and morphology, in human prostate cancer. Four well-established human prostate cancer...
TABLE I. Characteristics of the Four Human Prostate Cancer Cell Lines Used

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Histology of original tumor</th>
<th>Doubling time (hr)</th>
<th>Androgen responsiveness</th>
<th>PSA-producing</th>
</tr>
</thead>
<tbody>
<tr>
<td>LNCaP15</td>
<td>Lymph node</td>
<td>Moderately different</td>
<td>50.1 ± 8</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DU-14516</td>
<td>Brain</td>
<td>Poorly different</td>
<td>28.7 ± 2.9</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PC-317</td>
<td>Bone</td>
<td>Poorly different</td>
<td>35.2 ± 6.7</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>TSU-PR118</td>
<td>Lymph node</td>
<td>Poorly different</td>
<td>26.4 ± 4.5</td>
<td>–</td>
<td>±</td>
</tr>
</tbody>
</table>

cell lines were characterized with in vitro proton MRS, and metabolic differences were correlated with their biological behavior.

**MATERIALS AND METHODS**

**Tumor Cell Lines**

Four established human prostatic carcinoma cell lines, PC-3, DU-145, TSU-PR1, and LNCaP, were used in this study. [18-21]. The characteristics of the four cell lines are summarized in Table I. Cells were maintained in RPMI 1640 culture medium (Life Science, Breda, The Netherlands) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 nM dexamethasone at 37°C in 6% CO₂ at 99% relative humidity.

**Preparation of Tissue Extracts**

After reaching a confluency of 70–80%, cells were harvested for perchloric acid (PCA) extraction as described previously [14]. In short, single-cell suspensions were obtained by trypsinization (0.25% trypsin/0.1% EDTA) and immediately washed three times with ice cold 0.9% NaCl to remove all medium components. The total number of cells varied between 4–6 × 10⁶ cells. Viability, as determined by the trypan blue dye exclusion test, was always ≥95%. After centrifugation of the single-cell suspension, the cell pellet was frozen in liquid nitrogen and stored at −80°C until time of extraction.

The cell pellet was transferred into an all-glass homogenizer, and 4 ml ice-cold 0.25 M PCA were added dropwise. Cell homogenization was achieved at −80°C in approximately 30 min. After centrifugation (12,000 g, 15 min, 4°C) of the cell extract, the pellet was discarded and the pH of the supernatant was immediately adjusted to 7.5 with 2.5 M KOH. The PCA precipitate was centrifuged (12,000 g, 15 min, 4°C), and the supernatant was passed through a Chelex sample preparation disc (Bio-Rad Laboratories, Richmond, VA) and lyophilized. Lyophilizates were dissolved in 500 μl 40 mM potassium phosphate, pH 7.0. The pH was corrected to pH 6.90–7.10 if necessary by addition of HCl or KOH. The samples were lyophilized again and stored at −20°C.

Three-ml samples of culture media of all prostate cancer cell lines investigated were lyophilized and stored at −20°C.

Just before the ¹H MRS measurements, the lyophilizate was carefully thawed and dissolved in 500 μl D₂O containing 1.6 mM 3-(trimethylsilyl) propionic acid-d₄ sodium salt (TSP).

**¹H NMR Measurements**

¹H NMR spectra were acquired on a 500 MHz spectrometer (Bruker AM500) and were recorded with a standard 5 mm ¹H NMR probe. The spectra were recorded employing a 45° flip angle (6 μs) and a 7.28 sec pulse repetition time. The resonance of H₂O was suppressed by low-power continuous wave presaturation. For the spectrum of each PCA-extracted cell sample, 900 scans were accumulated. The chemical shifts were referenced with respect to the chemical shift position of the TSP resonance.

¹H NMR spectra were further evaluated employing the NMR1 package (New Methods Research, Inc., Last Syracuse, NY) on a SUN Sparc station 330 (Sun Microsystems, Inc., Mountain View, CA). Free induction decays (FIDs) were Fourier transformed after zero-filling from 16 K to 32 K and after the application of a Lorentzian to Gaussian transformation filter. The spectra were semiautomatically fitted to Gaussian lineshape model functions. Relative resonance integrals of proton metabolites of interest are expressed as ratios to the integral resonance of TSP.

**Statistical Analyses**

Differences in ratios of metabolites between the different cell lines were investigated by the Wilcoxon's rank test. Due to multiple testing, P<0.01 was considered as statistically significant.
**RESULTS**

**PCA-Extracted Cell Lines**

MR spectra of PCA extracts of four human prostate cancer cell lines were recorded, and for each cell line at least five different passages were investigated. A 500 MHz proton MR spectrum of a PCA extract of the LNCaP cell line, with two expanded parts, is shown in Figure 1. Resonances of some main metabolites could be identified with data from the literature [14, 22].

For each compound, the best resolved resonance(s) were selected for quantitation purposes. Table II lists relative peak area integral ratios involving the doublet of the CH3 group of lactate (Lac) at 1.33 ppm, the doublet of the CH3 group of alanine (Ala) at 1.49 ppm, the quartet of the CH2 group of citrate (Cit) at 2.54 ppm, the singlet of the CH3 group of (phospho)creatine at 3.04/3.05 ppm, singlets of the CH3 groups of phosphocholine (PC) and choline (Chol) at 3.23 and 3.22 ppm respectively, the triplet of the CH2 group of taurine (Tau) at 3.42 ppm, the singlet of the CH2 group of glycine (Gly) at 3.59 ppm, and the triplet of the CH group of inositol (Ino) at 3.63 ppm.

Several differences were found among MRS spectra of the PCA-extracted human cell lines.

The first prominent observation was of the relatively high amount of total creatine (TCr = PCr + Cr) in the PCA extracts of LNCaP cells (Table II). The TCr/TPS ratio was significantly higher for the LNCaP cells samples when compared with the other three cell lines (P < 0.01).

Secondly, citrate resonances were absent in the MR spectra of PCA extracts of the PC-3, DU-145, and TSU cell lines, whereas in only one of the five samples of the LNCaP cell line could citrate be detected in the MR spectra. Since citrate can rapidly diffuse from the prostate cell into the culture media, all media were also spectroscopically examined. 1H MR spectra of the culture media of the LNCaP cells showed citrate resonances, whereas in all the media of the other three cell lines no citrate resonances were detected (Fig. 2).

Finally, several differences in metabolic content of PCA extracts of DU-145, TSU, and PC-3 cells were found. The relative amount of taurine was significantly higher for the DU-145 PCA cell extracts when compared with the PC-3 and TSU cell extracts (all P<0.01). PC-3 and TSU cell extracts differed significantly from each other for inositol and creatine content, whereas the creatine content of DU-145 PCA extracts was also different from that of TSU PCA extracts (P<0.01, Table II).
TABLE II. Mean of Metabolite Ratios (Range Between Parentheses) of Four Human Prostate Cancer Cell Lines

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>LNCaP</th>
<th>DU-145</th>
<th>TSU-PR1</th>
<th>PC-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iac/tsp</td>
<td>94 (18–169)</td>
<td>34 (11–67)</td>
<td>25 (14–46)</td>
<td>12 (6–56)</td>
</tr>
<tr>
<td>Ala/tsp</td>
<td>84 (18–164)</td>
<td>11 (7–23)</td>
<td>6 (4–19)</td>
<td>5 (1–9)</td>
</tr>
<tr>
<td>Citr/tsp</td>
<td>0 (0–72)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TCr/tsp</td>
<td>60 (36–132)</td>
<td>9 (2–11)</td>
<td>0</td>
<td>10 (3–37)</td>
</tr>
<tr>
<td>Chol/tsp</td>
<td>40 (18–69)</td>
<td>12 (9–23)</td>
<td>22 (15–26)</td>
<td>16 (7–56)</td>
</tr>
<tr>
<td>PChol/tsp</td>
<td>6 (3–9)</td>
<td>5 (4–9)</td>
<td>4 (0–7)</td>
<td>3 (1–10)</td>
</tr>
<tr>
<td>Ino/tsp</td>
<td>0 (0–140)</td>
<td>156 (6–406)</td>
<td>0 (0–20)</td>
<td>44 (21–53)</td>
</tr>
<tr>
<td>Tau/tsp</td>
<td>11 (0–43)</td>
<td>84 (20–224)</td>
<td>0 (0–5)</td>
<td>0 (0–13)</td>
</tr>
<tr>
<td>Gly/tsp</td>
<td>48 (0–107)</td>
<td>7 (0–13)</td>
<td>5 (0–9)</td>
<td>6 (3–10)</td>
</tr>
</tbody>
</table>

Fig. 2. In vitro $^1$H MR spectrum of 3 ml culture medium of LNCaP human prostate cancer cell line. Citrate resonances are indicated.

DISCUSSION

Prostate tumors present sharp interindividual variations in biological behavior and response to therapy. At least 20–25% of the patients where hormonal treatment is the first choice therapy will not react, and an equal percentage become resistant to the treatment within 2 years [23,24]. Therefore, new diagnostic methods able to predict more accurately the biological behavior of the tumor are needed, since these may lead to a better basis for individual treatment. Several in vitro MRS studies have suggested a possible role for this noninvasive technique for improving diagnosis of prostate cancer [10–15]. Moreover, correlations were found for several metabolites and for the biological behavior of Dunning R-3327 rat prostate tumor sublines. In this study, four well-characterized human prostate cancer cell lines were studied with $^1$H MRS to substantiate these findings. An in vitro approach was chosen because $^1$H MRS of biological material may produce complex spectra, which are difficult to resolve in vivo. Assignments for the same main metabolites were made as found earlier for human prostate cancer [14].

In this panel of PCA-extracted human prostate cancer cell lines, the relative level of total creatine was found to be significantly increased for the hormone-sensitive LNCaP cell line compared to the three hormone-nonresponsive ones. This suggests a correlation for hormone sensitivity and the creatine content of the tumor. Vigneron et al. [16] found by in vivo $^{31}$P MRS, an increased PCR/ATP ratio in one hormone-sensitive Dunning tumor subline when compared to one hormone-insensitive subline. Androgen deprivation (orchiectomy) resulted in a significant decrease of this ratio [16]. However, in a recent in vitro MRS study, where three different hormone-sensitive and four different hormone-insensitive Dunning sublines were investigated, no specific correlation for hor-
monal sensitivity with relative metabolite levels could be made [15]. A correlation of increased PCR concentrations and hormone sensitivity has also been suggested for rat breast cancer [25]. Creatine levels can presently be detected in 1H MR spectra recorded with transrectal probes; hence, in vivo human MRS studies can now test the relevance of these in vitro observations [26].

Previous biochemical and MRS studies have suggested that the metabolism of citrate may be altered in prostate cancer [12–14, 27–30]. Citrate is a secretory product of the prostate, and its production and secretion are under androgenic control [28]. Testosterone stimulates the net citrate production and might also play a role in citrate secretion [28]. Normally citrate is present in human prostate tissue in high concentrations [27, 28, 30]. In culture, however, citrate can rapidly diffuse from the prostate cell in the culture media [27]. It is therefore obligatory to measure both citrate levels in prostate (cancer) cells, and in their culture media in studies with cultured prostate (cancer) cells. In the present study, citrate resonances were absent in the MR spectra of DU-145, PC-3, and TSU cells, and in their culture media. Kurhanewicz et al. [12] have already shown that citrate signals could not be observed in 3H MR spectra of DU-145 xenografts, either in vivo or in PCA extracts. In contrast, in one of the five LNCaP extracts, an MRS-detectable level of citrate was found, and all the media of LNCaP cells showed citrate resonances in the MR spectra, suggesting that the hormone-dependent LNCaP cells have a higher relative level of citrate as compared to the other three hormone-insensitive cell lines. Earlier studies already suggested that citrate is reduced in early prostate cancer and nearly absent in advanced disease, both of which are known to have only a small portion of hormone-sensitive cells [28–30]. Possibly there is a difference in citrate content between hormone-responsive and -nonresponsive prostate cancers. Our present findings seem to fit well with this hypothesis and the aforementioned studies. However, further in vivo MRS studies should be performed to compare the citrate concentrations in tumors of patients which are escaped from hormonal treatment with patients who do respond well, to validate this finding.

Finally, each prostate cancer cell line has its own metabolic fingerprint, i.e., several differences in metabolic content were found irrespective of their biological behavior. The most striking difference was the high taurine content of the DU-145 cell extracts as compared to both the PC-3 and the TSU cell extracts. Kurhanewicz et al. [12] also showed large taurine resonances in the in vivo and in vitro 1H MRS spectra of the DU-145 tumor. The difference in taurine content might be explained by differences in origin of the metastasis [18–21].

In conclusion, this MRS study of PCA extracts of four human prostate cancer cell lines demonstrates that, although each cell line has its own metabolic fingerprint, the total creatine/tsp and citrate/tsp ratios can be used to discriminate the androgen-dependent LNCaP cell line from the other androgen-independent cell lines. The extrapolation of these findings to the clinic will not be so easy. Clinical MRS studies with transrectal probes are ongoing, but are complicated by several difficulties. The minimum volumes (voxels) within the prostate which can be measured with transrectal MRS are relatively large. Heterogeneity of the measured lesion, i.e., contamination of the cancer with normal prostate tissue and/or BPH, cannot be excluded. Smaller volumes and spectroscopic imaging methods will probably solve these problems. Secondly, a proper absolute quantification of the metabolites measured with in vivo MRS is still not possible.

The question remains as to whether MRS will be clinically applicable in the detection and staging of prostate cancer. Our results suggest that MRS might possibly play a role in the identification of hormone-responsive prostate cancers.

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