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Proton MR spectroscopy of prostatic tissue focused on the etection of spermine, a possible biomarker of malignant behavior in prostate cancer

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\bstract

To investigate whether polyamines may be valuable diagnostic and prognostic markers in prostate cancer, the presence of olyamines was studied in various human prostatic tissues using both proton magnetic resonance (MR) spectroscopy and igh-pressure liquid chromatography (HPLC). The HPLC results showed that normal and benign hyperplastic prostatic tissues re characterized by a high content of spermine. Spermine levels were reduced in tumor tissue, especially in prostatic carcinoma h metastases, and in xenografts of human prostatic carcinoma cells. These preliminary results indicate that spermine may be d as a biomarker for malignant behavior. The MR spectroscopy study showed that it is possible to detect spermine resonances prostatic biopsy material by one-dimensional and two-dimensional J-resolved MR spectroscopy at high field (600 MHz). ocalized one-dimensional in vitro MR spectra obtained at the clinical field strength of 1.5 T showed spermine signals in the region between 3.0 and 3.3 ppm. In in vivo MR spectra of the human prostate, however, these signals were obscured by esonances of choline (3.2 ppm) and creatine (3.0 ppm). © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Spermine; Polyamine; Magnetic resonance spectroscopy; Prostate; Cancer; Malignancy

Introduction

Although prostate cancer is one of the most frently diagnosed malignancies in males [1], the num- of patients developing large invasive tumors with tastases is comparatively low [2]. The natural lyamines putrescine (Put), spermidine (Spd) and sperine (Spm) play an important role in the regulation of cell proliferation and differentiation [3,4]. With respect to the prostate, the high concentration of spermine present in prostatic tissue (10–20 mM) may be responsible for the relatively slow growth of cancer cells in the prostate [5]. Prostate cancer cell proliferation requires an increased rate of polyamine biosynthesis and increased levels of the two other polyamines Put and Spd [6]. This is in accordance with previous suggestions that a decrease in Spd level and/or an increase in Spm level usually indicates a shift of cells from a proliferation state into a state with a higher degree of differentiation [3]. Furthermore, spermine seems to be a marker for the functional secretory state of prostatic epithelium [7]. The prostate is a secretory organ with a large glandular and ductal volume where secretary products, like spermine and citrate, are concentrated. Morphologic changes in cell organization that result in decreased ductal volume consequently reduce the detection of secreted compounds. Hence, a dramatic decrease of the prostatic spermine content could indicate changes in cellular metabolism and/or changes in ductal morphology as a result of a conversion of prostatic tissue from a benign to a malignant phenotype.

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As disseminated prostatic carcinoma is hard to cure completely, much emphasis is put on early diagnosis. Prostate cancer can be identified by the detection of decreased levels of citrate using in vivo proton magnetic resonance spectroscopy (MRS) [8–10]. Unfortunately, markers to detect patients with a high risk for progressive growth of prostatic cancer are lacking. In vivo measurement of prostatic polyamine levels could provide a valuable new tool in early diagnosis and in predicting the biological behavior of prostatic cancer.

In the present study, the polyamine content of various human prostatic tissues was determined by high-pressure liquid chromatography (HPLC). In addition, we explored the possibilities of using MRS for the detection of prostatic polyamines, especially spermine, in a noninvasive way.

2. Materials and methods

Prostatic tissue specimens were obtained from patients undergoing radical cystoprostatectomy ($n = 2$), radical prostatectomy ($n = 9$) and transurethral resection of the prostate ($n = 3$). Three prostatectomized patients had disseminated prostate cancer. Tissue samples were harvested using a 5-mm skin biopsy punch, snap frozen in liquid nitrogen and stored at -80°C until further use. One tissue specimen was immediately subjected to MR spectroscopy before freezing and storage. Tissue specimens of xenografts derived from three human prostate carcinoma cell lines, i.e. LNCaP [11], DU-145 [12], and PC-3 [13], were also included in the study.

Concentrations of Put, Spd and Spm were measured by HPLC using a method modified from Kirschbaum et al. [14]. In short, tissue homogenates were deproteinized with sulfosalicylic acid (60% V/V in water). After centrifugation, supernatant was collected and neutralized with 6 M NaOH. After derivatization with *n*-9-fluorenylmethoxycarbonyl succinimide in borate buffer, metabolites were separated by reversed-phase HPLC (MicroSpher 3 C18, 100 × 4.6 mm i.d.; particle size, 3 μm; Varian Chrompack Benelux, Bergen op zoom, The Netherlands). The mobile phase consisted of a gradient from 0 to 30 min starting with buffer A (50% of 0.1 M NaAc and 50% acetonitrile) and changing to buffer B (100% acetonitrile). Eluting analysis was performed with a Waters 470 fluorescence detector at an excitation and emission wavelength of 265 and 315 nm, respectively. Experiments were performed twice in duplo. Polyamine concentrations are expressed as nanomoles per milligram protein and presented as means ± standard deviation. Comparison of polyamine levels between normal prostate and other prostatic tissues were statistically analyzed using Dunnett's Multiple Comparison post-test after one-way analysis of variance.

High-resolution proton MR experiments in one and two dimensions were performed at 25°C on a Bruker AMX 600 spectrometer. Measurements were carried out on five samples with predominantly healthy biopsy material of the human prostate and on solutions mimicking prostatic fluid. The biopsy material was put into the MR tube without being crushed and it was covered with phosphate buffered saline (PBS), pH 7.2, with 10% D_2O and a small amount of sodium 3-trimethylsilyl-(2,2,3,3-$^2H_4$) propionate (TSP) for referencing purposes. All samples with biopsy material were studied by one-dimensional MR and, of three samples, a two-dimensional (2D) J-resolved MR spectrum was also recorded. The solutions mimicking prostatic fluid contained 90 mM citrate, 18 mM spermine, 61 mM KCl, 18 mM $CaCl_2$, 15 mM $MgCl_2$, and 9 mM $ZnCl_2$ (pH ~ 7.0) [15]. In the sample used for a 2D J-resolved experiment, 9 mM choline and 12 mM creatine were also present. All solutions contained 10% D_2O, and a small amount of TSP. Acquisition parameters of one-dimensional pulse-acquire experiments comprised a spectral width of 6667 Hz, a nominal flip angle of 50–60°, two dummy scans, and 16–128 scans of 32 K data points. Water suppression was performed by frequency-selective irradiation during the relaxation delay of 6 s. 2D J-resolved MR experiments [16,17] were performed with a spectral width of 6667 Hz in the F2-dimension, and a spectral width of 100 Hz in the F1-dimension, two dummy scans, 12–16 scans and 64 t_1-increments of 4 K data points. The water resonance was suppressed by frequency-selective irradiation during the relaxation delay of 2 s. One-dimensional high-resolution proton MR data were processed using the Bruker WIN-NMR software and 2D J-resolved MR data were processed using NMRI software (New Methods Research, Inc., Syracuse, NY).

It was also tested whether spermine could be detected at the clinical field strength of 1.5 T using localized MR spectroscopy. In vivo and in vitro MR experiments were carried out on a Siemens Magnetom SP or Vision spectrometer. Measurements were performed in vitro on a phantom containing a solution with 90 mM citrate, 18 mM spermine, 61 mM KCl, 18 mM $CaCl_2$, 15 mM $MgCl_2$, and 9 mM $ZnCl_2$ (pH ~ 7.0) or a solution in which 9 mM choline and 12 mM creatine were also present. In vivo measurements were performed on the human prostate. In vitro proton MR spectra of the two solutions were obtained by single-voxel spectroscopy with a CP head coil both for excitation and signal reception. A volume of 24.4 cm^3 ($29 \times 29 \times 29$ mm^3) was selected using a PRESS localization scheme ($90°-\tau-180°-0.5TE-180°-(0.5TE-\tau)-Acq$) [18,19] with a delay τ of 11 ms and various echo times (TE). After four dummy scans, 64 scans of 1024 data points were acquired with a spectral width of 1000 Hz and a repetition time (TR) of 1.5 s. In vivo

proton MR spectra of the prostate were obtained from spectroscopic imaging (SI) data sets that were acquired using the body radio-frequency coil for excitation and a disposable endorectal coil (Medrad, Pittsburgh, PA) for signal reception (see [9,20]). A PRESS localization scheme ($\tau = 11$ ms and TE = 120 ms) was used for pre-selection of an axial slab through the prostate. The selected volume was generally 10 mm thick, 40 mm in the left–right and 30 mm in the anterior–posterior direction. TR was 1.2 s and three acquisitions were made for each of the 16 × 16 phase encoding steps in the two directions within the field of view of 144 mm. For the acquisition of both in vitro and in vivo proton MRS metabolite spectra, chemical shift selective (CHESS) water suppression [21] was used. In addition, reference spectra without water suppression were acquired for eddy-current correction according to Klose [22]. Data were processed using the Siemens spectroscopy post-processing software LUISE.

3. Results

As established by histology, the prostatic tissue specimens exhibited normal morphology ($n = 4$), benign hyperplasia ($n = 3$) and prostate carcinoma with a Gleason pattern score ranging from 5 to 8 ($n = 7$). Polyamine levels in the various prostatic tissues as determined by HPLC are displayed in Fig. 1. Comparing healthy with tumor tissue, a decrease of Put and Spm levels can be observed, while Spd levels were rather variable. Especially in tissue of tumors with metastases and in the panel of xenograft models, Spm content was dramatically reduced.

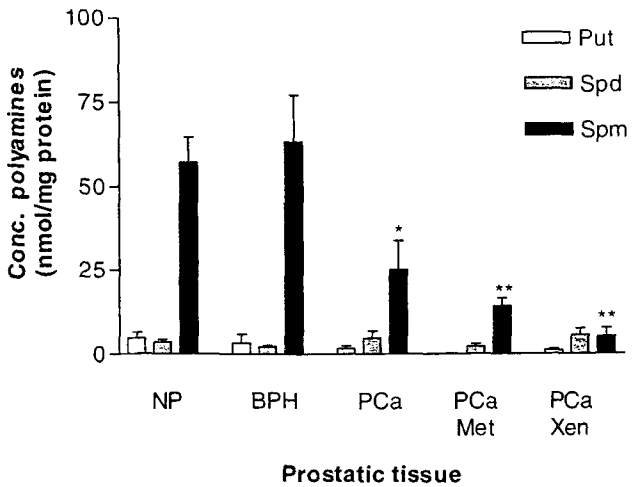

Fig. 1. Average polyamine concentrations (nmol/mg protein) with standard deviations in various prostatic tissues as determined by HPLC. * $P < 0.05$, ** $P < 0.01$ compared with control (NP). NP, Normal prostate; BPH, benign prostatic hyperplasia; PCa, prostate cancer; PCa Met, prostate cancer with metastases; PCa Xen, human xenograft models of prostate carcinoma.

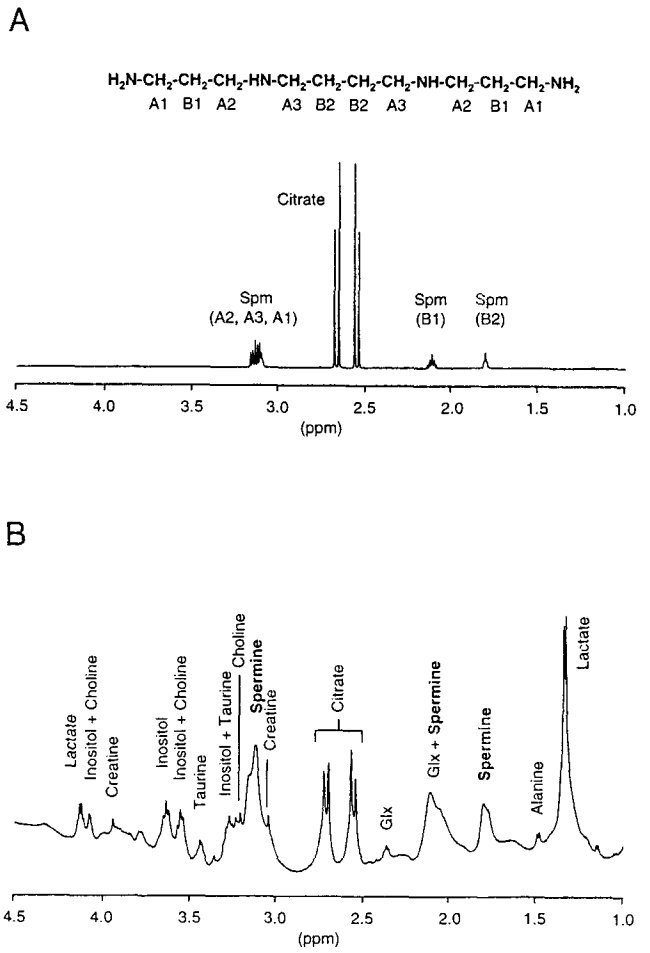

Fig. 2. (A) 600 MHz proton MR spectrum of 90 mM citrate and 18 mM spermine in a solution with various salts (see Section 2). The spermine resonances are labeled A1-3 and B1-2, assignments that correspond to various proton positions in the spermine molecule as indicated. (B) The 600 MHz proton MR spectrum of 260 mg fresh prostatic biopsy material in PBS (pH 7.2). Glx, Glutamine and/or glutamate.

One-dimensional 600 MHz proton MR spectra of spermine solutions and five samples with prostatic biopsy material were acquired to gain insight into the possibilities of using proton MRS for the detection of spermine in prostatic tissue. The in vitro MR spectrum presented in Fig. 2A shows that spermine proton MR signals consisting of several multiplets are present around 1.8, 2.1, and 3.1 ppm. Furthermore, a strongly coupled AB-type multiplet of citrate can be observed around 2.6 ppm. This spectrum closely resembles a 500 MHz proton MR spectrum of expressed prostatic fluid presented by Lynch and Nicholson [23], in which resonances of citrate, spermine and myo-inositol are present. A proton MR spectrum of prostatic biopsy material (Fig. 2B) shows more broadened spermine and citrate resonances. Spermine resonances are present between the singlets of (phospho)choline (3.2 ppm) and (phospho)creatine (3.0 ppm), at around 2.1 and 1.8

ppm. The spermine signal at 2.1 ppm partly overlaps with resonances of glutamine and/or glutamate. In addition, various other signals can be observed, which are assigned to choline, creatine, lactate, inositol, taurine, glutamine/glutamate (glx), and alanine. The spectrum shown was obtained from fresh prostatic biopsy material that had not been frozen.

Fig. 3A,B shows 1.5 T MR spectra of a solution with citrate and spermine obtained by a PRESS localization scheme with echo times of 30 and 120 ms, respectively. These two figures clearly illustrate the effect of J-modulation on the multiplet signals of citrate and spermine. The spectrum obtained with the short TE of 30 ms (Fig. 3A) shows all spermine resonances with dispersive line shapes. However, when using a TE of 120 ms (Fig. 3B), the spermine resonances between 3.0 and 3.3 ppm appear with an absorption-like line shape, while the spermine resonances in the region around 1.8 and 2.0 ppm are barely visible. In addition, the two outer lines of the citrate multiplet have disappeared at TE = 120 ms, a phenomenon which has been described previously [24]. Fig. 3C shows a spectrum of a solution of 90 mM citrate, 18 mM spermine, 9 mM choline, 12 mM creatine and various salts measured with the same TE as the spectrum shown in Fig. 3B. Although the spermine resonances in the spectral region between 3.0 and 3.3 ppm were clearly visible in Fig. 3B, they are not resolved from the resonances of choline (3.2 ppm) and creatine (3.0 ppm) in the spectrum shown in Fig. 3C. An in vivo proton MR spectrum of the human prostate (Fig. 3D) obtained with identical timing of the PRESS localization sequence ($\tau = 11$ ms and TE = 120 ms) shows a comparable resonance pattern in the region between 3.0 and 3.3 ppm. Generally, the peaks present at 3.0 and 3.2 ppm are assigned to creatine and choline. However, as the resonances of spermine also appear in this region (see Fig. 3B), some part of the signal intensity may have to be assigned to spermine. The resonance at 2.1 ppm in the in vivo MR spectrum is situated at a position where a spermine multiplet resonates in the high-resolution MR spectrum (Fig. 2A), but where hardly any signal intensity is present in the in vitro 1.5 T MR spectrum (Fig. 3B). Therefore, this signal is expected to arise from another metabolite than spermine, e.g. from lipid or glutamine/glutamate.

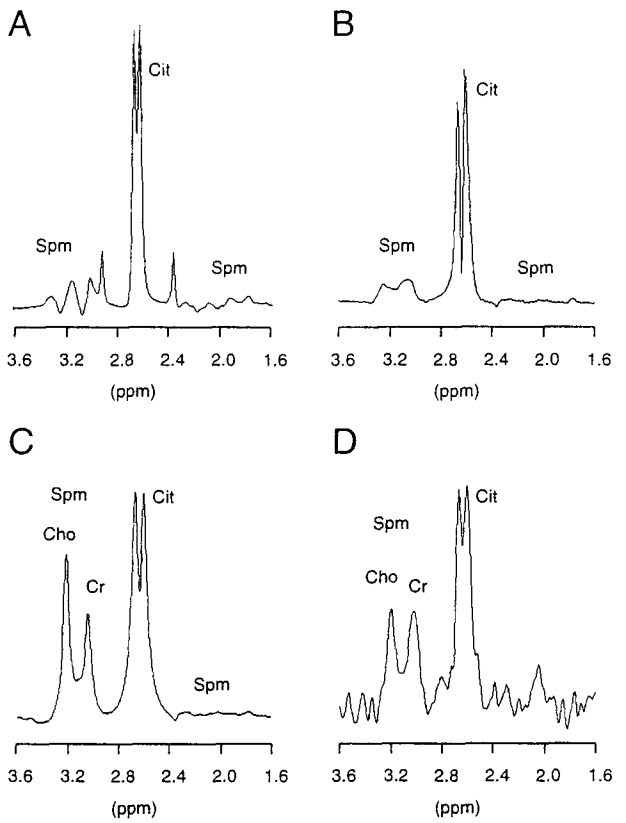

Fig. 3. Proton MR spectra measured at 1.5 T using a PRESS sequence with TE = 30 ms (A) and TE = 120 ms (B, C, D). Spectra were obtained from a solution with 90 mM citrate and 18 mM spermine (A, B), from a solution with 90 mM citrate, 18 mM spermine, 9 mM choline and 12 mM creatine (C), and in vivo from healthy tissue in the peripheral zone of a human prostate (D). Spm, Spermine; Cit, citrate; Cho, choline; Cr, creatine.

As already shown, it is difficult to measure spermine resonances in the prostate by localized single voxel spectroscopy due to J-modulation and overlap with choline and creatine resonances. For that reason, it was tested on biopsy material whether spermine resonances could be observed by 2D J-resolved proton MR spectroscopy, a technique that has been shown to be applicable in vivo [25–27]. In a 2D J-resolved MR spectrum, the multiplet pattern of each resonance along the F1-axis is separated from its chemical shift along the F2-axis. 2D J-resolved MR experiments were successfully carried out at high field on a solution with 90 mM citrate, 18 mM spermine, 9 mM choline and 12 mM creatine (Fig. 4A), and on biopsy material of the human prostate (Fig. 4B). The spectrum of the solution shows the resonance patterns of spermine, citrate, choline and creatine (all labeled in Fig. 4A). The 2D J-resolved MR spectrum of the biopsy material shows, besides these resonances, signals of several other metabolites like inositol, taurine, lactate and glutamine or glutamate. As this spectrum is very crowded, only the resonances of citrate and spermine are labeled in Fig. 4B. In addition, a homonuclear-decoupled spectrum (Fig. 4C) was obtained by summation of the subspectra of the 2D J-resolved spectrum of the biopsy material. In this spectrum, individual multiplet peaks are combined into one resonance (e.g. a single peak at 4.1 ppm corresponds to the quartet of lactate), which results in higher resolution and S/N as compared with a conventional one-dimensional MR spectrum. However, the line widths in this spectrum are relatively broad as resonances in a 2D J-resolved MR spectrum are displayed in magnitude mode.

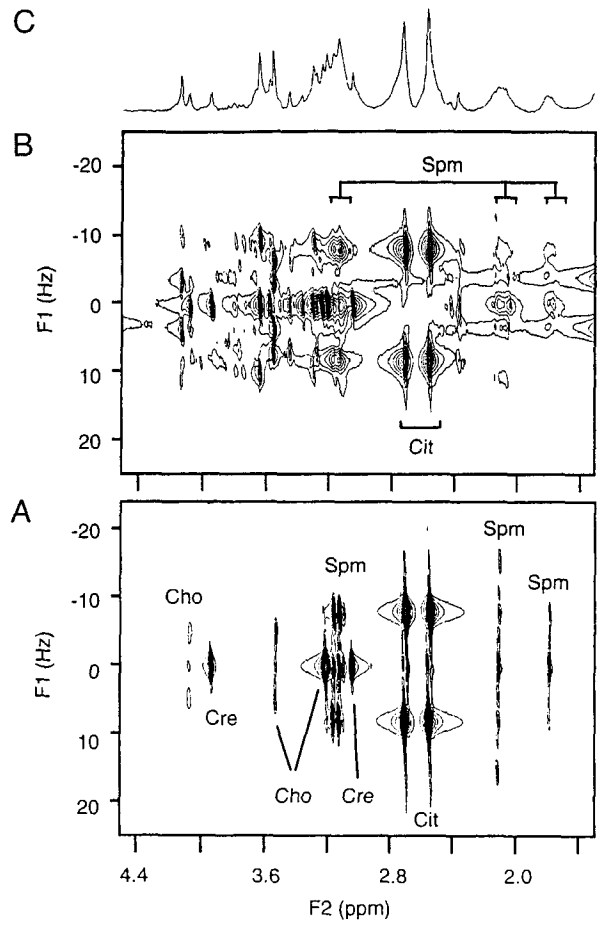

Fig. 4. Sections of 600 MHz 2D J-resolved proton MR spectra of (A) a solution with 90 mM citrate, 18 mM spermine, 9 mM choline and 12 mM creatine, and of (B) 260 mg prostatic biopsy material. (C) The 600 MHz homonuclear-decoupled proton MR spectrum derived from (B). Spm, Spermine; Cit, citrate; Cho, choline; Cr, creatine.

4. Discussion

In this preliminary study, the polyamine content in various prostatic tissues was determined by HPLC and it was investigated whether the presence of spermine in prostatic tissue could be measured by proton MR spectroscopy. Although it has been shown that prostate cancer is characterized by a decreased level of citrate and an increased level of (phospho) choline (for example, [9,28]), we only focused on polyamines in the present study. The reason for this was that the polyamines directly play a role in the regulation of cell proliferation and differentiation (see Section 1). It may be investigated in a future study how changes in spermine concentrations correlate with differences in citrate and choline.

Although only a limited number of tissue specimens have been examined, these results as well as data from others [29] show that normal and benign hyperplastic prostatic tissue contain high Spm levels, which are decreased in tumors. This reduction in Spm may result from shifts in polyamine homeostasis and/or from morphologic changes reducing the ductal volume. Comparing the three polyamines, the Spm concentration is the highest and shows the largest variation. Therefore, the spermine content may be a valuable marker to discriminate prostate cancer from benign prostate hyperplasia and normal prostate. The number of tumor specimens studied was too limited to correlate Spm levels with the degree of malignancy. However, the lowest Spm levels were found in tissue derived from primary tumors that were metastasized and in the human xenograft models, which rarely form ducts. Recent studies in new xenograft models of human prostate carcinoma [30] also showed low Spm levels, in particular in the less differentiated tumors (Dr J.C. Romijn, personal communication).

The results of the one-dimensional high-resolution MR experiments show that resonances of citrate and spermine can be observed in spectra of prostatic biopsy material. However, the relative signal intensities of the citrate and spermine resonances clearly differ from those in the spectrum obtained from a solution with 90 mM citrate and 18 mM spermine. The relative concentrations of citrate to spermine in this solution (5:1) had been chosen in agreement with values reported for human prostatic fluid [15,23]. The relatively lower citrate resonance could be the result of a relatively lower amount of citrate present. Histological analysis of this sample indeed showed the presence of some tumor tissue. Another possibility is that the resonances of citrate are more broadened than those of spermine. In Fig. 2B, the chemical shift δ_{cit} of the midpoint of the citrate multiplet has a slightly higher ppm value, and the chemical-shift difference Δ between the two resonance groups is larger than in Fig. 2A. It has been shown that the values of δ_{cit} and Δ increase by the binding of positively charged cations to the negatively charged groups of citrate [31]. Therefore, positively charged (possibly paramagnetic) cations may be bound to the negatively charged citrate in the biopsy material, resulting in citrate lines that are more broadened than the resonances of the positively charged spermine. Biochemical analysis that determines both the amount of spermine and citrate is necessary to find out the real concentration ratio present in the biopsy material.

Also, in the MR spectra measured at the clinical field strength of 1.5 T, the shape of the citrate signal varies with the absence or presence of other metabolites. Although the field homogeneity was comparable during the measurement of the spectra shown in Fig. 3B–D, the two central lines of the citrate multiplet become less well separated going from a solution with only citrate and spermine, via a solution with also choline and creatine present to the situation in vivo. Overall, the in vitro spectrum in Fig. 3C closely resembles the in vivo spectrum in Fig. 3D. Only the choline peak is increased

compared with in vivo spectra of the healthy prostate, which suggests that the concentration of choline in the solution (9 mM) was relatively too high compared with the in vivo situation. The in vitro spectrum in Fig. 3C was obtained from a solution in which 18 mM spermine was also present giving rise to signal intensity in the spectral region between 3.0 and 3.3 ppm (see Fig. 3B). However, in this spectrum, the spermine resonances are not resolved, but they overlap with the resonances of choline and creatine. This may also be the case in the in vivo spectrum shown in Fig. 3D. Peaks present at 3.0 and 3.2 ppm are generally assigned to creatine and choline, respectively, but some part of the signal intensity may have to be assigned to spermine. In a study by Vigneron et al. [32], it was found that the intensity of the choline peak in in vivo MR spectra of prostate tumor tissue increases going from Gleason pattern score 5 to 8. However, the intensity of the choline resonance in spectra of prostate tumors with Gleason pattern score 5 was slightly lower than in spectra of the healthy prostate. Cell densities can be lower in tumors of intermediate grade than in normal tissue due to mis-organization, but the reduction of the measured choline area may also be affected by changes in the underlying polyamine resonances.

Although we have been able to identify spermine resonances in MR spectra of prostatic tissue by 2D J-resolved spectroscopy, it is not trivial to extend these experiments to the prostate in vivo. First, the resolution at the clinical field of 1.5 T is much lower than the resolution of the spectra obtained at 600 MHz (14.1 T). Second, the low sensitivity at 1.5 T requires long measurement times. Finally, a 2D J-resolved MR measurement in vivo of the prostate is expected to be more complicated than that of the human brain [26,27] as it resides in a less homogeneous environment and it is prone to increased mobility. Some of these problems may be solved with the advent of clinical MR spectrometers with a higher magnetic field strength.

As the spermine concentration in prostatic tissue is much higher than the concentrations of the two other polyamines Put and Spd, the polyamine resonances in the MR spectra shown are labeled as 'spermine'. However, Put and Spd may give rise to some signal intensity at the same resonance positions. It has been shown to be possible to distinguish these three polyamines in cell extracts and biofluids by a $^{1}H/^{13}C$ inverse two-dimensional MR method at high field [33,34]. For prostatic biopsy material, this discrimination may be more difficult or even impossible due to increased line widths.

Narrow linewidth spectra may be achieved by the use of extracts. The disadvantage of this method, however, is the sample preparation method. Metabolites may be lost or degenerated during the extraction procedure. For example, polyamine signals were virtually absent in high-resolution MR spectra of perchloric acid extracts of human prostate tissue measured by our group [35], while signals of polyamines could be observed in high-resolution MR spectra of perchloric acid extracts of prostatic tissue reported by Kurhanewicz et al. [8]. Another possibility is the use of magic angle spinning (MAS) MR spectroscopy. It has been shown by Tomlins et al. [36] and Cheng et al. [37] that high-resolution MAS proton MR spectroscopy can be successfully applied to prostatic biopsy material to obtain narrow linewidth spectra with well-resolved resonances. Presently, our group is studying the possibilities of this method.

5. Conclusions

Although only a limited number of tissue specimens were examined, the HPLC results indicate that normal and benign hyperplastic prostatic tissues are characterized by high levels of spermine, while in human prostatic carcinoma, spermine levels are low. This reduction in spermine level may result from shifts in polyamine homeostasis concomitant with changes in cellular behavior and/or morphologic changes reducing the ductal volume. Hence, a strong decrease in the high concentration of spermine in the prostate could indicate a conversion of prostatic cells from a benign into a malignant phenotype.

The results of the MR experiments show that spermine in prostatic tissue can be detected by MR, in particular by one- and two-dimensional high-resolution MR of biopsy material. Noninvasive determination of spermine in the prostate by in vivo MR is still very difficult but may be easier in the future. The presence of spermine resonance intensity between 3.0 and 3.3 ppm in localized 1.5 T MR spectra complicates the interpretation of signal variations in this region. An increased intensity of the choline signal in spectra of tumor tissue may be obscured by a decreased underlying spermine signal.

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