Metabolic Response of the CWR22 Prostate Tumor Xenograft after 20 Gy of Radiation Studied by \(^1\)H Spectroscopic Imaging


Citigroup Biomedical Imaging Center, Department of Radiology, Weill Cornell Medical College, New York, New York 10021; Departments of Medical Physics [K. L. Z., W. M. S., C. M., Y. C., J. A. K.], Radiology [K. L. Z., J. A. K.], and Medicine [J. A. K.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021; and Hatch NMR Research Center, College of Physicians and Surgeons, Columbia University, New York, New York 10032

ABSTRACT

**Purpose:** The ability to determine the spatial and metabolic distribution of prostate cancer is essential in assessing initial stage, prognosis, and treatment efficacy. Current markers of tumor progression such as prostate-specific antigen (PSA) do not provide spatial information about tumor extent or regions of high metabolic activity.

**Experimental Design:** This study used the androgen-dependent CWR22 human prostate tumor xenograft in mice to characterize metabolic, PSA, and tumor volume changes that occurred with untreated growth or radiation therapy (XRT). One cohort of mice was studied as the tumor grew to 400 mm\(^3\), whereas a second cohort was treated with a single 20-Gy fraction of radiation and studied before and 1, 2, and 4 days after XRT. In both cohorts, tumor volume, PSA, and choline:water ratios measured by nuclear magnetic resonance were monitored.

**Results:** The CWR22 tumor had an untreated tumor-doubling time of 2.6 ± 0.6 days (\(n = 7\)). In untreated mice, PSA strongly correlated with tumor volume (\(P < 0.01, R^2 = 0.99\)). The untreated tumor cohort had a PSA-doubling time of 3.2 ± 0.6 days. Administration of 20 Gy produced a regrowth delay of >15.8 ± 4.8 days (\(n = 6\)). PSA values after XRT were not correlated with post-XRT tumor volume (\(P < 0.20, R^2 = 0.02\)). A constant level of the choline:water ratio (0.010 ± 0.001; \(n = 22, R^2 = 0.007, P < 0.3\)) was observed during the course of untreated tumor growth. A statistically significant (\(P < 0.04, \text{one-tailed} \ t\text{ test}\) 42% decrease in the choline:water ratio at 24 h after administration of XRT preceded observable changes in PSA.

**Conclusions:** Nuclear magnetic resonance spectroscopy provided a method with which to monitor metabolic changes of tumor response to XRT that preceded and predicted PSA and tumor volume changes.

INTRODUCTION

Prostate cancer is the most common malignant tumor in American men excluding basal and squamous cell cancers. An estimated 189,000 men will die of prostate cancer in 2002 representing 11% of all cancer deaths in men, second only to lung cancer. Localized prostate cancer is diagnosed in 70% of confirmed cases, yielding a 5-year relative survival rate of 100% (1). If the tumor has spread outside the prostate, the 5-year survival rate is 34%. These statistics emphasize the need for both nonlocalized markers of early detection such as PSA, as well as markers that provide information concerning specific tumor location and spread. The addition of localized metabolic information via NMR spectroscopy may allow more accurate mapping of tumor extent and characterization of tumor sensitivity to different therapies, providing an early prediction of tumor response (2).

Xenograft implants of the CWR22 tumor growing in nude mice represent a well-characterized animal model of human prostate cancer. The CWR22 prostate tumor line was taken from a stage D, Gleason grade 9 prostatic carcinoma with osseous metastases and is the first reported serially growing primary prostatic carcinoma (3). Among the standard prostate tumor lines, CWR22 has the least deviated set of chromosomal aberrations from human prostatic carcinomas (4). The CWR22 prostate tumor mimics the pattern of human prostate cancer regression in both size and PSA after androgen withdrawal with tumor shrinkage followed by a subsequent regrowth stage (5). This model allows the study of tumor behavior in both androgen-dependent and -independent states. The CWR22 prostate line regresses up to 50% in tumor volume and 3000-fold in PSA after androgen withdrawal. This tumor has not been examined for response after XRT.

XRT is an effective antineoplastic treatment for prostate cancer, although its clinical efficacy decreases with increasing stage. Advances in intensity-modulated radiotherapy, three-dimensional conformal radiotherapy, as well as high-dose-rate brachytherapy have allowed higher doses to be delivered to...
specific tumor regions while sparing healthy tissues (6, 7, 8). This emphasizes the need to be able to detect and localize tumor noninvasively. Various reasons for failure of XRT include extensive tumor bulk, micrometastatic disease, tumor microenvironment, and inherent cell radioresistance (9). Studies of tumor metabolism, PSA, and growth in the CWR22 line may provide predictive information on tumor physiology and prognosis during therapy that may eventually be useful in evaluating patient response to XRT.

Metabolic changes in prostate tumors in clinical NMR studies after XRT may be better understood by studying similar responses to therapy in human tumor xenografts transplanted in nude mice. Three primary metabolites with biochemical significance have been detected in human prostatic cancer: choline, creatine, and citrate (10). The choline resonance detected by proton NMR contains contributions from multiple species including choline, phosphocholine, and glycerophosphocholine (11, 12). The increase of phosphocholine and decrease of glycerophosphocholine in relation to tumor growth may be caused by up-regulation of choline kinase in response to increased phospholipid metabolism demands (13). Increased choline levels are measured in malignancies compared with normal tissue (14). The combined peaks of creatine and phosphocreatine are directly related to energy metabolism. The healthy human peripheral zone of the prostate exhibits a large citrate resonance because of secretion and accumulation of citrate by epithelial cells that is caused by limited oxidation in the Kreb cycle. Kurhanewicz et al. (15, 16) have determined in the in vivo human prostate, (choline + creatine)/citrate may be used to detect cancer with up to 98% accuracy within the peripheral zone of the gland. NMR spectroscopy also potentially provides an opportunity to noninvasively monitor changes in tumor metabolism that occur in response to therapy.

MATERIALS AND METHODS

Tumor Model. The tumor line studied was the androgen-dependent CWR22 prostate tumor line, a gift from Dr. Thomas Pretlow at Case Western Reserve University. The tumor arrived as frozen tissue and was subsequently immersed in a solution containing 20% bleach for 5 min and then washed with sterile water and placed in a solution of 75% ethanol for 5 min. The tissue was then minced with a scalpel and abraded against a 70-μm pore size nylon cell strainer immersed in RPMI 1640 containing 20% bleach for 5 min and then washed with sterile water. The excised tumor was crushed to a powder in a liquid nitrogen-cooled mortar and mixed with ~3 times the volume of 10% perchloric acid. The mixture was thawed and refrozen three times and centrifuged for 15 min. The freezing/thawing steps taken in the extract procedure were performed as an added step to ensure that any remaining particulate matter would precipitate out of the sample before centrifugation. The resulting mixture was buffered with potassium carbonate and titrated to pH 7.3 using potassium hydroxide. The precipitate was removed by centrifugation, and the sample was combined with Chelex-100 (Sigma-Aldrich, St. Louis, MO). After removal of the Chelex via centrifugation, the sample was frozen and lyophilized. The extract was reconstituted with 99.8% deuterium oxide and 2,2-dimethyl-2-silapentane-5-sulfonate as a 1H NMR chemical shift reference.

PSA Measurements. With a nonheparinized capillary tube or pipette, 100 μl of blood were collected from the mice via the orbital venous sinus to obtain 50 μl of serum. Methoxyflurane (Metofane) was used as a general anesthetic during the procedure. An antibiotic ophthalmic ointment was applied after phlebotomy. The collected blood specimen was allowed to clot and centrifuged to collect serum. Serum PSA measurements were conducted using the 96-well PSA Enzyme-Linked Immuno Sorbent Assay ELISA Test Kit (ALPCO Diagnostics, Windham, NJ). The system used rabbit anti-PSA antibody against intact PSA that was conjugated to horseradish peroxidase. Absorbance was measured spectrophotometrically at 450 nm using an ELISA plate reader (Bio-Rad). Reference standards of 0, 2, 4, 15, 60, and 120 ng/ml were used to calibrate the results.

External Beam Irradiation. External beam irradiation studies were administered using a Phillips MG 324 X-irradiation unit (Mahwah, NJ). The operational parameters were 320 kVp at 10 mA with a 5-mm copper filtration sheet. The field size was 10 × 10 cm at a distance of 50 cm between the source and surface for a dose rate of 150 cGy/min. Calibration of the unit is regularly performed using a Victoreen ionization chamber at

\[ V = \frac{\pi}{6} d_1 d_2 d_3 \]
a 50-cm source to surface distance and an exposure rate of 0.0405 coul/kg/min. A dose of 20 Gy was used for a single high dose treatment for a total time of 13.4 min. Animals were anesthetized (for duration of the irradiation) using ketamine (100 mg/kg) plus xylazine (10 mg/kg) via an i.p. injection. They were placed in a shielded lead apparatus to specifically isolate the tumor while providing protection to the rest of the body.

**In Vivo NMR Methods.** In vivo experiments acquired on a 4.7 T Bruker Omega CSI scanner used a two-turn Teflon-coated solenoidal coil. The tumor was submerged in a water bath of 37°C to improve field homogeneity (FWHM <0.1 ppm or 20 Hz) and maintain core body temperature (18). The pulse sequence used for acquisition of spectral data were the BAnd-Selective Spin echo Acquisition for Localized Editing sequence, which achieves single-shot three-dimensional localization by using two-dimensional OVS followed by an orthogonal 90-degree slice-selective excitation pulse (19).

Before spectroscopic analysis, an axial localizer image was taken using a repetition interval of 300 ms and an TE of 20 ms with a 32-mm field of view and a 4-mm slice thickness. This image allowed graphical prescription of the slice offset in the read direction that was required to study the center of the tumor. Slice thickness was fixed at 4 mm for all subsequent studies. A coronal image was taken allowing the region of OVS, to be determined for the tumor. The OVS region included arising from extraneous skin, lipid, and water near the coil by saturating a band of set thickness every 45 degrees around the center of the tumor. Elliptical regions of suppression were also used to account for variations in tumor shape.

Experimental CSI parameters included a 2000-Hz spectral width, 512 points, 1.0 s/136 ms repetition interval/TE, a 4-mm slice thickness, and a 32-mm field of view. The TE of 136 ms allowed for additional decay of the lipid signal, reducing contamination in the metabolic region of interest. Two-dimensional chemical shift imaging in the coronal plane used a 16 × 16 matrix size and eight averages yielding a total scan time of 34 min. A water reference CSI set required two averages for an additional 8.5 min. In vivo studies were done without treatment up to an approximate tumor volume of 400 mm³. In addition, further studies were done before XRT and at 1, 2, and 4 days post-XRT on eight mice having an average initial tumor volume of 186.9 ± 13.1 mm³.

**In Vitro NMR Methods.** To accurately assign in vivo resonances for the CWR22 line, high resolution proton spectra were acquired using a 9.4 T Varian Unity magnet operating at 400 MHz at a temperature of 20°C. Experimental parameters used included a sweep width of 4000 Hz, 8192 points, a 90-degree flip angle of 13.8 °, and 32 averages for a total experiment time of 8 min. A 15-s repetition time allowed complete relaxation of the in vitro 1H resonances before consecutive excitation pulses to eliminate saturation effects. Single pulse suppression techniques were used to eliminate any residual water signal. Tentative peak assignments were based on published standard values (20–23). Choline and creatine resonances were identified in vitro, as were myo-inositol, citrate, and other amino acids (24). Citrate is found in healthy human prostate and has been detected only at low levels in animal models. Correct identification of resonances was verified by signal intensity increases on addition of high concentration samples of pure metabolites.  

**Spectral Analysis.** Spectra were transferred from the Omega 4.7 T magnetic resonance imaging to an SGI Octane workstation for analysis using XsOsNMR software (developed by X. Mao and D. C. Shungu, Columbia University). Data were converted from the Omega format to XsOs format and Fast Fourier transformation using 3-Hz exponential filtering. Voxel shifting in both x and y dimensions was performed before Fast Fourier transformation to place a 3 × 3 grid of voxels in the center of the tumor. The processed CSI set was overlaid on the OVS image of the tumor. Spectra were zoomed to between 2.0 and 4.0 ppm and autophased. Manual phasing was performed on voxels that appeared to have a shift in the baseline. Peak picking was performed manually on a single voxel in the center grid and then automatically for other voxels within a fixed frequency range in the central region. Frequency domain fitting with a Levenberg-Marquardt algorithm was performed using a Lorentzian function optimizing frequency, amplitude, and width. Peak area was calculated, and spectral accuracy was evaluated visually via the residual as well as by eliminating fits having peak widths <1 Hz or >30 Hz. Identical fitting procedures were used to calculate the peak area of the unsuppressed water spectra.

Both cohorts of mice were evaluated by analyzing a 5-voxel cross pattern in the center of the 3 × 3 grid. The data presented are based on 5 voxels instead of 9 as there was concern that the proximity of the outer volume suppression bands to the tumor rim would preferentially eliminate signal and cause an error in the choline:water ratio, particularly in smaller tumors. The choline peak area for each voxel was measured individually and divided by the peak area of the unsuppressed water spectrum for the identical voxel. These values were averaged to produce a single value for each tumor. A correction factor was derived to account for differences in the number of excitations (NEX) and receiver gains between the suppressed and unsuppressed acquisitions. The final ratio was calculated as:
results

Initial studies focused on determining the growth rate of the CWR22 tumor. Tumor volume was measured on an untreated cohort of mice from when the tumor was initially palpable until approximately 400 mm³ and plotted versus time in Fig. 1. Tumor-doubling time of 2.6 ± 0.6 days was measured. As shown in Fig. 1, the cohort of mice treated with 20 Gy had a pretreatment volume of 78.2 ± 4.2 mm³ (day 2), which then decreased to 53.9 ± 10.2 mm³ (day 5) and 48.2 ± 11.2 mm³ (day 8) before rising. Thus, tumor shrinkage was noted, and these changes are statistically significant between day 2 and day 5 (P < 0.014) as well as day 2 and day 8 (P < 0.012). A gradual regrowth stage was observed through day 19. Tumors continued to grow, but the data are not presented because by the subsequent day of measurement (day 22), two mice had been sacrificed. The regrowth time, i.e., the time for the tumor to grow to its pretreatment volume, was >15.8 ± 4.8 days. The regrowth delay as estimated from Fig. 1 appears to be <15.8 ± 4.8 days, but tumor volumes in the figure are dominated by two tumors that had grown very quickly; therefore, individual measurements provide more accurate assessment than the mean volume of the group. Because one tumor had not regrown to its initial volume at the time of sacrifice, the regrowth delay represents an underestimate.

Untreated tumor growth was compared with PSA changes in Fig. 2, yielding a statistically significant (P < 0.01) linear correlation given by:

\[
\text{PSA} = 0.74 \times 13.9 \times (\text{tumor volume} (\text{mm}^3))
\]

The equation reveals a γ-intercept corresponding to a tumor volume very nearly equal to zero, reconfirming that the CWR22 tumor model expresses increasing PSA levels with tumor burden as seen in prostate cancer patients. PSA was plotted versus tumor volume for the treated group (Fig. 2) after XRT; however, no significant correlation was found (P < 0.20, R² = 0.02).

PSA levels were plotted versus time for both treated and untreated cohorts as shown in Fig. 3. Untreated tumor growth showed an increase in PSA over time yielding a doubling time of 3.2 ± 0.6 days. The group treated with 20 Gy on day 3 showed an increase in PSA on day 9 (P < 0.035) with a slight (not significant) decrease in PSA on day 13. The PSA values reached a plateau on day 9, with subsequent values measured on days 13 and 20 not being significantly different from day 9. The PSA for the treated group on day 9 [141.1 ± 36.8 ng/ml (mean ± SE; n = 7)] did not show a statistically significant difference compared with the untreated group (126.2 ± 40.8 ng/ml; n = 6), indicating that there was no evidence of a PSA response on day 9. In comparison, on day 13 the PSA for the treated group was 118.0 ± 46.8 ng/ml versus 252.1 ± 28.0 ng/ml (day 12) for the control group which is significant (P < 0.028 one-tailed), indicating that by day 13 a PSA response was noted.

Fig. 4 shows a typical 3 × 3 spectral data grid from a single tumor before and 1, 2, and 4 days after single-dose 20-Gy XRT with the 5-voxel cross-region of analysis highlighted. To facilitate visual comparison of the time points for which NMR
observations were made, all spectra in Fig. 4 are scaled to that of the baseline scan. Proton resonances that were consistently seen in vivo were: myo-inositol (3.55 ppm); choline (3.22 ppm); and creatine (3.03 ppm). As demonstrated in Fig. 5, citrate was detected in several tumors in vivo and confirmed in two of nine high resolution NMR of in vitro perchloric tumor extracts. Twenty-four hours after administration of XRT, a decrease in metabolic activity was observed. Specifically, all peaks except choline were reduced 24 h post-XRT to the point that low signal:noise levels made them difficult to fit. Qualitatively in Fig. 4, the choline peak is also markedly reduced at 24 h post-XRT.

The effect of increasing tumor volume on the choline:water ratio taken from a 5-voxel cross on an untreated cohort (n = 22) is displayed in Fig. 6. The choline:water ratio shows no change over the volume range of 100–400 mm³. The ratio was not correlated with untreated tumor volume and had a mean value of 0.010 ± 0.001 (n = 22, R² = 0.007, P < 0.3). Fig. 7 shows the
time evolution of the choline:water ratios over time for the treated cohort of mice ($n = 8$) after XRT. A statistically significant decrease of 42% ($P < 0.04$; one-tailed $t$ test) was detectable 24 h after 20 Gy of XRT. The decreases at 48 and 96 h post-XRT were not significant ($P < 0.09$, $P < 0.26$, respectively). If all 9 voxels from the central $3 \times 3$ grid are individually analyzed and the average computed, a similar decrease between the baseline and 24 h post-XRT is observed.

**DISCUSSION**

Previously reported investigations have shown changes in $^1$H and $^{31}$P metabolites in tumor systems undergoing radiation and chemotherapy. Studies have shown that after a priming dose of radiation or after treatment with carbogen and perfluorochemical emulsions, $^{31}$P NMR data can be used to determine the treatment time wherein maximal response to a second dose of XRT is obtained (27, 28). Significant metabolic changes were also observed in the CWR22 tumor line after XRT in this study. The RIF-1 tumor line has previously been studied for changes in lactate and choline levels after 2, 4, and 20 Gy of irradiation (29). Decreases in lactate:water levels at 48 h were apparent for all doses of radiation. Only the 20-Gy XRT produced significant decreases in tumor lactate levels at 24 h post-XRT in the RIF-1 line. The study revealed no significant changes in choline:water levels. In comparison, our data reveal a 42% decrease in the choline:water ratio at 24 h post-XRT for the CWR22 tumor xenograft model. A similar study examined changes in lactate levels after 4, 10, and 20 Gy of irradiation in the EMT6 breast tumor line (30). No changes in metabolite levels were observable for the 4-Gy dose of XRT. Decreases of 21 and 40% in tumor lactate levels were shown at 48 h after 10 and 20 Gy of XRT, respectively. The variances in metabolic tumor response for identical XRT doses were attributed to differences in hypoxic fraction and radiation sensitivity between the lines.

Several clinical studies have shown the utility of monitoring brain metabolites with MRS after XRT (31, 32). These studies suggest the possibility of using MRS metabolic markers as indicators of tumor response to various forms of therapy. Furthermore, changes exhibited by these markers have been shown to precede those seen in tumor volume. This was observed in our study as a significant metabolic decrease after XRT in contrast to a lack of change in tumor volume and PSA for the same time frame in control mice. Examination of MRS metabolic markers may prove to have clinical utility in evaluating therapeutic efficacy.

The CWR22 tumor model has been studied by several groups in relation to changes seen after ADT (33, 34). Decreases in tumor PSA were shown to precede those in tumor volume after ADT. In untreated tumors, changes in PSA and tumor volume were correlated. However, in the case of response to radiation, there was no apparent correlation between tumor volume and PSA as shown in Fig. 2. It is difficult to compare the effects of ADT and radiation because in the former, an effect would be expected quickly because the tumor requires androgens for growth, and there is a direct relationship between tumor volume and PSA. In contrast, cell kill after radiation often requires several cell cycles until mitotic death ensues; thus, a delay in PSA decrease and tumor shrinkage would be expected (35). Because tumor growth is dependent on testosterone, one would expect a rapid PSA drop after androgen withdrawal. Nevertheless, in the study of Agus *et al.*, they did not detect a significant drop in PSA at 8 days post-ADT. In the current study, PSA did not stabilize until day 13, 10 days after radiation, suggesting that the temporal sequence of PSA reduction post-treatment with ADT or radiation in this model is unexpectedly similar. Both positron emission tomography and magnetic resonance spectroscopic imaging changes were noted to occur before changes in PSA with ADT and irradiation, respectively. Kim *et al.* (36) noted that changes in cellular proliferation and staining for androgen receptor occurred rapidly after androgen deprivation (days 6 and 2, respectively), although in their study decreases in PSA after castration were noted by day 4. Thus, the imaging modalities detected changes in tumor biology which are likely to be attributable to changes in cellular physiology that occurred before PSA changes. The magnetic resonance spectro-
scopics imaging data showed a rapid change posttherapy suggesting possible utility as an early indicator of tumor response.

The period of regrowth in tumor volume post-XRT was not reflected in the PSA values (Fig. 3). PSA values continued to increase after administration of XRT from day 3 to day 9 ($P < 0.035$) and eventually reached a plateau. This was unexpected, based on the assumption that serum PSA levels correlate with tumor volume, as is the case for the untreated CWR22 tumors. Because PSA values were not measured until 6 days post-XRT, it is possible that a transient early decrease in PSA was missed. This is unlikely because on day 9 there was no difference between control and treated groups, but a difference in PSA did occur by day 13 and subsequently, suggesting that the PSA response was delayed. This observation is of interest in clinical studies wherein PSA was transiently or mildly elevated during XRT, sharply decreased after therapy and then plateaued (37). This pattern is similar to this study of the CWR22 line after XRT, confirming the possibility of mimicking changes in PSA that are clinically relevant after XRT.

Spectroscopic analysis of data as shown in Fig. 4 displayed heterogeneity within the individual tumor as well as temporal changes within the same tumor in response to therapy. Analysis incorporated calculations of peak areas that corrected for variations in intervoxel shim quality. Division of the choline area by the corresponding unsuppressed water peak area for each voxel eliminated variances in the ratio because of field heterogeneities. Intervoxel changes in peak area may then be attributed to other sources of variation including true metabolic heterogeneity within the tumor and possible loss of signal due to the saturation profile of the outer volume suppression pulses.

Analysis of metabolic data as acquired via NMR reveals a constant choline:water ratio during untreated tumor growth (Fig. 6). This is important as changes in the ratio may then be attributed to therapy alone and not to variations in tumor volume. The treated cohort exhibited a statistically significant decrease of 42% in the choline:water ratio 24 h after administration of 20 Gy of XRT (Fig. 7). The ratio subsequently increased slightly at both 2 and 4 days after XRT, although it did not reach its pretreatment value during the period of measurement. The increase of the choline:water ratio toward that of pre-XRT values suggests that 20 Gy produced an incomplete tumor response as reflected by subsequent tumor regrowth. The MRS data may predict changes in tumor response before that shown by PSA. Multivoxel proton spectroscopy of the prostate is a noninvasive method of evaluating localized tumor response (38). Future studies with the CWR22 tumor line and MRS may provide further insight into questions of tumor response to androgen withdrawal and chemotherapy.

**Conclusion**

Our data suggest that current clinical questions concerning metabolic decreases as a result of XRT may be explored using the CWR22 tumor model, PSA levels, and $^1$H NMR spectroscopy. This model allows one to measure concurrent volume regression and PSA expression in conjunction with measured metabolic changes attributable to therapy. Metabolic changes as studied with MRS were shown to precede those seen in PSA.

**ACKNOWLEDGMENTS**

We are grateful for the gift of the CWR22 tumor line from Dr. Thomas Pretlow of Case Western Reserve University.

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