Abstract. Background: Increased uptake and metabolism of glucose is a characteristic of malignant transformation. Overexpression of glucose transporters, especially Glut-1, is a common event in human malignancies. To date, little is known about the role of Glut-1 in human prostate cancer (PC). The aim of this study was to investigate the expression of Glut-1 both in PC cell lines and clinical specimens of primary PC. Materials and Methods: The PC cell lines DU145, PC3 and LNCaP were assessed for Glut-1 mRNA expression by Northern blot analysis. In a total of 45 primary PC specimens, radioactive (35S) in situ hybridizations (RISH) for Glut-1 mRNA expression were performed on frozen sections. Quantification of Glut-1 expression was obtained by use of an image analysis system. Results: Glut-1 expression was detected in all 3 cell lines. Expression in the more poorly-differentiated cell lines DU145 and PC3 was even higher than in the hormone-responsive LNCaP cell line. In situ hybridizations in primary PC revealed Glut-1 expression just above the detection limit in well-differentiated tumors. Significantly increased Glut-1 expression was detected in moderately- to poorly-differentiated PC. Conclusion: Glut-1 is expressed in PC cell lines and primary PC. The level of expression increases with advancing grade of malignancy. These findings support a role for Glut-1 in PC proliferation.

In 1930, Warburg first described increased glucose metabolism in cancer cells. Key regulatory glycolytic enzymes have been found overexpressed in tumor cells (1,2). One of these enzymes is glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In the Dunning Prostate Cancer Model, consisting of different cell lines with a range of metastatic potential, a correlation between GAPDH expression and cell motility and metastatic potential was reported (3).

Glucose uptake across the cell membrane is considered the rate-limiting factor for glucose metabolism in tumor cells (4). Glucose uptake from blood is mediated by facilitated-diffusion transporters (Glut). Several isoforms have been isolated, all being more or less specific for certain types of cells. The human erythrocyte glucose transporter (Glut-1) appears to have a particular role in malignant transformation and progression. In liver tumors, a shift in glucose transporter gene expression during neoplastic transformation was noted. Glut-1 expression has been found to be significantly increased during the late stages of hepatocarcinogenesis (5).

Investigators have studied the expression of different glucose transporters in human malignancies and overexpression of Glut-1 has been reported for several types of cancer. Among these are tumors of the lung, stomach, thyroid, colon, esophagus, bladder and breast (2, 6-11). Glut-1 overexpression has been found to be associated with tumor progression (2, 12-14).

In the clinical setting, glucose uptake by tumor cells is evaluated by means of positron emission tomography (PET) with 18fluorine (18F)-labelled deoxyglucose (18F-FDG). Increased FDG-uptake has been successfully used for both detection of malignancy as well as an indicator of adverse prognosis in different tumors (9,15).

To date, there has been only one study evaluating the expression of Glut-1 and Glut-12 in prostate carcinoma (16). Immunohistochemistry failed to detect the expression of Glut-1 in 3 clinical specimens of primary PC.

To further evaluate the role of Glut-1 in PC, three PC cell lines and an extended series of primary PC specimens, as well as benign prostatic tissues, were included in the study presented here.

Materials and Methods

Cell lines and tissues. The prostate cancer cell lines DU145, PC3 and LNCaP are maintained at the Urological Research Laboratory in Nijmegen, The Netherlands. A total of 45 evaluable prostate adenocarcinoma specimens were obtained from patients...
undergoing radical prostatectomy. Benign prostatic tissue was retrieved from cystoprostatectomy specimens in 2 patients undergoing radical surgery for bladder cancer. Tumor samples and benign tissues were excised from the gland, snap-frozen immediately in the operating room and stored at -80°C until further examination. All specimens were examined by a pathologist (S.H.) after hematoxylin and eosin staining of frozen sections. Prostatic adenocarcinomas were classified according to the Gleason grading system (17).

**RNA isolation and Northern blot analysis.** The basis for the cDNA probe we used in these studies was the 2400 bp fragment found in clone lambdaGT25, described by Mueckler and coworkers (18) and isolated after cloning a cDNA library isolated from the HepG2 cell line. The original cDNA probe was used in Northern analysis of human normal prostatic tissue as well as human PC cell lines, representing different states of differentiation with regards to tumorigenicity in nude mice and androgen-dependency.

Total RNA from tissues was isolated using the lithium-chloride/urea procedure as described by Auffray and Rougeon (19). For Northern blot analyses, 10 µg of total RNA was glosylated, size-fractioned on 1% agarose gels (illumination of gels at 254nm for analysis of banding intensity confirmed equal RNA load) and transferred to Hybond-N+ nylon membranes (Amersham, Buckinghamshire, England). Hybridization was carried out with the random prime labelled 2400 bp cDNA probe from the plasmid lambdaGT25. Hybridization experiments were performed according to Church and Gilbert (20). In short, the membranes were preincubated in hybridization buffer (7% SDS, 1% BSA, 0.5M sodium phosphate buffer pH 7.2, 1mM EDTA and 100 ng/ml salmon sperm DNA) for at least 5 hours at 65°C. Hybridization was carried out in hybridization buffer without salmon sperm DNA and the [32P]-labelled probes were added to a maximum of 1x10⁶ dpm/ml (2-5 ng probe/ml) and the membranes were hybridized overnight at 65°C. After hybridization the membranes were washed in buffers containing 1% SDS, 1mM EDTA and decreasing concentrations of sodium phosphate buffer pH 7.2; (0.5M, 0.25M and 0.1M, resp.). Each step was performed at 65°C for a period of 30 minutes. After autoradiography of the membranes, dehybridization of the membranes was performed as recommended by the manufacturer (0.1% SDS).

**Preparation of frozen sections for hybridization.** Serial sections from frozen tissues were cut on a cryostat at 6 µm, mounted on poly-L-lysine slides and heated in a microwave oven for 30 seconds at 750 W, 2450 MHz for fixation (21). The sections were rinsed 2x1 minute in PBS (1xPBS = 137mM NaCl, 2.7mM KCl, 8.1mM Na2HPO4, 1.5mM KH2PO4, pH 7.0), washed for 5 minutes in glycerine/PBS and incubated in 0.3% Triton X-100/PBS for 10 minutes. After rinsing for 1 minute in PBS, the sections were post-fixed in 4% parformaldehyde/PBS for 5 minutes, rinsed in PBS and acetylated in freshly prepared 0.25% acetic anhydride/0.1M triethanolamine (pH8) for 10 minutes. The slides were then finally dehydrated in gradually increasing concentrations of ethanol prior to hybridization.

**Probes used for hybridization.** Recloning the 2400 bp cDNA fragment into pGEM-3zf (Promega) containing SP6 and T7 phage promoters, resulted in the plasmid pTB-1, enabled us to synthesize sense- and anti-sense mRNA, which was used in the RNA-RNA hybridization protocols described below.

To test the preservation of RNA, we used a 28S ribosomal RNA (rRNA)-specific probe cloned in pGEM-II (Promega).

**Preservation of RNA.** In order to judge for RNA preservation in the tissues, samples were hybridized with sense- and anti-sense 28S rRNA probes. Samples with poorly or no preserved RNA were rejected from analysis, i.e. the number of silver grains after radioactive hybridization with the 28S rRNA anti-sense probe had to exceed 10x background.

**Preparation of [35S-UTP]-labelled RNA probes and hybridization.** The plasmid pGEM-3zf, containing the 2400 bp GLUT 1 fragment (pTB1), was digested with Xba to generate a template for anti-sense transcription (anti-sense RNA) by means of T7 RNA polymerase. Alternatively, digestion with Bst-X1 resulted in a template for sense probe synthesis (used as negative control) by means of SP6 RNA polymerase (sense RNA). [35S]-UTP-labelled single-stranded probes were prepared according to Melton (22) and Brysch (23), degraded to an average length of 200 nucleotides by alkaline hydrolysis (24), precipitated in ethanol and resuspended in 100 ml TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA). The specific activity of the radio-labelled probes, aliquoted and stored at -80°C, was approximately 1x10⁷ cpm/μg template DNA. Twenty-five μl of the hybridization buffer (50% deionized formamide/2xSSC (300mM NaCl, 30mM sodium citrate)/10% Dextran Sulphate/5x Denhardt’s= 5g Ficoll 400, 5g polyvinyl pyrrolidone, 5g bovine serum albumin in 500 ml H2O)/10mM DTT/1mg/ml tRNA) was mixed with 2x10⁶ cpm of [35S]-UTP-labelled probes and applied to the sections. After covering the sections with cover-slips, overnight hybridization was performed at 46°C. Cover-slips were removed in 2xSSC buffer at room temperature, washed at 46°C in decreasing concentrations of SSC (2x20 min. 2xSSC, 2x30 min. 1xSSC, 2x30 min. 0.5x SSC). In order to reduce background due to non-specific binding, 1mM DTT, 1% thiosulphate and 14mM β-mercaptoethanol was added to the washing solutions. The sections were finally dehydrated, dried and dipped in LM-1 film emulsion (Amersham) for high resolution microautoradiography diluted 1:1 in Milli-Q water. After an exposure time of 5 days in tightly closed boxes at 4°C, the microautoradiographs were developed in Kodak D-19 developer (4 minutes), washed in water (20 sec), fixed in 24% thiosulphate (w/v, 4 min), rinsed in water, counterstained briefly (1 min) in hematoxylin and mounted in Permount medium before microscopic examination.

**Quantitation by image analysis.** The quantitation by image analysis, as well as the statistical analysis, were essentially as described before (25). The image analysis system consisted of a video camera mounted on a routine light microscope and a personal computer equipped with a framegrabber board (VFG Visionplus-AT, Imaging Technology Inc., Bedford, MA, USA). The output image was presented on a video. Software was written in TIM-image analysis language (TEA, Dordrecht, The Netherlands). For each tumor area 10 images were recorded at 40x magnification. A Laplace filter was applied for grain detection. The mean number of grains per image was calculated for each slide. From these data the following score for in situ staining was derived:

\[ \text{Score } S = \text{mRNA Glut}^+ \times \text{mRNA Glut}^- \]
Thereby the expression of Glut(+) mRNA (anti-sense hybridization) minus its negative control Glut(-) (sense-hybridization) is calculated.

Statistical analysis. For a comparison of the means corresponding to three groups (Gleason grade 1-2, 3 and 4-5), the analysis of variance (F test) was performed on the S score (see above).

Results

GLUT-1 expression in normal human prostate and cell lines. Northern analysis was performed with RNA samples derived from normal human prostate as well as from three different human prostatic tumor cell lines in order of decreasing differentiation, i.e. LNCaP, a hormone-sensitive cell line and the DU-145 and PC-3 = hormone-independent cell lines. No detectable expression in normal prostate. Glut-1 expression is noted in all PC cell lines, higher expression in hormone-independent cell lines DU-145 and PC3.

GLUT-1 expression in primary PC. GLUT-1 mRNA expression was evaluated in 45 primary human prostatic adenocarcinomas divided into three groups (A, B and C) according to their state of differentiation. Group A consisted of 6 well-differentiated tumors (Gleason grade 1-2). Group B included 25 moderately-differentiated tumors (Gleason grade 3) and Group C consisted of 14 poorly-differentiated tumors (Gleason grade 4-5).

On the basis of rRNA hybridizations (anti-sense rRNA), 96% of the frozen samples showed adequate preservation of RNA. Table I summarizes the GLUT-1 expression illustrated as the mean ± standard deviations of S values for Gleason grade 1-2 (Group A), Gleason grade 3 (Group B) and Gleason grades 4,5 (Group C) tumors, respectively.

Gleason grade 1-2 tumors (Group A): All but 1 Gleason grade 1-2 tumors studied showed signals just above the detection limit. Signals were located in the tumor cells within the glands and the mean expression level was 149±124. Signals obtained by RISH in nonmalignant parts did not exceed that of background. We concluded that GLUT-1 expression was below the limits of detection in these areas.

Gleason grade 4-5 and anaplastic tumors (Group C): GLUT-1 RISH in this group of tumors resulted in higher ‘grain density’ as compared to Gleason grade 1-2 and Gleason grade 3 tumors (Figure 3). The mean S value for this group was 1518±597.

Statistical analysis. Table I summarizes the statistical analysis of these data, and significance was found for differences in expression levels for Groups A and B (Gleason grade 1-2 / Gleason grade 3) and Groups A and C (Gleason grade 1-2 / Gleason grade 4). No statistically significant difference was found between expression levels in Groups B and C (Gleason grade 3/ Gleason grade 4-5) (p<0.1).

Discussion

Analysis of glucose transporter expression in different tumor systems has revealed an important role for Glut-1 during

Table I. GLUT-1 expression as determined by RISH and statistical analysis.

<table>
<thead>
<tr>
<th>Gleason grade</th>
<th>No. of tumors</th>
<th>S-score (mean values ±S.D.)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G 1-2</td>
<td>6</td>
<td>149 124</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>G 3</td>
<td>25</td>
<td>891 582</td>
<td></td>
</tr>
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<td>G 1-2</td>
<td>6</td>
<td>149 124</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>G 4-5</td>
<td>14</td>
<td>1518 597</td>
<td></td>
</tr>
<tr>
<td>G 3</td>
<td>25</td>
<td>891 582</td>
<td>p&lt;0.1</td>
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<td>G 4-5</td>
<td>14</td>
<td>1518 597</td>
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neoplastic transformation and tumor progression (10,13,26,27). Up-regulation of Glut-1 expression may reflect the increased glucose consumption required for rapid proliferation of tumor cells. The overexpression of Glut-1 on Northern analysis of the PC cell lines demonstrated here would appear to be in line with this concept. Furthermore, even higher Glut-1 expression was found in the hormone-resistant DU-145 and PC-3 cell lines compared to the hormone-sensitive LNCaP cell line.

Stimulation of Glut-1 expression may also be a consequence of hypoxia in the tumor microenvironment. Mediated by HIF-1, the expression of a number of genes is regulated by a decreased concentration of oxygen in the extracellular environment (28).

Figure 2. Expression of Glut-1 in a Gleason grade 3 prostatic adenocarcinoma as determined by RISH (x 50).

- **a**: RISH-Hybridization with sense Glut-1 RNA probe.
- **b**: RISH-Hybridization with anti-sense Glut-1 RNA probe.
One of these is Glut-1. Experimental studies have shown increased transcription of Glut-1 in response to hypoxia (29,30). Overexpression of Glut-1 has been documented in various human malignancies, a correlation with adverse histopathological parameters having been reported for tumors of the thyroid, breast, colon, bladder and lung (2,10,13,31,32). Moreover, when disease-free and overall survival were analysed, increased expression of Glut-1 was associated with poorer clinical outcome in carcinomas of the breast, hypopharynx, esophagus, rectum and oral squamous cell carcinoma (9,14,31,33,34). In addition, suppression of Glut-1 mRNA expression resulted in reduced tumorigenicity in nude mice (35).

Figure 3. Expression of GLUT-1 in a Gleason grade 4 prostatic adenocarcinoma as determined by RISH (x 50).

a: RISH- Hybridization with sense Glut-1 RNA probe.
b: RISH- Hybridization with anti-sense Glut-1 probe.
We are aware of only one study by Chandler and coworkers evaluating the expression of Glut-1 and Glut-12 in PC cell lines and 3 clinical specimens each of benign and malignant prostatic tissue (16). The PC cell lines were reported to express both Glut-1 and Glut-12 at the mRNA and protein level. In clinical PC specimens, however, immunohistochemistry failed to detect any expression of Glut-1. While our findings in the PC cell lines are in agreement with the results of these investigators, they strongly differ with regard to Glut-1 expression in primary PC. All tumors examined in our study showed expression of Glut-1; increasing tumor grade was associated with increasing Glut-1-mRNA expression. Methodological differences may explain these discordant findings. Our approach of detecting Glut-1 expression at the mRNA-level may yield a higher level of sensitivity. Also, it allows for an investigator-independent quantification by image analysis. Surprisingly, however, Chandler and coworkers reported detection of Glut-1 expression in 3 benign prostate specimens. This would imply down-regulation of Glut-1 upon malignant transformation in prostatic tissue which, to our knowledge, has never been reported for any tumor system.

Different lines of evidence would appear to support our finding of increased expression of Glut-1 in clinical specimens of PC. First, a correlation between FDG-uptake by tumor cells in PET studies and expression of Glut-1 has been demonstrated recently (9,32,36). Imaging of PC by PET has been successfully performed. Increased FDG-uptake was detected in PC and metastases (37,38). Therefore, up-regulation of Glut-1 in malignant prostatic tissue seems a likely event. Furthermore, neovascularization in malignant tissues in response to hypoxia was found associated with adverse prognostic factors. In PC, a number of studies have demonstrated the relevance of angiogenesis for tumor growth, progression and metastasis (39-41). Since hypoxia induces expression of Glut-1, increased expression of Glut-1, as found in our study, would be expected to parallel neovascularization. Finally, VEGF has been identified as an important inducer of angiogenesis in PC. Since induction of both VEGF mRNA and Glut-1 mRNA has been demonstrated in experimental systems after hypoxic stimulation, further support for our findings of increased Glut-1 expression is provided (42).

In summary, the findings obtained in our study, along with increased glucose uptake demonstrated by PET-imaging, as well as evidence for the important role of angiogenesis inducers and neovascularization in PC, strongly suggest a role for Glut-1 in PC proliferation.

Acknowledgements

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