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Research Article

Metabolic alterations in bladder cancer: applications for cancer imaging

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ABSTRACT

Treatment planning, outcome and prognosis are strongly related to the adequate tumor staging for bladder cancer (BC). Unfortunately, a large discrepancy exists between the preoperative clinical and final pathologic staging. Therefore, an advanced imaging-based technique is crucial for adequate staging. Although Magnetic Resonance Imaging (MRI) is currently the best *in vivo* imaging technique for BC staging because of its excellent soft-tissue contrast and absence of ionizing radiation it lacks cancer-specificity. Tumor-specific positron emission tomography (PET), which is based on the Warburg effect (preferential uptake of glucose by cancer cells), exploits the radioactively–labeled glucose analogs, i.e., FDG. Although FDG-PET is highly cancer specific, it lacks resolution and contrast quality comparable with MRI. Chemical Exchange Saturation Transfer (CEST) MRI enables the detection of low concentrations of metabolites containing protons. BC is an attractive target for glucose CEST MRI because, in addition to the typical systemic administration, glucose might also be directly applied into the bladder to reduce toxicity-related complications. As a first stage of the development of a contrast-specific BC imaging technique we have studied glucose uptake by bladder epithelial cells and have observed that glucose is, indeed, consumed by BC cells with higher intensity than by non-transformed urothelial cells. This effect might be partly explained by increased expression of glucose transporters GLUT1 and GLUT3 in transformed cells as compared to normal urothelium. We also detected higher lactate production by BC cells which is another cancer-specific manifestation of the Warburg effect. In addition, we have observed other metabolic alterations in BC cells as compared to non-transformed cells: in particular, increased pyruvate synthesis. When glucose was substituted by glutamine in culture media, preferential uptake of glutamine by BC cells was observed. The preferential uptake of glucose by BC cells gives an opportunity to develop NMR based imaging procedures where glucose or its derivatives can serve as a contrasting agent. In addition, metabolic alterations observed in BC cells could provide the basis for development of new anti-cancer therapeutics.

1. Introduction

BC is the second most common urological malignancy. There are 2 major subtypes of BC: non-muscle-invasive cancer, which frequently recurs, but often has good prognosis, and muscle-invasive form with poor prognosis. Treatment planning, outcome and prognosis are strongly related to adequate tumor staging for BC. Unfortunately, a large discrepancy exists between the preoperative clinical and final pathologic staging [1]. Therefore, a low-invasive, high resolution imaging technique is essential for preoperative staging. Magnetic Resonance Imaging (MRI) is currently the best *in vivo* imaging technique for BC staging because of its excellent soft-tissue contrast and absence of ionizing radiation [2]. However, existing MRI methodologies lack cancer-specificity and are inadequate for selective contrast in BC. Tumor-specific positron emission tomography (PET), which is based on the Warburg effect (preferential uptake of glucose by cancer cells), exploits the radioactively–labeled glucose analogs, i.e., FDG [3]. High-uptake of ^{18}F-2-fluoro-2-deoxy-D-glucose (FDG) in urothelial cancer of the bladder was already reported [4]. In this study FDG was introduced intravenously into rats that developed an orthotopically transplanted, locally metastasizing bladder tumor. Substantial accumulation of FDG after 2 h indicate potential usage of FDG/ PET imaging of bladder tumor and metastasis. And, indeed, FDG/PET scan of a patient with biopsy proved recurrent intravesical BC after radiation therapy [4]. However, FDG excretion in the urine, potentially could prevent the use of FDG/positron emission

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tomography (FDG/PET) scanning for localized BC [4]. Therefore, instillation of contrast agent directly into the bladder followed by lavage can provide improved conditions for imaging of local bladder tumor. In a more recent study evaluation of delayed pelvic (18) F-2-fluoro-2-deoxy-D-glucose-positron emission tomography combined with the computed tomography (FDG-PET/CT) imaging showed that delayed pelvic FDG-PET/CT imaging after pre-hydration and forced diuresis detects more primary bladder tumors than standard FDG-PET/CT protocols [3].

Although FDG-PET is highly cancer specific, it lacks resolution and contrast quality comparable with MRI [5]. However, there is no MRI procedures that utilize cancer-specific contrasting agents. Therefore, a new MRI based technique combining high resolution and cancer cell-specific contrast is highly demanded. Chemical Exchange Saturation Transfer (CEST) MRI enables the detection of low concentrations of metabolites that can be selectively saturated and the saturated spin can be transferred to the tissue water via chemical exchange [6]. Glucose analogs have been used for CEST MRI in several preclinical studies as cancer-specific contrasting agent for solid tumor imaging [7–9]. BC is an attractive target for glucose CEST because, in addition to the typical systemic administration, glucose or its analogs might also be directly applied into the bladder to reduce toxicity-related complications related to the systemic administration and to control presence of the contrasting agent directly in the proximity of bladder tumor.

The application of the MRI based technique CEST using glucose analogs as contrasting agents for cancer cells might be highly promising in BC staging and as a result in disease prognosis and planning of adequate treatment. As a first step in this development it is necessary to prove that BC cells indeed uptake glucose with a higher rate than normal urothelial cells. Although effective uptake of glucose by BC cells were shown in *in vitro* system [10] and in an experimental animal model of BC [4], we propose herein the analysis of glucose and glutamine uptake by BC cells presenting wider range of tumors stages in comparison with normal urothelial cells.

Cancer development is accompanied with substantial metabolic alterations. The high glycolytic flux is an intrinsic feature of cancer cells and is known as the Warburg effect [11]. Otto Warburg observed a hallmark of cancer cells: they produce high levels of lactate and use glycolysis to generate energy, even in the presence of oxygen [12].This phenomenon is exploited in the field of cancer–specific imaging. However, this is not widely applied yet for BC even for imaging techniques with contrasting agents that are currently in use for other malignant disease [5]. Although the Warburg effect is observed for many cancers limited information was presented about switching to glycolysis during bladder carcinogenesis [4,10,13].

Therefore, the major aim of this study was to examine alterations in glucose metabolism in relation to cancer progression and to find proof that glucose-based compounds can be utilized as cancer cell-specific contrasting agents and, possibly, to find additional entry points for new therapeutic modalities. In addition, we plan to study metabolic alteration in BC cells when glucose was substituted with another major energy metabolite–glutamine.

2. Materials and methods

2.1. Cell lines

Primary cultures were established from surgical samples of normal urothelium. Cell culture was started and maintained basically as described [14]. Primary urothelial cells (PUC) were used at 5 first passages. The collection and analysis of all samples were approved by the Institutional Review Board and written informed consent was obtained from each subject. Established human cell lines represented different types of transitional cell carcinoma (TCC): The transitional papilloma carcinoma cells RT4 cells maintain transitional epithelial appearance, are highly differentiated, and derived from recurrent TCC of low grade [15]; 5637 cells were established from primary TCC of grade 2 and T24 cell line were derived from highly aggressive undifferentiated and invasive TCC [16,17]. These cell lines were purchased from ATCC (Manasas, VA) and were maintained in RPMI media with 10% FBS and 1% penicillin–streptomycin solution. For metabolites analysis experiments cells were maintained in Dulbecco's Modified Eagle Medium (D5030, Sigma, St. Louis, MO) supplemented with 10% FBS. This media does not contain pyruvate, glucose or glutamine. For particular experiments we added 10 mM glucose, 2 mM L-glutamine or both as indicated and as was described before [18,19]. Cells were maintained in an incubator with 5% CO_2 at 37 °C and were grown until they reached a confluence of 75–85%.

2.2. 2-NBDG

For direct visualization and measuring glucose uptake in living cells we have used a fluorescent D-glucose analog 2- [N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG). Cells were grown in 8-well chambers to subconfluence and after overnight glucose starvation, were incubated with 2-NBDG for 30–120 min as described [20]. After washing cells with PBS images were captured with fluorescent microscopy. For quantitative analysis of accumulated 2-NBDG cells were lysed in 0.1 mL cell lysis buffer (1% sodium deoxycholate, 40 mM KCl, and 20 mM Tris [pH 7.4]) for 10 min). Cells were scraped off and homogenized with 10 passages through a 27-gauge needle. Immediately following centrifugation at 12,000 × *g* for 5 min at 4 °C, fluorescence of aliquots from supernatants was measured with a fluorescence microplate reader. To quantify the intracellular concentration of 2-NBDG (2-NBDG), standard curve graphs were generated by measuring the fluorescence of 2.5–50 μM 2-NBDG in lysis buffer [21]. To adjust fluorescence intensity, lysates were normalized to cell number by adjustment of protein concentration of samples measured with BCA assay (Pierce, Rockford IL).

2.3. Proton nuclear magnetic resonance (^{1}H NMR) and spectra analysis

^{1}H NMR spectroscopy was performed to determine glucose and glutamine consumption, lactate production and variations in other substrates, such as pyruvate and alanine, during the 24 h of cell incubation in DMEM containing glucose (10 mM), glutamine (2 mM) or both. Samples of the extracellular medium were collected from each plate and analyzed by ^{1}H NMR, using routine methods [10,22]. In brief, spectra were acquired at 20T, using a Bruker Avance 850 MHz spectrometer equipped with a 5-mm TCI probe (Bruker Biospin, Karlsruhe, Germany). For each sample, 64 scans were acquired at 25 °C, using a presaturation water suppression pulse sequence with a 45-degree excitation pulse and a 14 s repetition delay to insure full proton relaxation. The relative areas of ^{1}H NMR resonances were quantified using the curve-fitting routine supplied with the NUTS pro NMR spectral analysis software (Acorn, NMR Inc., Fremont, CA, USA). Signal amplitude was adjusted to cell density. The referred metabolites were identified in the spectra using the chemicals shifts described in the literature: glucose, doublet located at 5.22 ppm; lactate, doublet located at 1.33 ppm; alanine, doublet located at 1.46 ppm; pyruvate, singlet at 2.36 ppm as described [22], glutamine doublet located at 2.46 ppm [23,24]. Fumarate (final concentration of 5 mM) was used as an internal reference (6.50 ppm, singlet) to quantify metabolites in solution [22].