Human Cancer Biology

Regional Cell Proliferation in Microdissected Human Prostate Specimens after Heavy Water Labeling In Vivo: Correlation with Prostate Epithelial Cells Isolated from Seminal Fluid

Gregory M. Hayes1, Jeff Simko2, Daniel Holochwost1, Kyle Kuchinsky2, Robert Busch1, Lisa Misell1, Elizabeth J. Murphy1,4, Peter Carroll3, June Chan3, Katsuto Shinohara3, and Marc K. Hellerstein4,5

Abstract

Purpose: Prostate cancer is detected with increasing frequency but has a highly variable natural history and prognosis and active surveillance of men with low-risk prostate cancer would benefit greatly from minimally invasive methods to identify progression. We describe here two novel in vivo metrics of cell proliferation in men with prostate neoplasia.

Experimental Design: Three groups of men drank heavy water, a nonradioactive, stable isotopic tracer for 14 to 28 days: (i) healthy men, (ii) men scheduled for transrectal core needle biopsy, and (iii) men scheduled for radical prostatectomy. Prostate epithelial cells (PEC) were isolated from ejaculated seminal fluid in all subjects. Histologically graded lesions were microdissected from tissue slides obtained from subjects undergoing surgery and proliferation rates were measured from isolated cells via mass spectrometry.

Results: Proliferation rates of seminal PEC in healthy men (0.10%-0.27%/d) were stable on repeat sampling. Rates above 0.34%/d were seen only in patients with cancer where rates increased progressively from normal tissue through benign prostate hyperplasia, prostate intraepithelial neoplasia, and tumor grades III and IV in all subjects. Seminal PEC kinetics correlated highly with the most proliferative microdissected region in each subject ($r^2 = 0.94$).

Conclusions: Prostate cell proliferation can be measured in vivo from microdissected histopathology sections or noninvasively from seminal fluid where the latter reflects the most proliferative region of the gland. This approach may allow monitoring of progression in men with low-risk prostate cancer. Clin Cancer Res; 18(12); 3250–60. ©2012 AACR.

Introduction

Prostate cancer represents the most common male malignancy in developed countries and the second leading cause of cancer-related death (1, 2). Postmortem studies of men more than 50 years of age reveal, however, that approximately one out of 3 has histologic evidence of clinically insignificant prostate cancer (3), indicating that most prostate cancers will not progress to a stage that necessitates treatment. It is likely that low-risk prostate cancer at present is being overtreated (4, 5) and a better understanding of who will experience morbidity or mortality from prostate cancer is therefore critical for effective treatment of this disease.

The most effective current method of prostate cancer diagnosis is pathologic analysis following transrectal biopsy. However, only about 1 of 3 men who undergo this invasive procedure are confirmed to have such malignancies (6). In addition, patients face substantial psychologic stress and the risk of overtreatment as a result of a diagnosis of low-risk prostate cancer.

An optimal management approach has yet to be established for favorable-risk prostate cancer. Active surveillance, consisting of repeated follow-up observations to identify those with higher risk phenotype or progression to more aggressive disease, has emerged as a potential solution to overdiagnosis and overtreatment. Optimal active surveillance, however, would require methods to identify a cancer’s potential for evolution into an aggressive phenotype and to monitor its progression over time. In practice, such a method would be most useful if it was noninvasive. The “watch and wait” approach would almost certainly be better accepted by patients if a reliable test was available to detect...
any transition of their malignancy to a more aggressive phenotype.

Histologic analysis of prostate tissue may reveal several phenotypes, ranging from benign tissue through benign prostate hyperplasia (BPH), prostate intraepithelial neoplasia (PIN), and cancers of different grades (7, 8). Histologic grading of prostate tissue (Gleason score) is standard practice but does not predict prognosis for intermediate-grade, favorable-risk cancer and is invasive. Less invasive tests have been evaluated, including prostate-specific antigen (PSA) density (PSAD; ref. 9), PSA velocity (PSAV; ref. 10, 11), and prostate-specific antigen (PSA) in urine (PCA3; ref. 12), but none of these tests has shown an ability to improve surveillance to identify progression.

We describe here for the first time methods of measuring in vivo prostate cell proliferation rates in men using stable isotope labeling and mass spectrometry. Microdissection of prostate tissues following heavy water intake and subsequent biopsy or prostatectomy revealed progressive increases in proliferation rates in benign, prostate intraepithelial neoplasia, and tumor grade III and IV histopathologic regions where turnover rates of the most proliferative areas of the gland correlate highly with epithelial cells shed into and isolated from the seminal fluid. These methods illustrate a novel, noninvasive means of measuring prostate epithelial cell proliferation as a quantifiable metric of prostate phenotype that may allow monitoring in men with low-risk cancer who would benefit from minimally invasive surveillance to identify progression.

Materials and Methods

Subject enrollment and 2H2O labeling protocol

Healthy men (n = 3) were recruited via advertisement. Prostate disease patients (n = 11) scheduled for core needle biopsy due to high PSA levels and/or prostate ultrasound or those scheduled for radical prostatectomy due to previously diagnosed cancer (n = 10) were enrolled at the UCSF Helen Diller Cancer Center. Protocols were approved by BioMed Institutional Review Board (KineMed, Inc.) and the UCSF Committee on Human Research. All participants provided written informed consent before entry into the study. Subjects were given heavy water aliquots (2H2O; 70% enriched, Spectra Gases Inc.) to ingest daily (50 mL twice or 3 times a day) for 2 to 4 weeks, with the goal of reaching approximately 1.0% to 2.0% body 2H2O enrichment at the end of the labeling period. These levels of 2H2O enrichment have no adverse effects in humans (16). During 2H2O administration, weekly saliva samples were obtained from all subjects at home via a Salivette kit (Sarstedt) to monitor compliance. Ten milliliters of blood samples was obtained in desiccated heparin from normal subjects at their week 3 visit and from prostate disease patients at their surgical appointment and used for monocyte isolation. Body water content was determined from saliva and serum samples via cycloidal mass spectrometry (Monitor Instruments) as previously described (21). Body water enrichment area under the curve was calculated by trapezoidal method to determine mean 2H2O levels over the labeling period for each study subject.

Semen and tissue collection

Healthy volunteers provided one semen sample each week over 4 weeks of 2H2O administration. Healthy subjects’ ejaculated seminal fluid samples were processed for cell isolation within 2 hours of collection. Prostate disease patients provided a single semen sample (ejaculation within 2 hours before clinical visit) at their surgical appointment that was processed immediately for cell isolation and analysis. Prostate tissue was collected by either core needle biopsy or radical prostatectomy and was formalin-fixed/paraffin-embedded for histologic characterization via standard tissue collection procedures. Following histologic grading, the benign, hyperplastic (BPH), PIN, and tumor tissues (grades III and IV), where available, were microdissected and provided blindly for analysis. PSA levels for
healthy subjects were conducted using the human PSA ELISA kit from Abnova as per manufacturer’s protocol.

Prostate tissue microdissection

Five-micron-thick sections were taken from the top of each paraffin-embedded prostate tissue for hematoxylin and eosin (H&E) staining, followed by 6 unstained sections of 10 µm each, and a final H&E section. The first and last H&E sections were evaluated by a pathologist and areas where tumor concentration exceeded 80% based upon surface area were marked and assigned a Gleason score. The corresponding areas of the unstained sections were microdissected and shipped to KineMed, Inc. for analysis. Similarly, areas of high-grade preinvasive neoplasia (HGPIN), atrophy, and benign tissue were marked on the guide slides and microdissected for analysis. Infiltrating immune cells were assessed in each area before microdissection and only those tissues exhibiting minimal inflammation were analyzed.

PEC isolation from seminal fluid

Epithelial cells shed into the seminal fluid were separated using a combination of gradient centrifugation and fluorescence-activated cell sorting (FACS), as summarized in Fig. 1.

- **Percoll gradient enrichment of PEC.** Semen was incubated with SpermPrep Viscolytic Enzyme mixture (ZDL Inc.) for 30 minutes at 37°C with gentle agitation. Seminal fluid cells were washed with 10 mL warm RPMI-1640 media (Sigma), pelleted at 800 x g, and resuspended in 2 mL RPMI before being loaded onto a room temperature 42.5% Percoll (GE HealthCare) gradient previously spun at 21,000 x g for 30 minutes (5.5 mL RPMI-1640, 4.5 mL Percoll, 0.5 mL 10x Hanks balanced salt solution; Sigma). The seminal fluid cell-containing Percoll gradient was centrifuged at 21,000 x g for 20 minutes, allowing for removal of the majority of spermatocytes that band near the bottom of the column. The top 7 mL of the gradient containing a PEC-enriched fraction was washed with 10 mL RPMI. Any residual contaminating squamous epithelial cells were removed by passing this fraction through a 20-µm Celltrics filter (Partek GmbH).

- **Immunostaining and sorting of PSMA⁺/PAP⁺ PEC.** To achieve more than 90% PEC purity for subsequent measurement of ²H enrichment in DNA, cells were fixed in 10% formalin overnight (Sigma-Aldrich), permeabilized in PBS containing 0.5% Tween 20 (Sigma-Aldrich), stained, and sort-purified by flow cytometry. Two prostate markers were used: prostate-specific membrane antigen (PSMA; clone 5E10 from Chemicon and clone 7E11 from Cytoxen) and prostate alkaline phosphatase (PAP; ChromoProbe). PSMA antibodies were labeled with Zenon-FITC reagent and the PAP antibody labeled with Zenon-phycocerythrin reagent (both from Invitrogen) as per manufacturer’s protocol. Dual positive PSMA⁺/PAP⁺ cells were isolated on a Beckman Coulter Epics Elite sorter (Beckman Coulter Inc.). Purities were confirmed by cytocentrifugation and immunostaining of an aliquot of the sorted population with a pan-cytokeratin (CK) antibody (clone C-11; Abcam). Cytospun and stained sort fractions were scored for CK⁺ cells, residual spermatocytes, and any remaining lymphocytes or squamous epithelial cells.
Quantification of stable isotope enrichment in DNA

Genomic DNA was purified from cells via modification of previously described methods (16) using Qiagen DNeasy columns following the manufacturer’s formalin-fixed, paraffin-embedded tissue procedure. Because of the relatively low number of PEC purified from semen, at best approximately 3,000 cells, a modified DNA extraction and derivatization method for low cell counts was developed for reliable gas chromatography (GC)-MS analysis, as previously described (16). Isolated cell pellets were thawed on ice, 200 µl phosphate-buffered EDTA (10 mMol/L) solution was added to each, and heated to 100°C for 10 minutes. Samples were cooled on ice before the addition of 20 µL Proteinase K solution (Qiangen) and digested overnight at 55°C with gentle agitation. Digested cell solutions were transferred to 10 kDa molecular weight filter columns (YM-10; Millipore) and centrifuged at 5,000 × g for 60 minutes until the solution passed completely through the column. The purified DNA remaining on the filter was resuspended in 50 µL dH2O before being enzymatically hydrolyzed to free deoxyribonucleotides by addition of 5 µL hydrolysis buffer (16) and incubated for 16 hours at 37°C. The deoxyribose (dR) moiety of purine deoxyribonucleosides was converted to the pentfluorobenzyl triacetate derivative in 3 independent derivatization runs for each microdissected tissue DNA. Analysis was conducted via GC-MS in negative chemical ionization mode, as previously described (22). Because of the relatively low number of PEC isolated from each seminal fluid sample, samples were concentrated and one analysis was conducted for each triplicate derivatization method for low cell counts was developed for reliable gas chromatography (GC)-MS analysis, as previously described (16). Isolated cell pellets were thawed on ice, 200 µl phosphate-buffered EDTA (10 mMol/L) solution was added to each, and heated to 100°C for 10 minutes. Samples were cooled on ice before the addition of 20 µL Proteinase K solution (Qiangen) and digested overnight at 55°C with gentle agitation. Digested cell solutions were transferred to 10 kDa molecular weight filter columns (YM-10; Millipore) and centrifuged at 5,000 × g for 60 minutes until the solution passed completely through the column. The purified DNA remaining on the filter was resuspended in 50 µL dH2O before being enzymatically hydrolyzed to free deoxyribonucleotides by addition of 5 µL hydrolysis buffer (16) and incubated for 16 hours at 37°C. The deoxyribose (dR) moiety of purine deoxyribonucleosides was converted to the pentfluorobenzyl triacetate derivative in 3 independent derivatization runs for each microdissected tissue DNA. Analysis was conducted via GC-MS in negative chemical ionization mode, as previously described (22). Because of the relatively low number of PEC isolated from each seminal fluid sample, samples were concentrated and one analysis was conducted for each triplicate derivatization reaction. Ions with mass to charge ratios of 435 and 436 (m/z), representing the M0 and M1 mass isotopomers, were monitored and 2H incorporation in deoxyribose from purine deoxyribonucleosides was calculated from the increase over background (natural abundance) of the M1 peak (EM1), using unlabeled DNA from calf thymus as the natural abundance reference.

Ki67 analysis

Immunohistochemistry for Ki67 was carried out using 5 µm formalin-fixed paraffin sections. Antigen retrieval was carried out using 0.1% trypsin at 37°C for 15 minutes followed by heat retrieval in 0.01 mol/L citrate buffer at pH 6 in a pressure cooker. Sections were incubated with the Ki67 mouse monoclonal antibody (Clone MIB-1, Dako#M7240, dilution 1:100) overnight at 4°C and detected by ABC-HRP system (Vector Labs #PK6100). Stained sections were scanned for an area with highest occurrence of Ki67-positive cells and all epithelial cells were counted within a 0.79 cm² area. The benign areas scored occurrence of Ki67-positive cells and all epithelial cells were Stained sections were scanned for an area with highest detected by ABC-HRP system (Vector Labs #PK6100). All Ki67 scoring was carried out blindly.

Data analysis and statistics

• Calculation of percentage of new cells. The proliferation rates of PEC and prostate tissues were calculated based upon the precursor-product relationship, comparing measured EM1 values in labeled cells to asymptotic or maximal EM1 (EM1*) values, based on 3H enrichments in the body water pool. The asymptotic (EM1*) values were calculated using mass-isotopomer distribution analysis (MIDA), from the mean body 2H2O enrichment during the preceding labeling period, as previously described (16, 23–25).

Percentage of new cells (% f) = \frac{dR \text{ enrichment (EM1), PEC} \times 100}{EM1^*}

The % f was divided by days of labeling to determine the fraction of new cells per day for each sample.

• Correlation of seminal PEC and prostate tissue proliferation rates. The percentage of new cells per day for each prostate disease patient’s seminal PECs was compared with the same metric from the highest proliferative compartment isolated from biopsy or radical prostatectomy tissue. The Pearson correlation coefficient was calculated for these data. Statistical comparison among groups was conducted via one way ANOVA test with 95% confidence interval.

Results

Proliferation kinetics of healthy seminal fluid PEC

Three young healthy male volunteers were recruited for initial evaluation of proliferation rates of PECs shed into the seminal fluid and the stability of this measurement over time (Table 1). Each subject was provided heavy water to ingest daily for 4 weeks and body water 2H2O enrichments were determined by MS analysis of saliva samples obtained weekly. The 2H2O levels, graphed in Fig. 2A, illustrate good compliance with the dosing regimen. The approximately 1% to 2% 2H2O enrichments achieved over the labeling period are consistent with previous studies using similar labeling protocols (17–20). Blood was collected at week 3 of the study for confirmation of heavy water labeling from enrichments in monocyte DNA, a fully turned over cell population (data not shown), whereas sera was used for determination of PSA levels, which were normal (<2 ng/mL) in all the subjects (Table 1).

Each healthy subject provided a weekly seminal fluid sample over the 4 weeks of heavy water labeling, for isolation of PEC as illustrated (Fig. 1). Individuals were asked to abstain from ejaculation for 24 hours before sample collection. Cell isolation occurred within 2 hours of sample collection to minimize any PEC loss due to decreases in viability. The majority of seminal spermatoocytes were removed by density gradient centrifugation before the PEC-enriched fraction was stained for cell surface PSMA and intracellular PAP. PSMA+/PAP+ PEC cells were sorted by FACS as previously described (26, 27) and cell number and purity of each isolated PEC sample confirmed via cytospin analysis (listed in Table 1). Criteria of more than
90% purity and more than $10^3$ cells (16) to which were adhered.

Values of $^2$H enrichment in DNA from isolated PEC (Fig. 2B) increased over the study period for each of the 3 healthy subjects, as expected. The percentage of new cells produced per day (Fig. 2C), illustrates a stable turnover of PECs within healthy individuals in the range of 0.10% to 0.27% new cells produced per day, reflecting a relatively low birth rate of these cells.

**Heavy water labeling of prostate disease patients**

A total of 21 prostate disease patients (Table 1) were enrolled by the UCSF Helen Diller Family Cancer Center for entry into the heavy water labeling study. The first cohort (biopsy) consisted of 11 men scheduled to undergo core needle biopsy in response to elevated PSA levels, although subject (#202) had previously diagnosed prostate adenocarcinoma and treatment before biopsy. One subject from this group (#208) withdrew before the labeling period began. The second cohort consisted of 10 men scheduled for radical prostatectomy due to prior diagnoses of cancer. Men were provided aliquots of heavy water to ingest daily before their surgical visit (range 14–29 days of labeling). Labeling compliance and body water enrichments were determined from weekly saliva samples obtained by the patients at their residence.

#### Table 1. Subject demographics

<table>
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<tr>
<th>Subjects</th>
<th>Age</th>
<th>PSA, ng/mL</th>
<th>PSA range, ng/mL</th>
<th>Days of labeling</th>
<th>% $^2$H2O enrichment</th>
<th># SF-PEC (% purity)</th>
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<td></td>
<td>1.68</td>
<td>3431 (95.1)</td>
<td>n/a</td>
</tr>
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</table>

| Biopsy   |     |            |                  |                  |                      |                     |         |
|          | 201 | 57         | 7.8              | 5.8–12.1         | 15                   | 1.24                | 1530 (61.4) | —       |
|          | 202 | 54         | 13.6             | 10.1–15.0        | 18                   | 1.23                | 1087 (23.8) | 6       |
|          | 203 | 57         | 7.4              | ND               | 16                   | 1.13                | 766 (96.6)  | —       |
|          | 204 | 64         | 10.4             | 5.4–8.9          | 19                   | 1.05                | 2987 (88.9) | 7       |
|          | 205 | 60         | 5.1              | ND               | 22                   | 1.24                | 1889 (94.7) | 7       |
|          | 206 | 55         | 0.34             | 0.34             | 14                   | 1.26                | 1972 (84.1) | —       |
|          | 207 | 60         | 7.6              | 6.2              | 20                   | 1.05                | 2585 (93.5) | —       |
|          | 209 | 53         | 4.5              | 4.3              | 27                   | 1.35                | 1950 (90.5) | 7       |
|          | 210 | 70         | 5.7              | 2.8–3.8          | 29                   | 1.86                | 2520 (93.6) | —       |
|          | 211 | 69         | 6.6              | ND               | 29                   | 1.73                | 1962 (91.2) | —       |

| Radical prostatectomy |     |            |                  |                  |                      |                     |         |
|                      | 301 | 72         | 3.6              | 2.3–5.4          | 18                   | 1.52                | 685 (84.4)  | 8       |
|                      | 302 | 65         | 4.3              | 2.3–5.3          | 17                   | 1.30                | 2600 (91.8) | 7       |
|                      | 303 | 63         | 8.1              | 4.0–6.3          | 29                   | 1.68                | 6504 (83.8) | 6       |
|                      | 304 | 64         | 7.6              | ND               | 19                   | 1.92                | 3079 (91.7) | —       |
|                      | 305 | 60         | 5.7              | 4.2              | 21                   | 1.64                | 4554 (92.5) | 7       |
|                      | 306 | 44         | 5.8              | 3.5              | 20                   | 1.45                | 4036 (92.4) | 7       |
|                      | 307 | 50         | 7.9              | ND               | 27                   | 1.37                | 3942 (93.5) | 7       |
|                      | 308 | 53         | 8.5              | ND               | 20                   | 1.40                | 4169 (90.0) | 6       |
|                      | 309 | 53         | 5.5              | 3.2–4.8          | 20                   | 1.37                | 3247 (82.9) | 6       |
|                      | 310 | 68         | 4.9              | ND               | 20                   | 1.40                | 2741 (93.3) | 7       |

Abbreviations: ND, not determined; n/a, not available.
Final body water enrichments for biopsy (Fig. 3A) and radical prostatectomy patients (Fig. 3B) were in the expected range of 1% to 2%, similar to that showed in the healthy control subjects (Fig. 2A).

**Proliferation rates of prostate tissues**

Following biopsy and radical prostatectomy surgical procedures, tissues were processed via standard histopathologic methods and 8 serial slides were produced from each; the first and last slides were H&E stained (5 μm thick), pathologically scored, and used to identify those regions for dissection among the remaining intermediate and unstained 10 μm thick slides. Benign sections were identified and microdissected for determination of baseline proliferation rates whereas BPH, PIN, and tumor (grade III and/or IV) areas were microdissected, where available. Gleason scores determined for each patient’s tissues are listed (Table 1).

DNA was purified from microdissected samples and used for analysis of deuterium enrichment by GC-MS, as previously described (16). The calculated percentage of new cells produced per day for each specimen obtained from biopsy and radical prostatectomy patients is graphed in Fig. 4A and B, respectively. In the biopsy cohort, there were 4 subjects with cancer tissue identified and analyzed whereas in those undergoing radical prostatectomy, there were 8 subjects with cancer tissue.
identified and analyzed. Two subjects (#304 and 309) had previously shown histologic evidence of cancer by biopsy but remaining tumor tissue was not detected. Rates of proliferation in benign tissues among biopsy patients and those undergoing radical prostatectomy (range: 0.07%–0.59% newly divided cells per day) were not significantly different (Fig. 4C). In almost all patients, however, there were elevated rates of proliferation for BPH, PIN, and/or tumor regions compared with benign tissue, with a clear proliferative gradient correlating with histopathologic grade (Fig. 4A–C). Significant differences in proliferation rates were seen between benign tissues derived from subjects with no clinical evidence of malignancy compared with tumor-associated hyperplastic regions or malignant regions (grade III and IV; Fig. 4C). These data also show considerable interpatient variability in proliferation rates for the benign as well as neoplastic prostate tissues examined.

Figure 4. Prostate cell proliferation rates determined by $^2$H DNA labeling in microdissected tissues and seminal PEC fractions. A, cellular rates of proliferation in benign, HGPIN, and tumor areas (where available) were determined in subjects undergoing core needle biopsy. Proliferation of seminal PEC derived from each patient is graphed alongside their tissues. B, cellular rates of proliferation exhibited by tissues and seminal PEC in subjects undergoing radical prostatectomy. Because of maintenance of tissue architecture during procedure, BPH regions as well as grade III and grade IV areas were also evaluated. C, comparison of tissue kinetics among all subjects undergoing surgical evaluation illustrating an increase in proliferation from benign to BPH and tumor regions. D, comparison of Ki67 rates among tissues obtained from subjects undergoing surgical evaluation.
Tissue Ki67 results

Measurement of Ki67 expression of prostate tissue samples was carried out blindly for those undergoing biopsy or radical prostatectomy. In histologically benign tissue, Ki67 expression ranged from 0.4% to 4.3%. Interestingly, tissue from histologic BPH exhibited uniformly lower percentage of Ki67 expression (Fig. 4D), in contrast to increased cell proliferation in this subset by stable isotope labeling (Fig. 4C). Ki67 expression was highly variable in the tumor grade III and in the tumor grade IV tissue (Fig. 4D). The BPH group was significantly different from all 3 other histologic areas, but other groups were not significantly different from each other.

Proliferation rates of seminal fluid PEC derived from prostate disease patients

Each biopsy and radical prostatectomy patient was instructed to obtain a seminal fluid sample within 2 hours of their surgical visit at the end of the labeling period. Patients 202 and 301 were unable to produce an adequate volume of semen before surgery, whereas the number or purity of PEC from subjects 201 and 203 fell below the criteria of >90% purity and >1,500 cells required for analysis. The number of PEC and purity for each of the remaining 7 biopsy and 9 radical prostatectomy subjects are listed in Table 1. The calculated percentage of SF-PEC that had divided each day during the labeling period is graphed according to tissue analysis for each patient (Fig. 4A and B). No significant difference in the rate of seminal PEC proliferation was observed between healthy donors and those patients exhibiting elevated PSA who had no evidence of cancer (Fig. 5A). No subject in these groups had PEC values greater than 0.34%/d. In contrast, in this pilot study, SF-PEC proliferation rates exceeding 0.34% new cells per day were observed in 10 of the 12 patients (>80%) with identified tumor tissue. These proliferation rates were significantly higher than those exhibited by either the healthy donor or high-PSA no-tumor groups (P < 0.05).

Correlation between proliferation rates of seminal PEC and patient tissues

To evaluate the potential use of seminal PEC as a noninvasive biomarker for prostate tissue kinetics, the percentage of newly divided cells per day for seminal fluid PEC is graphed against the proliferation rate for the most highly proliferative tissue region analyzed (Fig. 5B). Notably, the rate of seminal fluid PEC proliferation correlated closely with the highest proliferative compartment in prostate tissue for each of the patients analyzed, regardless of the tissue histologic type that had the highest proliferation rate. Pearson correlation between these patient-matched samples yielded a within-subject R value of 0.969.

Discussion

Metrics, or biomarkers, for managing patients with low-risk prostate cancer are of critical importance to determine those individuals who need treatment and to establish the optimal timing of treatment. In particular, the active surveillance approach requires measurements to monitor the progression of disease over time and provide reliable criteria for treatment decisions. We describe 2 novel methods here for measuring in vivo a key physiologic parameter integrally related to prostate biology and tumorigenesis: cell proliferation and turnover rate. The microdissection tissue-based method requires prostate tissue and, thus, is invasive but is relatively straightforward to couple with current clinical evaluation processes, whereas the seminal fluid method is noninvasive.

- Tissue-based biomarker. This is the first time, to our knowledge, regional cell proliferation kinetics have been measured in humans from histopathologic sections based on in vivo metabolic labeling with stable isotopes. Technically, sufficient amounts of DNA could be isolated from specific regions of microdissected tissues to facilitate reproducible measurements of deuterium isotope enrichment by GC-MS, using recently developed low cell count methods (16). Moreover, the degree of deuterium labeling was
adequate using a simple outpatient heavy water–labeling regimen that we have previously used in several human studies (16, 17, 20).

We report that normal PEC proliferation rates in vivo in men are low, in the range of 1% to 3% new cells per week. Our finding of a distinct proliferative gradient according to histologic grade of the prostate tissue (benign, BPH, PIN, cancer grade III, cancer grade IV) within each subject supports indirectly the biologic and prognostic informational value of cell proliferation rates. One might expect that cells more advanced along the neoplastic scale should exhibit higher rates of mitosis in vivo, and this expectation was met with direct staple isotope measurement of cell proliferation rates, at least within each individual subject. In contrast, Ki67 labeling was lower in BPH regions than in either benign or tumor areas, which a priori seems unlikely. Because Ki67 expression reflects cells that are in cycle rather than cells that have progressed through mitosis, these results suggest that Ki67 may not accurately reflect progression rate through the cell cycle in this setting (28, 29).

Substantial intersubject variability for all histologic grades of prostate tissue was also observed, and is important. Such variability is likely to prove informative relative to both underlying molecular mechanisms and clinical prognosis. Interestingly, although the results in this small study population need to be interpreted cautiously, the proliferation rates of normal tissue were not significantly different in cancer and noncancer patients. It will be important in future work using this approach to test this question more extensively.

- **Noninvasive (seminal fluid-based) method.** The ability to isolate from ejaculated seminal fluid adequate numbers of PEC with sufficient purity for analysis of DNA replication by GC-MS represents an important technical achievement. The recent development of PCR-like techniques for preventing and ensuring lack of contamination by ambient cells during preparation of ultralow cell number samples (16) was essential for achieving consistent measurements from 2,000 to 4,000 cells. The demonstration of a stable rate of PEC proliferation within healthy subjects over a 4-week labeling period supports the reproducibility and internal consistency of the method.

A remarkable correlation was observed between seminal fluid PEC and the most proliferative region of prostate tissue isolated from histopathologic slides (Fig. 5B). This correlation is congruent with biologic considerations: the highest turnover region in the prostate gland might be expected to dominate the number of cells sloughed into the lumen, although other explanations are possible. More importantly, this finding supports the use of seminal PEC to represent the most aggressive and advanced grade region of the gland both for kinetic analysis and other potential measurements such as proteomics, genetic, or epigenetic changes that occur during tumorigenesis. These data further suggest a potential cutoff point for proliferation rate of SF-PEC (~0.34% new cells per day in this pilot data set) may be able to differentiate cancer from noncancer individuals, or at least increase the likelihood of cancer being present.

Together, these findings support the value of SF-PEC proliferation rate as a quantifiable metric of prostate phenotype for active surveillance and management of low-risk prostate cancer. The tissue-based method may be compatible for testing as an adjunct strategy for grading and staging prostate patients who are receiving prostate biopsies. Applications might include stratifying patients at the time of diagnosis, neoadjuvant trials examining regional cell proliferation rates in prostate tissues, or coupling with more sophisticated types of microdissection to characterize the proliferation rates of molecularly defined cell subtypes. The tissue microdissection method may also prove informative for other malignancies where samples can be collected and direct measurement of tumor grade proliferation rates would be clinically informative. The noninvasive method has great promise for monitoring patients with proven or suspected prostate cancer where repeated follow-up measurements may be able to identify the presence of or progression to a more aggressive, higher risk phenotype.

The safety, compliance, and cost of heavy water labeling are worth mentioning. Heavy water has been given to humans for more than 70 years (30). The double-labeled water technique ($^2$H$_2$O/$^1$H$_2$O) has been used in tens of thousands of human subjects. There have been no reports of any serious or long-term adverse effects (reviewed in ref. 30). Extensive animal studies affirm the safety of heavy water. Rodents raised for serial generations on $^2$H$_2$O in drinking water show no phenotype—including normal body weight, appetite, hematopoiesis, reproductive function, general health, longevity, and no teratogenicity or carcinogenicity—at body $^2$H$_2$O levels of up to 15% to 20% (i.e., more than an order of magnitude above the levels here). With regard to compliance, we have given heavy water to more than 1,000 subjects in the community without this being an issue. Indeed, we just completed an NCI-supported multicentered outpatient study in >150 men with CLL that involved daily $^2$H$_2$O intake for 12 weeks (31). No subject within these 150 generally elderly men (a very similar demographic as patients with prostate cancer) had to be excluded because of poor compliance or because of an adverse effect. Finally, with regard to cost, 100 to 150 mL of 70% $^2$H$_2$O for 7 to 14 days is sufficient to label PECs and generate reliable clinical labeling data. This comes to a total of approximately 700 mL to 2.0 L $^2$H$_2$O, for a cost of ~$165 to $470 at the current bulk price of ~$235/L.

In summary, 2 methods are described for measuring in vivo prostate cell proliferation rates in men with prostate neoplasia. Microdissection of prostate histopathologic slides after heavy water intake in vivo revealed progressive
increases in proliferation rates in graded regions from benign through BPH, PIN, tumor grade III, and tumor grade IV in all subjects examined. Substantial intersubject variability was observed for both normal and neoplastic tissue. Proliferation rates of PEC isolated from seminal fluid correlated highly with the most proliferative region of histologically graded tissue where SF-PEC turnover rates above 0.34% new cells per day were only seen in patients with cancer. Thus, a noninvasive stable isotope PEC method is available in vivo to establish the most proliferative region of the gland and this approach, or a variant of it, may allow monitoring of progression in men with low-risk prostate cancer.

Disclosure of Potential Conflicts of Interest

G.M. Hayes, E.J. Murphy, and M.K. Hellerstein have ownership interest (including patents) for KineMed, Inc. R. Busch and M. K. Hellerstein are consultants/advisory board members for KineMed, Inc. L. Misell and M.K. Hellerstein are employed by Kinemed, Inc. No potential conflicts of interest were disclosed by the other authors.

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Gregory M. Hayes, Jeff Simko, Daniel Holochwost, et al.


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