Early Diagnosis of Prostate Cancer by Citrate Determination in Urine with Europium Oxytetracycline Complex

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Normal prostate tissue contains high levels of citrate. In the presence of prostate cancer, the citrate level is diminished. In this paper we show that it is possible to use Europium-Oxytetracycline complex as a citrate fluorescent probe and consequently of prostate cancer probe. We analyzed normal Nude male mice urine and urine from Nude male mice in which the prostate cancer was induced by DU145 cells intraprostatic inoculation. The urine samples were collected of the animals at 7th, 14th, 21st and 35th day after surgery procedures. The intensity of Europium emission at 615 nm in Europium-Oxytetracycline complex in the presence of Citrate increases linearly. From a calculated calibration curve were determined the Citrate concentrations in malignant prostate urine decrease from the normal (PBS group) urine value from ~8.0 mM to ~2.4 mM (tumor group at 35th day). The obtained results indicated that Europium Oxytetracycline provides a significant biomarker for prostate cancer detecting with a direct, accurate, non-invasive and non-enzymatic method for measurement of Citrate in biological fluids.

Keywords: Citrate, Europium, Tetracycline, Fluorescence, Prostate Cancer
1. INTRODUCTION

Located below the bladder, next to the urinary and genital tubes, the prostate can be affected by various diseases – infection (prostatitis), benign hypertrophy (BHP) and cancer. Among cancers in men, prostate cancer is the second cause of mortality [1].

Prostate cancer is diagnosed using a variety of tests including prostate biopsies[2], digital rectal examination (DRE)[3], transrectal ultrasonography (TRUS)[4], and assaying prostate-specific antigen (PSA)[5]. DRE and TRUS are very limited in their ability to diagnose prostate cancer since do not provide the ability to distinguish between BHP and prostate cancer [6-8]. PSA is frequently high in the event of cancer, but increases also when BHP or infection is present[9].

Only biopsies of the prostate can confirm the presence of cancer and its microscopic characteristics[10]. But despite the reliability of the method it is evident that this is a method widely invasive.

Kurhanewicz et al [11] demonstrated the potential of citrate as an in vivo marker for discriminating prostate cancer from normal and BHP using proton spectroscopy technique. In general, citrate quantification is based on enzymatic assays, but these methods involve complicated and expensive procedures. Development of citrate biosensors has become of the great importance in clinical analysis because the citrate concentration in body fluids is a parameter for diagnosis of some disorders such as prostate cancer and kidney poor function.

Citrate, the generic name of the salts of citric acid, is essential for cell metabolism and normal behavior, providing energy to cells. Normal prostate tissue contains high levels of citrate. In the presence of prostate cancer, the citrate level is diminished or undetectable because of a conversion from citrate-producing to citrate-oxidating metabolism [12-15].

Pal et al [16] developed ratiometric methods for the selective determination of citrate in microliter samples of body fluids following comparison of anion binding affinities for a family of luminescent europium(III) complexes, varying complex charge and ligand steric demand in order to modulate affinity for citrate. But the synthesis of these lanthanide complexes is quite complex, requiring multiple steps and equipments, thus making it a costly and time consuming.

Europium-tetracyclines complexes are prepared in few minutes and are stable for months[17]. It was shown that the europium luminescence of europium-tetracyclines complexes increase in the presence of citrate [18-20]. When Europium Tetracycline complex is excited in a wavelength resonant with the tetracyclines absorption band (400nm), Europium ion luminescence is observed (615nm) [17, 18]. This is due to the ligand broad absorption and an antenna-effect [21] that transfers the absorbed energy to the Europium through an intramolecular process.

The main objective of this paper is to verify the possibility of using the complex europium-oxytetracycline (EuOTc) to map the citrate present in urine and its application in the detection of prostate cancer and the determination of prostate tumors stages.
2. MATERIALS AND METHODS

2.1 Cell Line and Cell Culture Conditions

DU145 cells were cultured in DMEM containing high Glucose amounts (4.5 g/L at 25 mM) and supplemented with 100 units/mL Penicillin, 50 mg/mL Streptomycin, and 10% FBS. The cells were maintained in a humid chamber at 37 °C in an atmosphere of 5% CO₂.

2.2 Animals and tumor induction

The orthotopic tumor model of prostate cancer was used in 20 male Nude mice, where 1x10⁵ cells (DU145) were inoculated into the prostate gland in a volume of 10 µL of sterile phosphate buffered saline (PBS). The PBS group, 5 animals, has received just PBS (without cells). Before the surgery procedures, the 25 Nudes had their urine collected and this group was called Control.

2.3 Urine collection

The urine samples were collected of the animals at 7th, 14th, 21st and 35th day after surgery procedures by the application of gentle massaging pressure over the bladder and direct collection of the urine in a clean tube. Not all animals urinated on all collection days, but at least 10 of them (randomly) at each day.

2.4 Europium Oxytetracycline complex

The EuOTc complex in the ratio 1Eu:1OTc was prepared starting from inorganic salts with analytical purity, obtained from Sigma Aldrich and Molecular Probe according to procedures adopted in the reference [22].

2.5 Citrate calibration Curve

Solutions of Tri-Basic Sodium Citrate (Sigma) were prepared by dissolving 0.00148g of Sodium Citrate in 1mL of bi-distilled water and a series of dilutions were done to obtain concentrations of 0.05, 0.125, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 10.0 mM. Sodium Citrate solution was added in EuOTc solution to perform a calibration curve.

2.6 Fluorescent spectral analysis

The emission spectra were obtained by exciting the samples, inside a 1 mm optical path cuvette. The emissions of the samples were analyzed with a Fluorimeter Jobin Yvon exciting samples at 405 nm. Emission spectra were obtained between 580 and 700 nm.
2.7 Tumor excision and histological analysis

At the 7th, 14th and 21st three animals with prostate tumor were sacrificed and at 35th day, the remaining animals used in the experiment, including animals from PBS group, were sacrificed following the American Veterinary Medical Association guidelines for euthanasia. The prostates were excised and washed in PBS, fixed in 10% PBS-buffered formalin for 24h, and then routinely processed for paraffin-embedding. Histological analysis was performed in 4 µm sections stained with hematoxylin and eosin.

2.8 Statistical analysis

Statistical analysis of the differences between the experimental groups was performed by applying ANOVA test. Significance was set at p < 0.05. Data are expressed as mean ± standard deviation (SE).

3. RESULTS

To quantify the concentration of citrate in urine samples, a EuOTc:citrate complex calibration curve was obtained and it is shown in the Figure 1. In this curve the maximum intensity (at 617 nm) of the emission spectra were plotted as a function of the citrate concentrations. In the inside of the figure 1 it is shown the europium emission spectra in Oxytetracycline complex without and with 3 mM of citrate. The intensity of europium emission in Europium-Oxytetracycline complex in the presence of citrate shows a linear dependence, \( Y = a + b \times c_C \), where \( Y \) is the maximum intensity and \( c_C \) is the citrate concentration. The linear coefficient \( a = (2.18 \pm 0.11) \times 10^5 \) represents the background of the experiment. The angular coefficient \( b \) was found to be \((1.84 \pm 0.03) \times 10^5 \) (mM). The citrate concentration (\( c_C \) in mM) of unknown samples can be calculated from this equation since the value of the maximum intensity is experimentally determined. For these results it was possible to obtain the LOD (limit of detection) of 0.45 mM and the SD (standard deviation) of 2774.54. This procedure allows us to determine the concentration of citrate in urine samples solutions prepared according to our procedure.

Figure 1

Figure 2 shows the EuOTc + urine fluorescence intensity from animals of Control, PBS and Tumor (at 7, 14, 21 and 35 days) groups. Data are presented as mean ± standard error (SE). The means were calculated from the average of each spectrum of all animals from each group, and the maximum intensity peaks were plotted as a function of days of tumor progression. The samples were prepared by addition of the urine of each animal with EuOTc complex solution.

Control group corresponds to all animals before inoculation procedures and this group shows the highest intensity, followed by PBS group (the PBS animals have received just PBS (without DU145 cells) to exclude enhancement of EuOTc complex from possible inflammation, infection or surgery reaction, that presents lower intensity then control animals due to the damage caused by the needle insert into the prostate.
glands (these 5 animals had their urine collected at 7, 14, 21 and 35 days and there were no significant difference between these days). All maximum intensity value, independently of the collection day, was inside between the intervals, mean ± SE, of PBS group. As noted in figure 2 the citrate concentration, expressed in mM in the samples, was reduced in the prostate tumor animals urine.

Table 1 shows the europium fluorescent intensity of the control, PBS and tumor groups, shown in Figure 2. Data were expressed as mean ± standard error (SE). Citrate concentration is also shown in this table, where citrate concentration was calculated from the standard curve of citrate using the linear fitted curve equation. The calculated areas of tumors at 14, 21 and 35 days are also show in Table 1. To obtain these areas, histological analysis of the samples were done.

A panel of histological prostate slides (a, b, c, d, e, f) of control (inoculated PBS) and tumor (inoculated DU145 cells after 7, 14, 21 and 35 days) groups are shown in Figure 3. The PBS group samples (Fig 3.a) show normal prostate gland, and any inflammation characteristics were observed. Fig 3.b represents animals’ tumor group at 7 days from tumor induced. At this point of tumor staging no evidence of tumor cells could be observed by HE staining. Tumor progression was observed at 14th (Fig 3.c), 21st (Fig 3.d) and 35th (Fig 3.e) day evidenced proliferating tumor cell areas.

4. DISCUSSION

The aim of this study was to examine the correlation between citrate in urine of control and tumor induced groups, at different stages, in order to design a novel method to distinguish normal from cancerous specimens, since citrate concentration is diminished in prostate tumor cells. For this purpose the urine of nude mice in which prostate carcinoma cells (DU145) were inoculated was collected for the quantification of citrate concentration, using complex EuOTc.

Urine in water and pure citrate aqueous solution have no fluorescence in this analyzed spectral region. This is the reason for the use of EuOTc complex. When citrate binding to EuOTc complex, citrate replaces water molecules in the vicinity of Europium. With less water molecules binding to Europium ions, the energy transfer to water molecules is minimized and the energy is mainly kept in the Eu(III) ions, increasing its luminescence and lifetime intensity, resulting in an emission enhancement.

We observe from the figure 2 that the europium fluorescence intensity decreases with tumor progression, since less citrate is produced by prostate glands in the presence of the tumor. Anova test, considering \( p < 0.05 \), shows that PBS group result is
statistically significantly different from 14 days after tumor induction, when citrate concentration is analyzed in urine. In the comparison with control group and tumor animals, p<0.05 since the first week after inoculation procedure. Analysis of tumor cells in situ, using HE (Hematoxylin and Eosin) staining, confirm the growth of the tumor area during the experiment as it can be seen in the Figure 3.

By our results, citrate concentrations in malignant prostate urine decrease from the normal urine value from approximately 9.5 - 8.0 mM to 6.9 - 2.4 mM. This result is similar to described by Kline et al [23] that found around 2-fold difference in mean citrate concentrations between semen or expressed prostatic secretions in no cancer and cancer specimens.

It is our understandings that this is the first study that analysis Citrate concentration in the urine of animals with prostate cancer using EuOTc complex as the biosensor.

We believe that the analyses of semen or prostatic fluid would improve the results and make this method more efficient and sensitivity.

5. CONCLUSIONS

EuOTc complex fluorescence at 617 nm was analyzed in the presence of citrate and an enhancement of europium emission was observed. The samples of PBS and tumor urine groups were prepared by addition of the urine of each animal with EuOTc complex. It could be seen that the intensity means decay in the tumor samples, indicating that less citrate is produced for prostate cells. Citrate concentrations in malignant prostate urine decrease from the normal (PBS group) urine value from ~8.0 mM to ~2.4 mM (tumor group at 35th day).

The obtained results indicated that Europium Oxytetracycline provides a significant biosensor for prostate cancer detecting with a direct, accurate, non-invasive and non-enzymatic method for measurement of Citrate in biological fluids.

Acknowledgments

We thank Neide from IPEN–USP Bioterium and Paulinho from UNIFESP Histological Department.
Figure Captions

Figure 1. Calibration curve of EuOTc – Citrate : sodium citrate aqueous solutions of 0.05, 0.125, 0.25, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 10.0 mM were added to EuOTc solution obtained from emission spectra. In the inside figure it is shown the europium emission spectra in Oxytetracycline complex without and with 3 mM.

Figure 2. The maximum intensity emission peak of EuOTc + animals’ urine, in the range of 580 – 700 nm, plotted as a function of the days of tumor growth). Days 7, 14, 21 and 35 correspond to Tumor Groups at these respective days of tumor growth.

Figure 3 - Histological prostate slides of all points of tumor progression. A) The PBS group prostate slide; b) Prostate Tumor at 7th day; c) Prostate Tumor at 14th day; d) Prostate Tumor at 21st day; and e) Prostate Tumor at 35th day. Arrows indicate the tumor.
Table 1. Europium fluorescence Intensity and Citrate concentration measured for cancer at different stages of tumor progression and no cancer (PBS) samples. Tumor area measured from histological analysis were also shown.
Figure 1

Intensity ($10^5$ CPS) vs. Citrate concentration ($\mu$g/mL) graph showing a linear regression line with the equation $Y = A + B \times X$.

Linear Regression: $Y = A + B \times X$

Parameter | Value | Error
--- | --- | ---
$A$ | 21759.746 | 1074.0596
$B$ | 18439.171 | 285.82235

$R = 0.99892$
$SD = 2774.5403$

Figure 1
Figure 2.
<table>
<thead>
<tr>
<th>Groups</th>
<th>Intensity (a.u.) Mean ± SE ($10^6$)</th>
<th>[Citrate (mM)] Mean ± SE(µg/mL)</th>
<th>Tumor Mass Area (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.967 ± 0.169</td>
<td>9.51 ± 0.015</td>
<td>-</td>
</tr>
<tr>
<td>PBS</td>
<td>1.686 ± 0.178</td>
<td>7.98 ± 0.016</td>
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<tr>
<td>Tumor 7 Days</td>
<td>1.491 ± 0.096</td>
<td>6.91 ± 0.007</td>
<td>-</td>
</tr>
<tr>
<td>Tumor 14 Days</td>
<td>1.301 ± 0.094</td>
<td>5.93 ± 0.007</td>
<td>1420.4</td>
</tr>
<tr>
<td>Tumor 21 Days</td>
<td>1.103 ± 0.143</td>
<td>4.79 ± 0.013</td>
<td>4131.7</td>
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<tr>
<td>Tumor 35 Days</td>
<td>0.652 ± 0.065</td>
<td>2.38 ± 0.005</td>
<td>18520.2</td>
</tr>
</tbody>
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5. REFERENCES


