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ORIGINAL PAPER

Study of ProtoPorphyrin IX Elimination by Body Excreta: A new Noninvasive Cancer Diagnostic Method?

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Abstract This paper describes the elimination of porphyrins by feces. It was demonstrated that porphyrin accumulates substantially more in tumors than in normal tissues, and consequently more PPIX reaches the blood of patients and animals with tumors, and then, it needs to be eliminated. The fluorescence of feces revealed that there are large amounts of PPIX in the excreta of animals with cancer comparing with healthy animals. The autofluorescence of feces porphyrin extracted with acetone was analyzed using fluorescence spectroscopy of animals inoculated with DU145 cells into the prostate and healthy animals to monitor the PPIX concentration. Emission spectra were obtained by exciting the samples at 405 nm. Significant differences were observed in autofluorescence intensities measured in the 575–725 nm spectral regions for the studied groups. The results showed a noninvasive, simple, rapid and sensitive method to detect cancer by feces analysis.

Keywords Cancer · Feces · Porphyrin · Fluorescence · Photodiagnosis

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Introduction

The excess production of porphyrins in the organism may result in its elimination by feces and urine, as it has been reported in case of patients with the Porphyria disease [1], where even the diagnosis of such diseases comprises the analysis of increased urinary excretion of coproporphyrin and uroporphyrin [2]. In the case of cancer subjects the porphyrin, that increases in blood and tissue, is the protoporphyrin IX or PPIX. The selective accumulation of PPIX in malignant tissue provides a strong color contrast between the intense red fluorescence of malignant lesions and the weak fluorescence of normal tissue [3–5]. Abnormal metabolism of PPIX has also been observed in total blood, plasma and erythroid cells of cancerous patients [6, 7]. Since the excess of heme and PPIX is harmful to the organism, it is supposed that this excess can also be eliminated in the feces and urine.

The autofluorescence of blood protoporphyrin IX has already been analyzed using fluorescence and excitation spectroscopy on healthy male NUDE mice and in those with prostate cancer induced by inoculation of DU145 cells [6, 8]. A significant contrast between the blood of normal and cancer subjects could be established. Blood PPIX fluorescence showed an enhancement on the fluorescence band around 632 nm following tumor growth. The excess of PPIX is transferred into blood before it is eliminated by feces.

In this paper we analyze the elimination of the excess of PPIX synthesizes by tumorigenic cells.

Materials and Methods

Cell Line and Cell Culture Conditions

DU145 cells were cultured in DMEM containing high Glucose (4.5 g/L at 25 mM) and supplemented with 100 units/

mL Penicillin, 50 mg/mL Streptomycin, and 10 % fetal bovine serum (FBS). The cells were maintained in a humid chamber at 37 °C in an atmosphere of 5 % CO_2.

Animals

A total of 12 male NUDE mice, 6–8 weeks old on arrival, were obtained from the IPEN-USP and housed in laminar airflow cabinets under pathogen-free conditions with a 12-h light/12-h dark schedule and fed autoclaved standard chow and water ad libitum. These 12 animals were divided into 2 groups: 5 animals for the group without tumor (Control group) and 7 animals for the group with cancer (Tumor group). The control group refers to animals that have been inoculated with only sterile phosphate buffer solution (PBS), into prostate gland (without DU145 cells), to exclude enhancement of PPIX from possible inflammation, infection or surgery reaction. The orthotopic tumor model of prostate cancer [9] was used in the animals of tumor group, where 1×10^5 cells were inoculated into the prostate gland in a volume of 10 μL of sterile PBS.

Porphyrin Extraction

450 μL of analytical grade acetone were added to 150 μL of total blood collected and mixed well. The mixture was centrifuged at 4,000 rpm for 15 min. The clear supernatant of mixture was stored in a clean tube and spectrofluorometer analyses were carried out on the same day. Three times of the acetone's volume was added in the urine, following the same protocol used for blood. In the feces, it was added 200 μL of acetone to 10 mg of feces, macerating it and centrifuging the mixture at 4,000 rpm for 15 min. The supernatant was analyzed spectroscopically.

Fluorescent Spectral Analyses

The emission spectra were obtained by exciting the samples at 405 nm, inside of a 1 mm optical path cuvette. The fluorescence of the samples was analyzed with a Horiba Jobin Yvon Fluorolog 3 Fluorimeter in the range of 575–725 nm.

Results

The nude mice bearing prostate tumors were investigated by autofluorescence analysis of blood and feces and compared with healthy mice to study PPIX endogenous metabolism.

Figure 1 shows the results of blood porphyrin emission spectra, in the range of 575–725 nm, obtained from the blood extracted with acetone by excitation samples with a 405 nm Xenon lamp. In this figure each curve represents the average of signals obtained for Control and Tumor Groups.

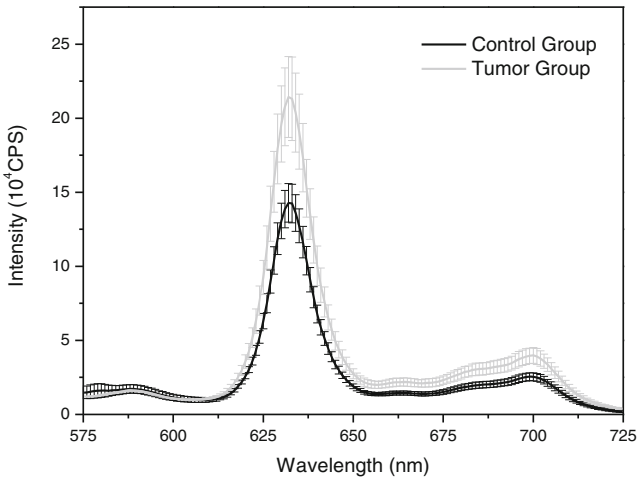

Fig. 1 The emission spectra of PPIX extracted from animals' blood, in the range of 575–725 nm, comparing the Control and Tumor Group, after 21 days of inoculation procedure

To verify our hypothesis that there is a higher porphyrin concentration in the animals' feces with more PPIX in the blood, i.e. tumor animals group, the excreta of animals was collected on the same day (21 days after the inoculation procedure) and extraction was performed before fluorescence analysis. The results of stool analyzed is shown in Fig. 2.

The emission spectra of the feces animals' group, Fig. 2, showed two bands, one with a maximum intensity at 632 nm and another at 671 nm. The peak at 632 nm refers to the PPIX emission, and the peak at 671 nm to the emission of coproporphyrin. The literature states that the bands between 616–625 nm and between 671–680 nm can be attributed to

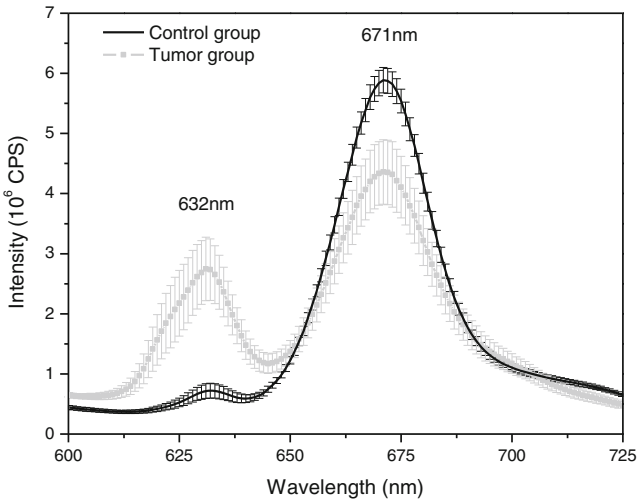

Fig. 2 Analysis of endogenous PPIX extracted from feces of control group and tumor (after 21 days of intraprostatic DU145 cells inoculation) group animals

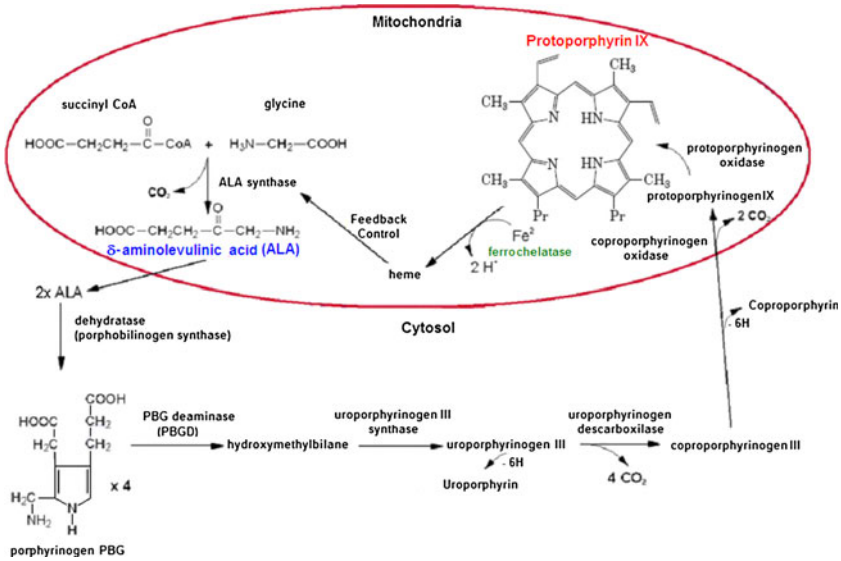

Fig. 3 Heme biosynthesis [Adapted from Ref. 11]

other soluble porphyrins such as coproporphyrin and uroporphyrin, or also to photoproducts formed by PPIX degradation [10].

In all feces samples, the most intense peak occurs at 671 nm, but it is clearly observed that in the animals with tumor the intensity of the band with a maximum peak at 632 nm has increased, while the band at 671 nm has decreased in emission intensity.

The coproporphyrin, as uroporphyrin and PPIX, is produced during the heme synthesis (Fig. 3). As in animals with tumor the PPIX synthesis is altered, it probably interferes in the synthesis of coproporphyrin as well. Thus, with the increase in the PPIX production, may there be less substrate for the production of coproporphyrin, and this is verified by the decrease in emission intensity at 671 nm.

Table 1 shows the mean value of the maximum intensity at 632 nm for both groups.

Statistically, there is a significant difference ($p < 0.05$) among the animals, for both, the blood and the stool analyzed. It is possible that the analysis of PPIX in stool can be used as a noninvasive diagnostic test for cancer, as it has already been done for porphyria. It is supposed the fecal occult blood is not an interference in this diagnostic method, since the use of acetone to extract the protoporphyrin from samples does not remove the iron (and other metals [12, 13]) responsible for quenching the fluorescence of heme groups. For measuring the nonfluorescing hemes of the possible fecal occult blood, it is necessary to incorporate a reduction step with oxalic acid to demetallation of heme [13].

Table 1 Maximums intensity of emissions at 632 nm of Fig. 2 spectra

Animals	Intensity at 632 nm (10^6 CPS)
Control group	0.72±0.12
Tumor group	2.73±0.51

In the urine emission spectra, shown in the Fig. 4, the PPIX emission band was not observed, since PPIX is poorly soluble in water and is excreted mainly in bile and feces [2]. In this figure it is possible to observe two emission bands: a more intense at 622 nm and another at 689 nm. Also, in these spectra, ranging between 575 and 610 nm, it is possible to find a strong scattering which occurs due to the crystals present in the urine. To avoid such interference in the spectrum of uroporphyrin, it is indicated a purification of the supernatant before spectroscopic analysis.

It is our understanding that this is the first study that analyzes the PPIX excreted by feces and urine of animals with cancer. The extra PPIX synthesized in tumor cells is

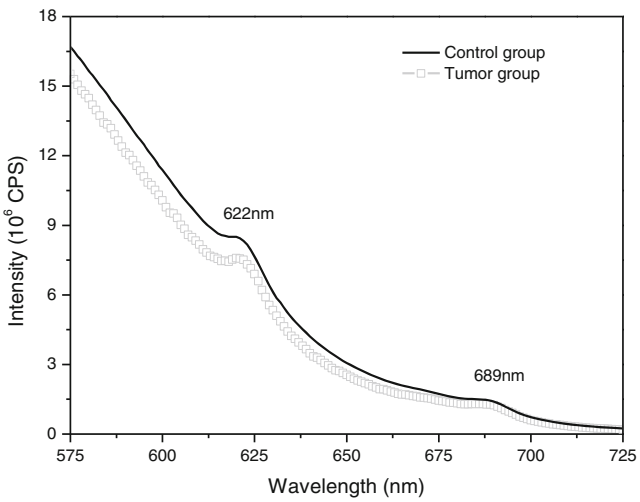

Fig. 4 Analysis of extracted urine porphyrins from animals with and without prostate tumor

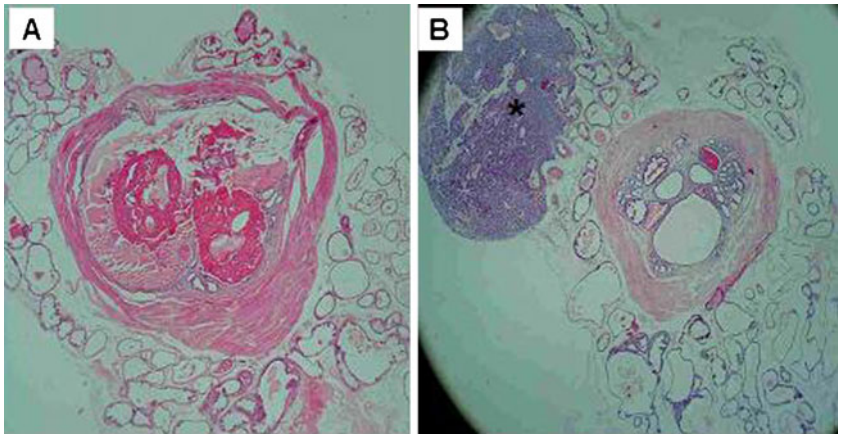

Fig. 5 Histological prostate slides of all animals. **a** The Control group prostate slide and **b** Prostate Tumor at 21st day

transferred to the blood. As the excess of heme and PPIX is harmful to the organism, it needs to be eliminated. In general, the porphyrinic fraction of the hemoglobin molecule, after iron has been removed and released into the blood, is converted by macrophages in several steps, in bile pigment bilirubin, released into the blood and later secreted by the liver in bile [14]. But, in animals with tumor, the higher concentration of PPIX is also excreted in feces, without suffering the conversion to bilirubin, probably due to a limitation of enzymes.

These significant differences can be used to diagnosis early prostate cancer. Although the study has been performed exclusively for identifying prostate tumor in an animal model, we believe this enhancement of PPIX fluorescence occurs in all kinds of cancer that accumulate endogenous PPIX, therefore this method can be used to diagnosis other cancers in an early stage, in animal and human models.

Larger and better-designed studies are being developed to elaborate more on this matter.

A panel of histological prostate slides of control (inoculated PBS) and tumor (inoculated DU145 cells) groups are shown in Fig. 5. The Control group samples (Fig. 5a) show normal prostate gland, and any inflammation characteristics were observed. Figure 5b represents animals tumor group at 21 days from tumor induced. At this point of tumor progression, the histological analysis of the prostate evidenced proliferating tumor cell areas, observed by HE staining.

Conclusions

The excreta of two groups of animals, one nominated Control group, and the other nominated Tumor group were studied of the point of view of tumor diagnosis. For this study the protoporphyrin IX was extracted of the excreta using acetone and the emission spectra between 575 and 725 nm were analyzed under excitation at 405 nm. This study shows that animals with prostate tumor presents an increase in the emission signal of PPIX extracted from their feces. With the increasing in the PPIX production, there is less substrate for the production of coproporphyrin, and this is verified by the decrease in emission intensity at 671 nm and an increase in the emission intensity of 632 nm band. In the urine emission spectra, no emission of PPIX was observed.

This work shows that fluorescence measurement in animal's feces is a noninvasive, simple, rapid, accurate and sensitive method for cancer diagnosis.

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