Spectrofluorimetric analysis *in vivo*: applications to kinetics of ALA induced fluorescence

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1. INTRODUCTION

Standard forms of PDT involve the administration of an endogenous photosensitizer. This photosensitizer accumulates preferentially in malignant tissues¹ and exposure to appropriate light wavelengths induces a phototoxic effect by producing active molecular species such as singlet oxygen (O₂) and free radicals². The advantage of photodynamic therapy (PDT) over conventional therapy is its dual selectivity³. First, increased selectivity is obtained by localization of the photosensitizer in tumor, and secondly toxicity is limited to the irradiated area, thereby avoiding serious damage to adjacent normal tissue. However, it remains difficult to document this selectivity routinely in patients. Selectivity is not likely to occur in all patients since tumors are heterogeneous and could also be transient. The capability of monitoring sensitizer concentration in tissues is thus of considerable importance. If that compound can be induced to fluoresce when exposed to photons of appropriate wavelength, then it may be possible to identify the compound of interest because of its characteristic fluorescence emission or excitation spectrum and then to follow its synthesis and/or degradation *in vivo* by measuring changes in fluorescence intensity. *In vivo* spectrofluorometry laser technique appears to have significant advantages over other methods. It is highly sensitive and can record complete fluorescence emission spectra so rapidly that it is now feasible to obtain a series of such spectra in anesthetized or non-anesthetized animals. An alternative approach to sensitizer administration for PDT is to achieve photosensitizer synthesis *in situ* using a precursor that is either preferentially localized or preferentially converted to active photosensitizer by target tissue or cells. ALA is an endogenous substance which is converted to heme via PpIX, an effective and well-known photosensitizer associated with the extreme skin phototoxicity seen in patients with porphyria^{4,5,6}. The present study was designed to investigate the possibility of monitoring PpIX fluorescence by the mean of a fiberoptic laser spectrofluorimeter designed in collaboration with INSERM U.281 (P.Lenz) after ALA injection. We also tried to enhance fluorescence by modifying PpIX concentration using the iron chelator desferrioxamine, also known as Desferal^R 7, as well as EDTA an other metal chelator.

2. MATERIALS AND METHODS

2.1 Fluorescence spectrometer

488 nm light from an argon laser was used to excite fluorescence which was then attenuated by reflection on a glass plate and fed into a 600 μ m core plastic-clad silica fiber with perpendicular polished end-faces by means of a small 45° metal mirror and an achromatic lens. The distal fiber end was brought into direct contact with the target tissue. This system provides simultaneous delivery of excitation light and gathering of fluorescence light. Light leaving the fiber is imaged onto a glass fiber bundle (ORIEL 77402) through the above-mentioned achromatic lens, a second achromatic lens located behind the metal mirror and a high-pass filter (SCHOTT OG 530) which virtually eliminates backscattered laser light. The fiber bundle converts the circular beam into a rectangular one. Thus convenient spectral resolution is obtained when this system is coupled to the monochromator (JOBIN YVON CP 200). Spectra are recorded using a cooled 1024-element diode array and a 386 computer (with JOBIN YVON QUIKVIEW software). The 488 nm line of an argon ion laser was used for excitation. Recording of a single spectrum took 20 ms, but we integrated over 3 s. Power density was 2 mW cm⁻² (488 nm). Light (excitation and reception) was then transmitted through a 600 μ m silica silicon step index optic fiber.

2.2. Animals

Six-weeks-old NMRI hairless male nude mice (CERJ) weighing approximately 30 g were used in this experiment. HT 29 tumor (human colic adenocarcinoma) was injected sub-cutaneously (0,2 ml of crushed tumor in solution in culture medium). All mice received a tumor graft [8]. Tumors were allowed to grow for 12 days until the experiment was performed. At that time, their mean diameter was 10 mm (SD +/-2 mm).

2.3. Chemicals

5-aminolevulinic acid (ALA) was obtained from Sigma Chemical Company (UK). Solutions containing 30, 60 or 90 mg of ALA per milliliter of isotonic saline were prepared immediately before use. 0,25 ml was injected intraperitoneally in order to reach final mouse concentrations of 250, 500 and 750 mg/kg of body weight. All mice were kept in the dark until spectrofluorometry was performed. Desferrioxamine was purchased in the lyophilized form of desferrioxamine mesylate (Desferal[®] [DFO]) from CIBA-Geigy (Basel,

Switzerland). This compound was administered subcutaneously to obtain concentrations of 20 and 40 mg/kg of body weight. EDTA was obtained from Grosseron (GROSSERON S.A., Nantes, France) and administered subcutaneously to obtain concentrations of 10 and 20 mg/kg of body weight.

2.4. Method

Kinetic fluorometric studies were performed after administration of etheroxide inhalant at intervals of 0; 30min; 60min; 90min; 2h; 3h; 4h and 6h and as well as in different tissues. We carried out measurements through the optic fiber placed directly in contact with the target. DFO and EDTA were injected subcutaneously 4 hours before ALA administration. Two, three and five hours after ALA injection, spectra were recorded directly in tissues. Thus, DFO or EDTA was injected at least 6 hours before spectrum recording. Muscle, tumor, liver, gall bladder, urinary bladder, kidney, heart and brain were successively studied. The same experimental conditions used to obtain sample material, anesthetic techniques and darkness were applied to the different groups. The fluorescence emission spectra of treated or untreated mouse tissue were recorded on 3 separate occasions in order to estimate mouse-to-mouse variation.

3. RESULTS

3.1. In vivo PpIX fluorescence spectra after ALA injection

Administration of a 250 mg/kg ALA solution to mice resulted in an increase in tissue fluorescence, with emission maxima at 632 nm and 702 nm. All mice produced comparable PpIX emission spectra for all tissues studied. The intensity of this fluorescence increased for approximately 180 min in lung and kidney but for only 120 min in muscle and gall bladder gradually decreasing over a 3-h period.

Figure 1 shows the spectral modifications of fluorescence in HT 29 adenocarcinoma (A) or normal liver (B) as a function of time. PpIX fluorescence was maximal after 180 min in liver but only after 90 min in tumors.

In addition, we noted an increased fluorescence emission at 545 nm in liver, gall bladder and urinary bladder. This emission wavelength, typical of metabolized porphyrins such as bilirubin, was present in controls but increased after ALA injection. In controls receiving no ALA, 545 nm fluorescence was 30 cts/s in gall bladder and urinary bladder but increased after 60 min (gall bladder) and 360 min (urinary bladder) to a mean value of 168 and 101 cts/s respectively. 632 nm fluorescence was also found in the urinary bladder less than 240 min after ALA injection but then decreased (Fig 1C).

Figure 1: Evolution of ALA-induced PpIX fluorescence as a function of time. Spectra were recorded in HT 29 human adenocarcinomas grafted in nude mice (1A), in healthy mice liver (B), in the gall bladder (C)

3.2. Changes in PpIX fluorescence after ALA injection

Tmax and fluorescence intensities differed from one organ to another. In addition, in some organs fluorescence increased and decreased in several phases. In heart, brain and lung, fluorescence intensity was relatively low (about 10-25 cts/s) but in elimination organs the fluorescence level was significantly higher, i.e. gall bladder with a recording of about 610 cts/s.

TABLE 1 : Evolution of PpIX after ALA injection

TISSUES	Tmax FLUO	CONTROL I*	INTENSITY	I/I*
632nm TUMOR	90min	3	31	10,33
MUSCLE	120min	2	11,66	5,83
LIVER	60-180min	7	82,25	11,75
GALL BLADDER	120min	9	610	67,8
BLADDER	60-120-240 300min	9	40	4,44
KIDNEY	180min	4	31,38	7,84
BRAIN	120min	3,5	13	3,71
HEART	60-120min	7	11	1,57
LUNG	180min	7	24	3,43
545nm GALL BLADDER	60min	30	168,5	5,62
BLADDER	60-360min	29	101	3,48

T MAX : TIME AT WHICH FLUORESCENCE INTENSITY IS MAXIMAL
AFTER ALA INJECTION
I* : BACKGROUND INTENSITY IN TISSUES
I/I* : INTENSITY IN TISSUE AFTER CHEMICAL ADMINISTRATION
TO BACKGROUND INTENSITY WAS STUDIED IN TISSUE.

3.4. Influence of DFO on ALA-induced PpIX fluorescence

Administration of 20 or 40 mg/kg DFO alone did not enhance background tissue fluorescence. DFO administered before ALA induced different effects on PpIX fluorescence changes. Tissues can again be divided into 3 groups (Table 2). In the first group, PpIX fluorescence intensity increased in HT 29 adenocarcinoma, muscle, heart and gall bladder, but the time required to reach maximum fluorescence was the same in these tissues as in mice injected with ALA alone. In the second group, DFO 20 or 40 mg/kg induced only slight changes in ALA-stimulated porphyrin production which was even somewhat decreased compared, to that of controls. However, in liver there was a shorter interval after ALA administration before maximal fluorescence was reached. In kidney and brain, on the contrary, Tmax was shifted to 5 h with DFO instead of 3 h as in controls. In the urinary bladder, both Tmax and fluorescence intensity were changed for DFO 40 mg/kg. The PpIX fluorescence intensity induced by ALA increased after DFO administration, and there was a shortening of the Tmax shifting from 5h to 3 h. After DFO 20 mg/kg, fluorescence intensity increased, but the kinetic was similar to that of controls.

TABLE 2 : Influence of DFO on ALA induced PpIX fluorescence

DFO*	0			20mg/kg			40mg/kg		
	Tmax	I _{max}	I/I*	Tmax	I _{max}	I/I*	Tmax	I _{max}	I/I*
ORGAN									
GROUP 1									
TUMOR	2h	29,33	9,78	2h	41,5	13,83	2h	34	11,33
MUSCLE	2h	14	7	2h	14,33	7,165	2h	17,5	8,75
HEART	2h	11	1,57	2h	20	2,86	2h	20	2,86
GALL BLADDER	2h	610	67,77	2h	987	109,66	2h	390	43,33
GROUP 2									
LIVER	3h	106	15,14	2h	72,66	10,38	2h	60,33	8,62
KIDNEY	3h	38	9,5	5h	31,5	7,87	5h	32,5	8,125
BRAIN	3h	20	5,71	5h	5	1,43	5h	4,5	1,28
GROUP 3									
BLADDER	5h	40	4,44	5h	84	9,33	3h	71	7,89

*DFO Desferrioxamin.
I_{max}: Maximal intensity.

Tmax: Time when fluorescence intensity is maximal since ALA injection.
I/I*: Intensity recorded in the tissue after chemical administration /
background intensity in tissue studied.

3.5. Influence of EDTA on ALA-induced PpIX fluorescence.

Administration of 10 or 20 mg/kg EDTA alone did not enhance background tissue fluorescence. Tissues can be separated into 3 groups in terms of EDTA effect : In the first group, EDTA decreased

PpIX fluorescence intensity to a non-significant level. In liver, fluorescence intensity was lower with EDTA and ALA than with ALA alone. However, fluorescence remained easily detectable. In liver and gall bladder, although T_{max} were unchanged, maximum fluorescence decreased for 10 mg/kg but increased for 20 mg/kg. Tumor tissues showed a T_{max} identical to controls but minimum fluorescence with 10 mg/kg and then increased amounts. EDTA decreased T_{max} and fluorescence intensity in kidney and urinary bladder.

4. DISCUSSION

We designed a laser spectrofluorometer to measure tissue sensitizer concentration since PDT efficacy is at least partly concentration-related. A new means of PDT sensitization was studied, involving administration of ALA to induce endogenous porphyrin synthesis. Kinetic studies of porphyrin biosynthesis in mouse tissues are presented as examples of the potential usefulness of the spectrofluorometer.

As ALA is non- or weakly fluorescent, the emission spectrum is related to the fluorescent material that appears in the mouse tissues after systemic administration which is thought to be PpIX.

This study showed a strong correlation between the intensity and location of ALA-induced PpIX fluorescence. In the absence of significant concentrations of quenching substances, that cell will begin to develop PpIX fluorescence and also will become photosensitized [11]. However, tissues such as liver may contain a high concentration of PpIX fluorescence but emit little because of internal quenching of the fluorescence. All of this indicates why fluorescence depends greatly on the organ involved as well as the beside ALA dose or the interval after injection.

We investigated the distribution of ALA-induced porphyrin fluorescence at various intervals after introduction of ALA, both in normal and tumor tissue in the mouse. Divaris et al.⁹ have shown that the intensity of ALA-induced PpIX fluorescence in tissue after systemic administration of ALA to mice is correlated with the amount of phototoxic damage. Maximum fluorescence is rapidly obtained with near-maximal tissue levels of PpIX reached at 2-3h. We also demonstrated a significant advantage of ALA-induced porphyrin over conventional sensitizers in that the sensitization level returns to nearly background fluorescence after 6 hours, except in urinary bladder, gall bladder and liver.

Modulation of ALA-induced porphyrin synthesis is also of interest as a means of eventually increasing PpIX concentration in tumors. Since desferrioxamine (DFO) is used as a therapeutic iron chelator in humans, we investigated whether tetrapyrrol synthesis may be stimulated in mammalian cells by DFO iron trapping. DFO is reported to be a very potent stimulator of ALA-driven photosensitization *in vitro*. In our study, DFO or EDTA administration before ALA injection induced no significant increase in PpIX fluorescence but rather an inhibitory action on ALA-induced PpIX fluorescence. In addition, it seemed to us that DFO and EDTA induced modifications in elimination pathways of endogenously synthesized porphyrins.

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