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Preliminary Note

In vivo photoproduct formation during PDT with ALA-induced endogenous porphyrins

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Abstract

The administration of 5-aminolevulinic acid (ALA) in tumor-bearing nude mice leads to the formation of the fluorescent, photounstable photosensitizer protoporphyrin IX in tumor tissue. On-line fluorescence spectroscopy during photodynamic therapy (PDT) shows the *in vivo* formation of chlorin-type photoproducts of protoporphyrin. The fluorescence of protoporphyrin as well as its photoproducts is bleached completely at the end of the PDT (100 J cm^{-2}, 630 nm). These findings were also verified using ultrashort laser pulses and time-correlated single-photon counting. A photoinduced shortening of the decay times and decrease in the integral fluorescence intensity were measured *in vivo* due to the photodestruction of the endogenous photosensitizer protoporphyrin IX in the tumor.

Keywords: ALA, Protoporphyrin, Photoproducts, PDT, Fluorescence

1. Introduction

5-Aminolevulinic acid (ALA) is a precursor in the biosynthesis of heme, cytochrome, chlorophyll, bile pigments and other porphyrins. The external administration of ALA leads to the formation of the fluorescent and photounstable photosensitizer protoporphyrin IX in certain types of mammalian cells [1]. Kennedy *et al.* [2] used the ALA-induced synthesis of protoporphyrin to treat superficial basal cell carcinoma.

The purpose of this study was to investigate the fluorescence of tumors and surrounding tissue after administration of ALA in tumor-bearing nude mice and to look for modifications of the fluorescence spectrum during photodynamic therapy (PDT).

2. Materials and methods

2.1. Experimental apparatus

Figure 1 shows a scheme of our experimental apparatus. The 407 nm line of a krypton ion laser was used for excitation. The background radiation of the laser consisting of spontaneous radiation and luminescence from the Brewster windows was separated by means of two quartz prisms. We then coupled the 407 nm radiation into the 0.2 mm central fiber of a fiber bundle. Eight peripheral fibers were used for detection, transmitting the fluorescence radiation into a polychromator and an optical multichannel analyzer. Registration of a single spectrum took 20 ms, but we integrated over 1 s.

Time-resolved measurements were carried out with a novel frequency-doubled laser diode for excitation (pulse duration, 40 ps; repetition frequency, 100 kHz, Hamamatsu Photonics). The kinetics of fluorescence decay were measured using a single-photon-counting unit, as described elsewhere [3], and evaluated by bi-exponential curve fitting.

A dye laser emitting at 630 nm served as light source for PDT. During the fluorescence measurement, a shutter stopped the PDT radiation for 1 s. Power densities on the surface of the tumor were 100 mW cm^{-2} (630 nm) for PDT and 5 mW cm^{-2} (407 nm) for fluorescence detection.

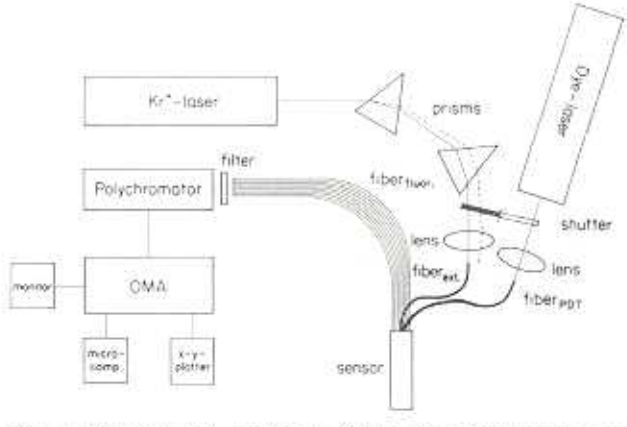

Fig. 1. Experimental apparatus for *in vivo* fluorescence measurements.

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The fluorescence measurements and PDT started 3 h after administration of ALA.

2.2. Tumors and animals

Human G2 bladder tumors were transplanted into female nude mice (NMRI nu-nu; body weight, about 25 g) subcutaneously. The skin covering the tumor was carefully removed for measurements 3 h after administration.

2.3. Chemicals

ALA (Sigma, Germany) was given intravenously (7 mg mouse^{-1}; solvent, phosphate-buffered saline (PBS); pH 7.3). In a first experiment we applied ALA topically (solvent, PBS–dimethylsulfoxide (1:1); a cotton swab with ALA was fixed on the skin above the tumor for 3 h.)

3. Results

No fluorescence of protoporphyrin was found in the tumor in the case of topical administration of ALA on the skin with intact stratum corneum. However, a strong fluorescence with bands around 635 and 710 nm appeared after systemic administration.

The fluorescence intensity in the tumor after intravenous injection was a factor of eight higher than in the surrounding muscle tissue, but lower than in the skin (see Fig. 2).

Fluorescence measurements of the xenotransplanted tumors were carried out during PDT. Figure 3 shows the modifications of the fluorescence spectrum during the treatment. The fluorescence intensities at 635 and 710 nm decreased due to the process of photobleaching. In contrast, the fluorescence intensity in the spectral region around 670 nm increased. During further light exposure the new band reached a maximum and then slowly decreased. The curves show the fluorescence for PDT irradiation doses of 0, 2, 4, 6 and 8 J cm^{-2}. At the end of the PDT, after a total radiant exposure of 100 J cm^{-2}, no fluorescence in the red spectral region could be measured as a result of complete photobleaching.

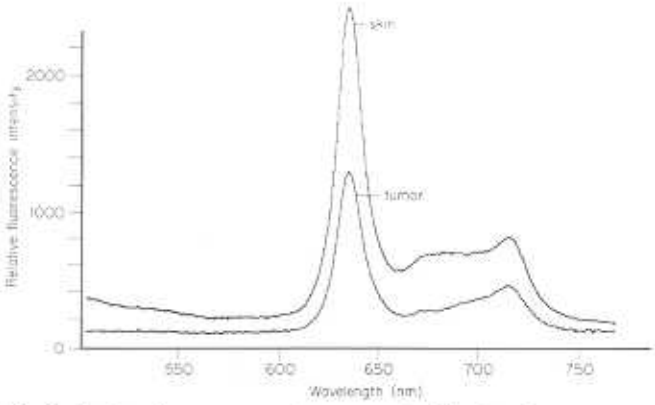

Fig. 2. *In vivo* fluorescence of tumor and skin after intravenous ALA injection (7 mg mouse^{-1}).

Protoporphyrin IX, resolved in dimethylsulfoxide, showed similar modifications during light exposure. The origin of the new fluorescence band can be explained by the formation of fluorescent photoproducts [4–8].

These *in vivo* findings were verified using time-resolved fluorescence measurements. First, the decay times of protoporphyrin IX and of an irradiated solution containing photoproducts were determined. The photoproducts showed decay times of 670 ps and 4.5 ns, whereas protoporphyrin exhibited a weak component with a decay time of around 3 ns and a main component with a decay time around 17 ns in dimethylsulfoxide. The long decay time is typical for porphyrin monomers [7, 9, 10].

Then, the *in vivo* kinetics of fluorescence decay of the tumors transplanted in nude mice were measured (see Fig. 4). After deconvolution of the decay curve of the tumor fluorescence before irradiation, decay times of about 230 ps and 17 ns were obtained. Irradiation by a high-pressure mercury lamp (10 J cm^{-2}) resulted in a significantly faster decay. The shortening of the decay times and the decrease in the integral fluorescence intensity were clearly demonstrated. The short-lived component in the picosecond range is superposed by scattered radiation; however, the decay time of around 5 ns is related to fluorescent photoproducts.

4. Discussion

These *in vivo* findings show that PDT using ALA-induced endogenous porphyrins leads to cytotoxic reactions and, in addition, to the photodestruction of these photosensitizers. The photodestruction results in photobleaching and the formation of fluorescent photoproducts leading to modifications of the fluorescence intensity, fluorescence spectrum and fluorescence decay kinetics.

Inhoffen *et al.* [4] described the possible photo-induced formation of two chlorin-type photoproducts of protoporphyrin. These photoproducts are created by the reduction of a double bond. Figure 5 depicts two types of isomer, formed by the action of light on protoporphyrin IX, which have been separated chromatographically [4].

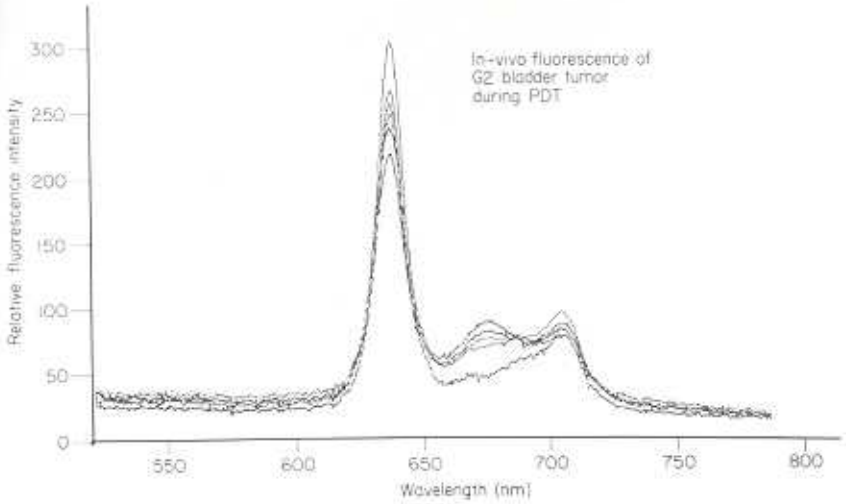

Fig. 3. Modifications of the *in vivo* fluorescence spectrum of the tumor during PDT (radiant exposure, 0, 2, 4, 6 and 8 J cm^{-2}; $\lambda_{PDT} = 630$ nm; $\lambda_F = 407$ nm).

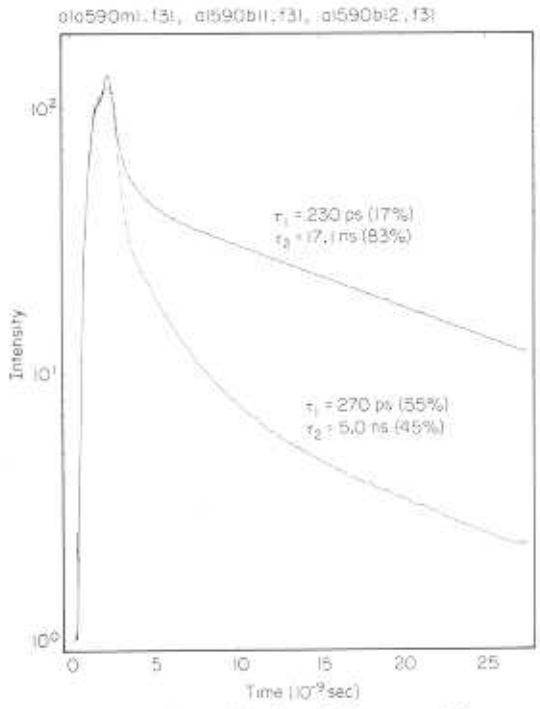

Fig. 4. *In vivo* decay kinetics of the tumor fluorescence: upper curve, before irradiation; lower curve, after irradiation (10 J cm^{-2}, high-pressure mercury lamp).

König *et al.* [5, 10] showed that the photochemical process of formation of photoproducts of protoporphyrin IX and of hematoporphyrin requires oxygen and that singlet oxygen is involved. Singlet oxygen is the main cytotoxic agent responsible for the photodynamic effect [11]. Therefore on-line *in vivo* fluorescence measurements during PDT may give information on the efficiency of the treatment.

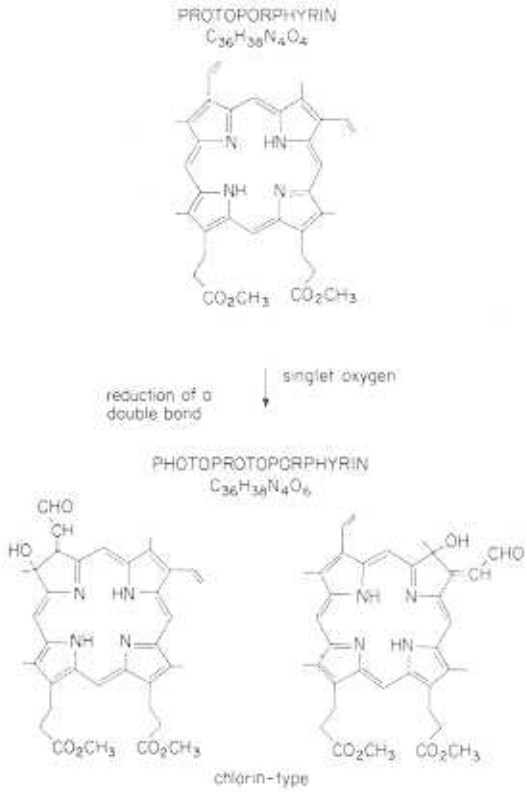

Fig. 5. Structure of the two chlorin-type photoproducts, separated chromatographically by Inhoffen *et al.* [4]. Singlet oxygen is involved in the formation of these isomers.

Acknowledgments

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