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Laser-induced fluorescence diagnostics of basal cell carcinomas of the skin following topical ALA application

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ABSTRACT

Fourteen patients with superficial basal cell carcinomas (BCCs) and fifteen patients with nodular BCCs were investigated by means of laser-induced fluorescence (LIF) in connection with photodynamic therapy (PDT). Topical application of δ -amino levulinic acid (ALA) was performed six hours prior to the treatment session. Fluorescence spectra were recorded, using a point-monitoring system with an excitation wavelength of 405 nm. The measurements were performed in scans over the lesion and the surrounding normal skin before application of ALA, and immediately before and after the laser treatment. The selective uptake of the photosensitiser resulted in a fluorescence intensity ratio of 2.4:1 for superficial BCCs and 2.5:1 for nodular BCCs. If the fluorescence intensity was divided by the autofluorescence, this resulted in a contrast enhancement of about a factor 6 for tumour tissue.

In seven patients (five with nodular BCC and two with superficial BCC), additional fluorescence measurements were performed two and four hours following the ALA application, and two hours after the PDT procedure. Thus, the kinetics of the transformation of ALA to protoporphyrin IX (PpIX) could be followed, which indicated that the synthesis of PpIX was more rapid in the tumour than in the normal tissue. After four hours, the PpIX level inside the tumour was saturated, while there still was an accumulation in the surrounding skin. The highest contrast between tumour and normal skin was reached within two hours after the ALA application.

Keywords: tissue diagnostics, fluorescence spectroscopy, non-melanoma skin malignancy, basal cell carcinoma, photosensitiser, ALA

1. INTRODUCTION

Photodynamic therapy (PDT) using non-ionising laser light in the visible wavelength region, in combination with tumour-sensitising drugs, is an example of a new non-thermal laser interaction with biological tissue. The method relies on the selective transfer of triplet to singlet oxygen, and the generation of free radicals or radical ions mediated by the laser excitation of the sensitising drug. It has been applied in the treatment of different types of tumours, including skin malignancies.¹⁻³ The drug, which normally is injected intravenously, is retained to a greater degree in the malignant tissue than in normal tissue. Thus, a selective necrosis of the tumour is obtained if the drug is activated by laser light in the wavelength region in which it has its light-absorption band. The tumour necrosis is due to cytotoxic effects on the tumour cells caused by the released singlet oxygen or the free radicals, but may also be an effect on the vessel wall endothelium in the tumour vascular system.⁴ A disadvantage with many of the photosensitising drugs administered intravenously is that they cause a transient photosensitivity, and the patients must take photoprotective measures against ambient daylight for about four weeks.

An alternative PDT procedure for treating superficial malignant tumours was described by Kennedy *et al.*^{5,6} In this procedure topical application of the non-fluorescent, photodynamically non-active haem precursor δ -amino levulinic acid (ALA) was used instead of an intravenously administered sensitiser. The highly fluorescent and photodynamically active

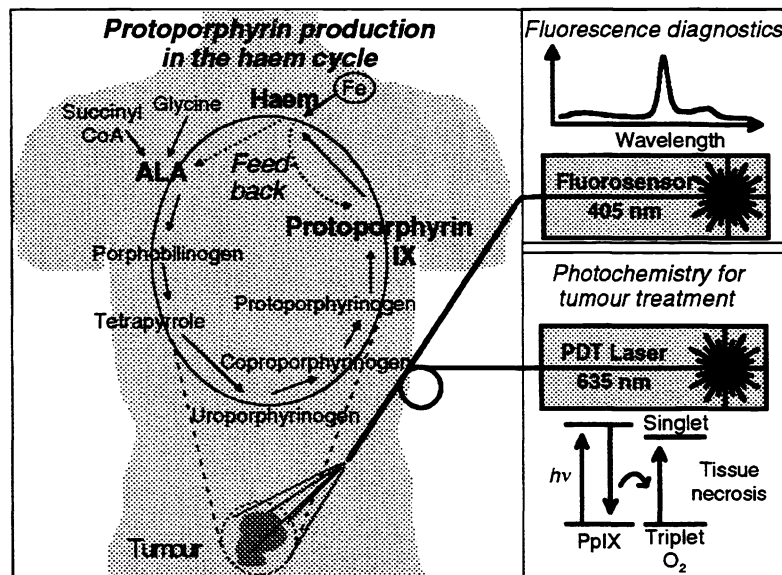


Figure 1. A schematic diagram of PDT using topical application of ALA. The biosynthesis of haem is shown to the left. A fluorescence spectrum of ALA-induced PpIX excited with 405 nm is shown in the top right corner. The bottom right corner shows a schematic of the photochemically induced energy transfer during PDT, leading to tissue necrosis.

substance protoporphyrin IX (PpIX) is synthesised via the haem cycle in cells exposed to ALA. This has made the modality more convenient, mainly because of the local and transient skin sensitisation with a short clearing time of about 24 hours.^{7,8} The method has proved to be effective on different types of non-melanoma skin malignancies.^{5,6,9}

Since PpIX is a highly fluorescent substance, the transformation of ALA to PpIX can easily be monitored by means of laser-induced fluorescence. In this study the spatial distribution of PpIX in basal cell carcinoma and the surrounding normal skin has been investigated *in vivo*, using a fibre-based fluorosensor to record the fluorescence spectra. The system used was equipped with an excitation light source, emitting light at a wavelength of 405 nm, and an optical multichannel analyser (OMA) system. Information on the kinetics of the synthesis of PpIX and the tumour selectivity in the superficial layers of the area planned for treatment was obtained.

2. MATERIALS AND METHODS

2. 1. Patients

A total of 29 ALA-PDT patients are presented in the present paper. Laser-induced fluorescence measurements were performed in 14 superficial and 15 nodular lesions. The median age of the patients was 69 years, ranging from 42 to 88 years. Of the 29 patients, 16 were male and 13 were female. The location of the lesion varied from patient to patient.

2. 2. Drug

The treatment procedure was initiated by applying a cream containing 20% ALA onto the lesion and a 10 mm margin in the surrounding normal skin. An occlusion pad (a thin adhesive plastic film) was used to prevent the cream from being smeared off, and a thicker dressing on top was used to protect from light. The application was made six hours prior to the laser treatment session.

ALA itself is a non-fluorescent and photodynamically non-active substance, which has the property of penetrating a few millimetres into abnormal keratin, and certain cells have the capacity of accumulating ALA. ALA is the first step in the haem cycle, which takes place in a number of cells in the body. The haem production within the cycle is regulated by different feed-back systems and the synthesis of different products is enzymatically dependent. The enzyme pattern involved in the haem cycle varies in different tissue types. Thus, the production of the highly fluorescent and photodynamically active PpIX is enhanced in malignant tissue due to higher enzymatic activity at the beginning of the cycle. Furthermore, the insufficient amount of ferrochelatase in malignant tissue results in a PpIX accumulation with only a low haem production.^{10,11} When ALA is distributed excessively, the feed-back mechanisms are overridden and PpIX is produced in malignant tissue and to a certain degree in tissues originating from the ecto- and endoterm, i.e. the epidermis, the mucous membranes, etc.⁶⁻⁸ The production of PpIX and the subsequent laser-induced photodynamic action leading to selective tissue necrosis are illustrated in Figure 1.

The transformation of ALA into PpIX takes place within a few hours, and can be visualised by means of laser-induced fluorescence recorded *in vivo*. The fluorescence from PpIX excited with laser light at a wavelength of 405 nm is characterised by a dual-peaked signal at about 635 and 700 nm.

2. 3. Fluorescence measurement equipment

A clinically adapted optical multichannel analyser (OMA) system, arranged on a mobile trolley, was used for the fluorescence measurements. The set-up is shown in Figure 2 and is described in detail elsewhere.¹² The excitation source of the system is a pulsed nitrogen laser pumping a dye laser, tuned to a wavelength of 405 nm. The laser system thus yielded excitation light with 10 μ J per pulse at a repetition rate of 10 Hz. This light was guided with mirrors through a lens and finally through an optical quartz fibre with a core diameter of 600 μ m. During the measurements, the fibre tip was held in close contact with and perpendicular to the tissue surface. The resulting fluorescence from the tissue and the photosensitiser was collected and guided by the same fibre to the detector; an intensified linear diode array connected to a polychromator. The full fluorescence spectrum was displayed in real-time, and stored on floppy disks for later evaluation.

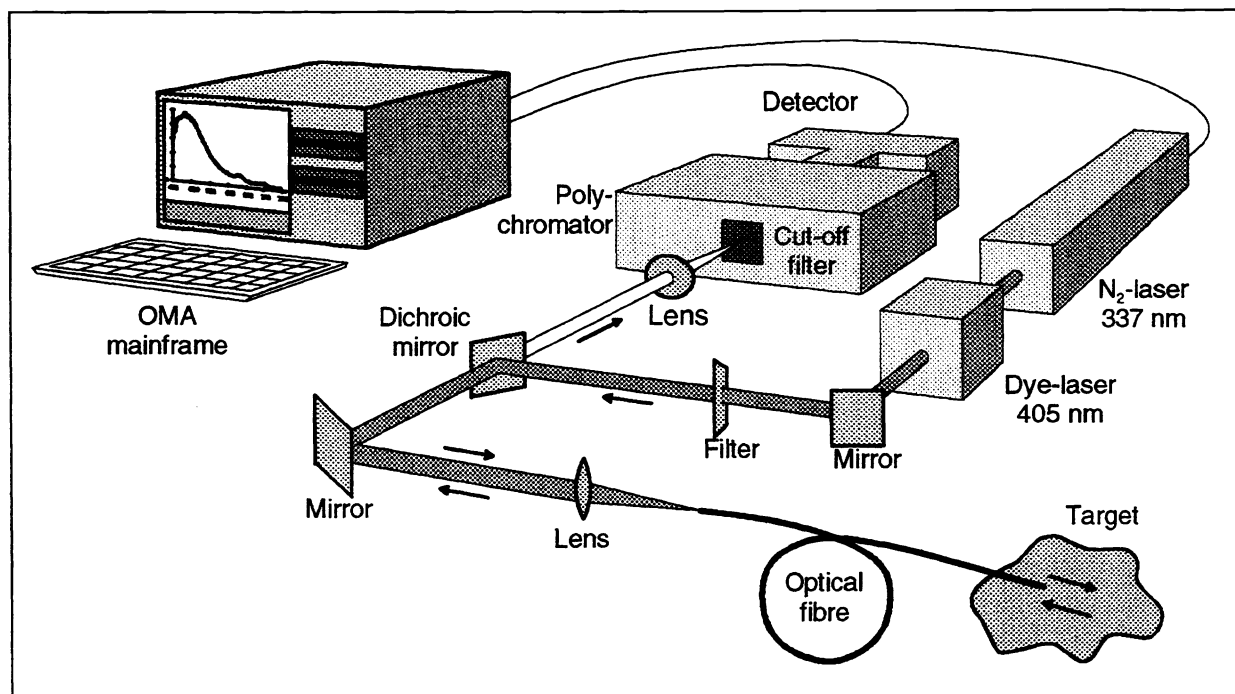


Figure 2. The set-up of the optical multichannel analyser (OMA) system for fluorescence measurements.

2. 4. Measurement procedure

The ALA-induced PpIX formation was measured *in vivo*, by means of laser-induced fluorescence (LIF), before the laser treatment procedures, in order to monitor the PpIX accumulation in the ALA-treated area. The fluorescence spectra were recorded in scans over the tumour areas, including the normal skin surrounding the lesions (Figure 3). The spot size for every measurement point was about 600 μm . The tumour fluorescence was monitored at the border, 2 mm inside the tumour and in the tumour centre. Fluorescence spectra were also recorded in the surrounding normal skin at 2, 5 and 10 mm from the visible border of the tumour. At each measurement point, two measurements were performed, resulting in twelve spectra from each patient at each occasion. In order to investigate the laser-induced photobleaching of the active substance, the fluorescence was measured again immediately after the laser treatment.

Additional fluorescence measurements were performed two and four hours after the ALA application, and finally at two hours after the treatment procedure in seven of the lesions. The two first measurements, in combination with those mentioned above, were used to study the kinetics of the transformation of ALA into PpIX. The last measurement indicated whether the PpIX synthesis continued after the PDT or not.

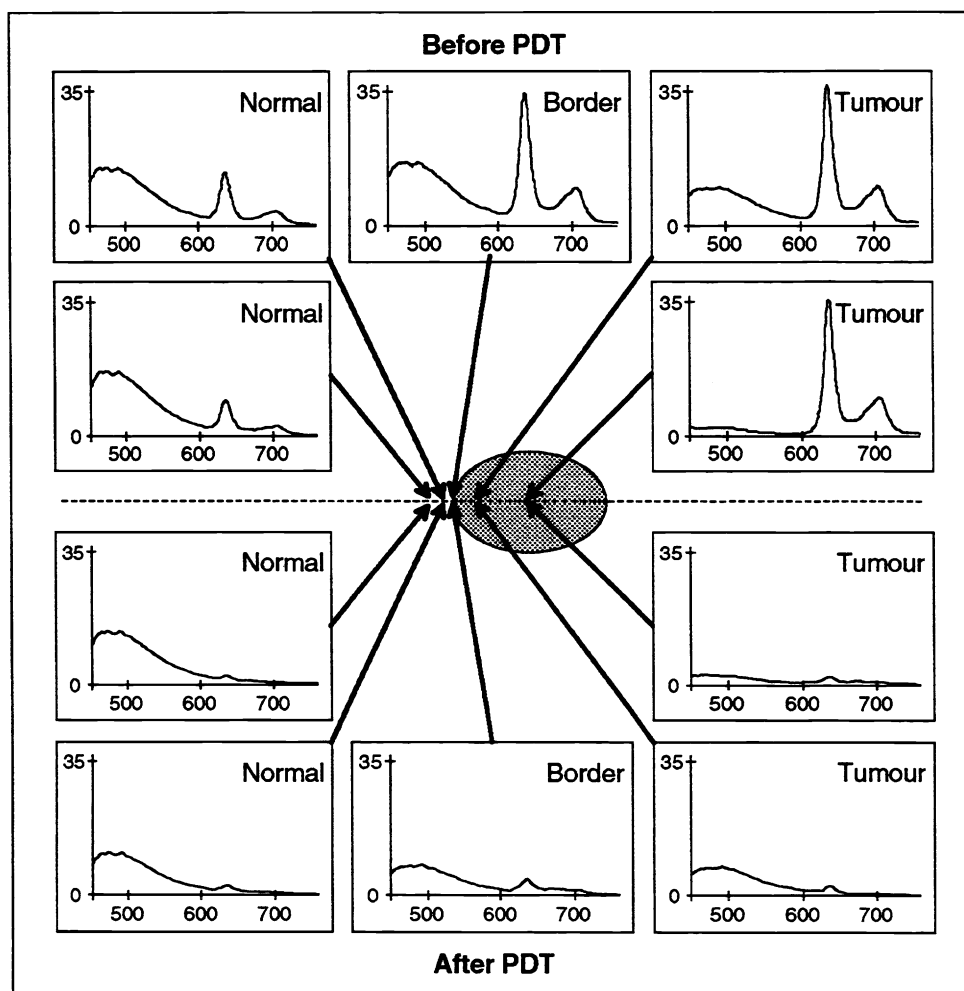


Figure 3. Fluorescence spectra recorded before PDT in a scan of a superficial basal cell carcinoma and the surrounding normal skin six hours after topical application of ALA (top). Similar recordings after PDT, indicating the laser-induced photobleaching of the sensitizer, are shown below.

2. 5. Data analysis

After the measurements, the fluorescence spectra were transferred to a personal computer for evaluation, using a computer program developed at the department.¹³ The PpIX-related fluorescence signal at 635 nm, was obtained as the total fluorescence intensity in the peak minus the background, originating from tissue autofluorescence. The background was given by fitting an exponential curve in the wavelength regions 550 to 600 nm, and 750 to 800 nm to the recorded spectrum. The background-free fluorescence at 635 nm is related to the amount of PpIX in the tissue, and was used to monitor the accumulation of the photosensitiser. The peak intensity of the autofluorescence at 490 nm was also evaluated. The intensity was used for diagnostics of the tissue, as it has been shown that the autofluorescence is lower in malignant tissue than in normal tissue.¹⁴ Forming the ratio between the PpIX-related fluorescence intensity and the autofluorescence will thus give an enhanced contrast between tumour and normal tissue. The ratio is a dimensionless quantity and is more insensitive to measurement geometry, fluctuations in the excitation light source etc.

3. RESULTS

3. 1. Selectivity

All lesions monitored by means of LIF exhibited a marked selectivity of the accumulation of PpIX in tumour compared to the surrounding normal skin six hours after ALA application (Figure 3). As seen in the different spectra, the tumour area is characterised by the dual-peaked fluorescence signal at 635 and 700 nm from PpIX, whereas the normal skin shows very low porphyrin-related fluorescence. The porphyrin fluorescence does, however, start to build up even in the visibly normal skin close to the tumour. In parallel, there is a decrease in the autofluorescence, peaking at about 490 nm. The autofluorescence usually has a lower intensity in malignant tumours than in normal tissue and represents the fluorescence from endogenous chromophores, such as NADH, NAD⁺, collagen, elastin and tryptophane. The decrease in the autofluorescence intensity inside the tumour was first reported in 1984,¹⁴ and is mainly due to a change in the relative concentrations of the highly fluorescent NADH and the less fluorescent NAD⁺.^{15,16}

The background-free PpIX-related fluorescence at 635 nm six hours after ALA application was evaluated. A ratio between the signal obtained in the tumour centre, and the corresponding signal obtained from an area 5 mm outside the visible border of the tumour was formed. This ratio represents a measure on the selective accumulation of PpIX in tumour compared to the surrounding tissue, and was 2.4:1 (SD=1.0) in superficial BCCs and 2.5:1 (SD=1.3) in nodular BCCs (Figure 4).

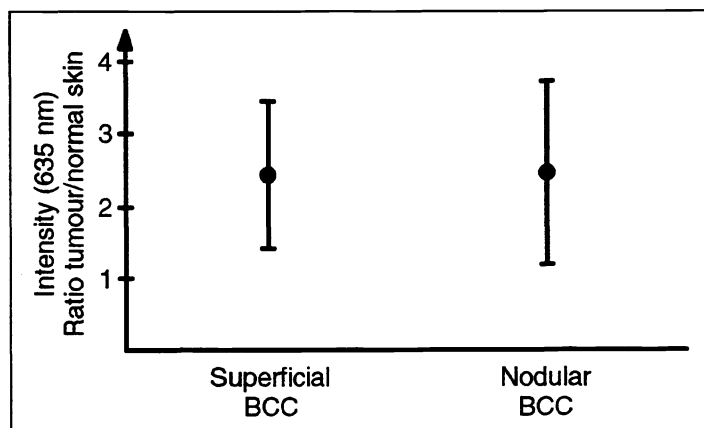


Figure 4. The background-free PpIX-related fluorescence intensity at 635 nm expressed as a ratio between the BCC lesion and normal skin. The fluorescence intensity is related to the concentration of the photosensitiser in the tissue.

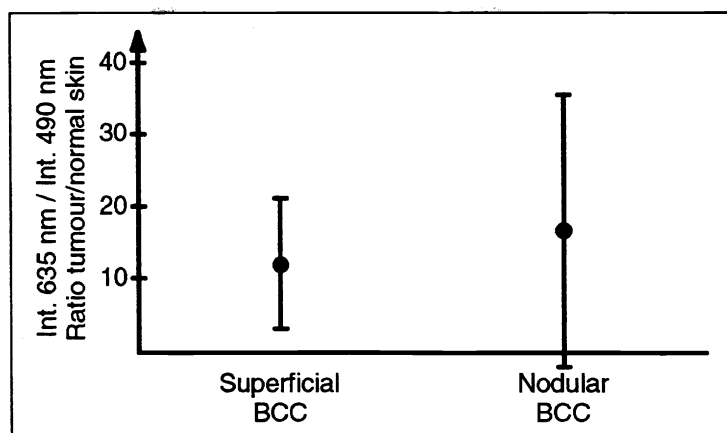


Figure 5. The demarcation between the tumour centre and the surrounding normal skin 5 mm outside the visible border of the tumour, when using the ratio of the background-free fluorescence intensity at 635 nm and the autofluorescence intensity at 490 nm.

If the tumour demarcation ratio is formed by dividing the PpIX-related fluorescence with the autofluorescence, a contrast enhancement of a factor of 5-7 is achieved. As seen in Figure 5, the ratio for superficial BCCs is 12:1 (SD=9) and for nodular BCCs 17:1 (SD=19). The nodular BCCs showed the highest standard deviations in both types of ratios, but the difference was more pronounced in the latter case.

3. 2. Kinetics

The result of the kinetics study performed in seven lesions (five nodular and two superficial BCCs) is shown in Figure 6. Two hours after the application of ALA, most of the PpIX formation has taken place inside the tumour. During the following four hours, the accumulation of the photosensitiser is proportionally greater in the normal skin. This results in a lower contrast between the lesion and the surrounding tissue, as can be seen in Figure 7.

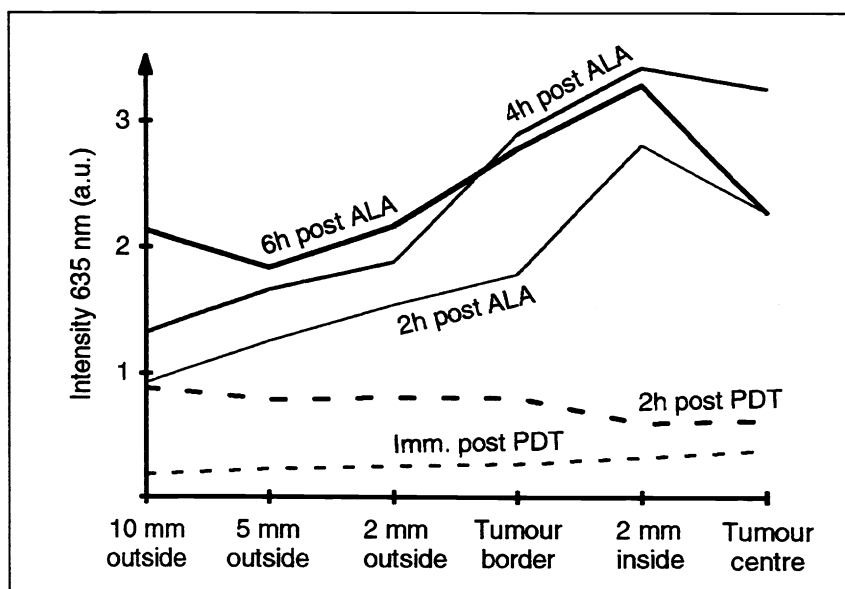


Figure 6. The kinetics of the synthesis of PpIX. The fluorescence intensity at 635 nm is shown for different positions and at different time intervals. In the tumour, the PpIX synthesis is completed within four hours, while it continues in the normal skin. The process continues even after the PDT session. The average values of 7 BCC lesions are shown.

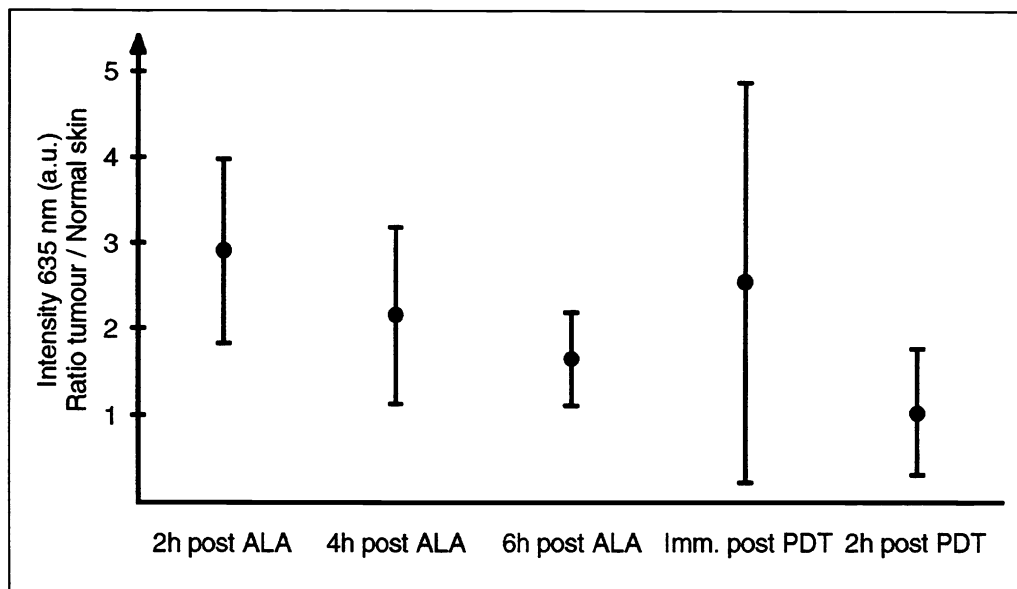


Figure 7. Ratio between the PpIX-related fluorescence intensity (635 nm) in the tumour and the surrounding skin. The higher value immediately after the PDT session is due to a higher bleaching of the active substance in the normal skin than inside the lesion.

The fluorescence was monitored immediately after the completed PDT session showed the result of the laser-induced photobleaching of the photosensitiser. In the normal skin, the PpIX-related peak at 635 nm was almost completely vanished. Inside the lesions, some amount of the photosensitiser usually remained. This was due to the higher concentration in the lesion before the treatment. The measurements performed two hours post PDT, indicated further formation of PpIX after the laser therapy. All measurement points showed an increase of the substance specific fluorescence. The increase was, however, larger in the normal skin than inside the lesion, resulting in a higher fluorescence outside the tumour. Since the synthesis of PpIX in the normal skin was slower, more ALA probably remained there at the time of the treatment, and the PpIX formation could continue. In the lesion, the PpIX concentration was saturated at an early stage, and not much ALA remained after six hours. Thus, a smaller amount of PpIX could be found some time after the treatment.

4. DISCUSSION

As can be seen in Figure 6, the production of PpIX inside the tumour is faster than in the normal skin. The maximum amount of PpIX is accumulated inside the lesion four hours after the ALA application, but already two hours earlier about 80% of that amount was found. During the period from four to six hours following ALA application, the amount of the photosensitiser is almost constant inside the tumour, while there is an accumulation in the normal surrounding skin. The treatment session should thus take place about four hours after the application. Longer application time will not give any beneficial effect in the treatment of the tumour. There might be an increase in the damage of the normal skin, though. This damage will show up as a sunburn and will be repaired by the formation of new epidermis. This repair mechanism does not take place in the tumour, and the necrosis will be selective. For diagnostic measurements, a high contrast would be desirable. Measuring the fluorescence shortly (about two hours) after the ALA administration, would give a better tumour demarcation than measurements performed later, as the synthesis of PpIX is proportionally higher in the normal skin after longer times. Six hours after the application, the contrast has decreased with almost a factor of two, compared to the contrast obtained after two hours (Figure 7).

In about 2/3 of all tumours, there is a decrease in the PpIX-related signal in the centre of the tumour as compared to the point two millimetres inside the visible tumour border. This decrease is probably due to an attenuation of the total fluorescence signal, since the intensity of the autofluorescence is reduced even more. As a result, the tumour demarcation

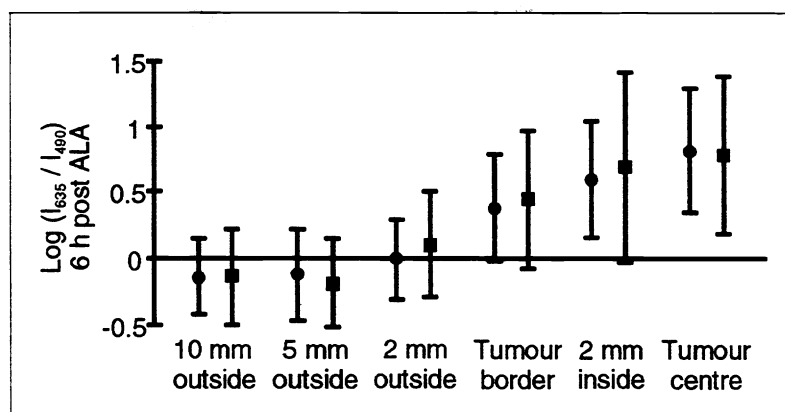


Figure 8. Ratio between the PpIX-related fluorescence and the autofluorescence six hours after topical application of 20% ALA. Superficial BCC are shown as circles, while squares indicate nodular BCC. Note the logarithmic scale on the y-axis.

ratio is higher in the tumour centre than in the border region. Thus, there might still be an increase of the PpIX concentration in the tumour centre. In Figure 8, the average values of the logarithm of the demarcation ratio six hours after the ALA application is shown for different positions in the scan.

The results of the study show considerably lower values of the contrast of the ALA accumulation in tumour and surrounding normal skin than reported earlier.^{9,17} The contrast enhancement factor, using the ratio between the PpIX-related fluorescence and the autofluorescence was about the same in both studies. This implies that the difference lies in the accumulation of the photosensitizer. One reason might be that in this study, the treatment was performed six hours after the ALA application. In the earlier study, results were obtained when the PpIX synthesis had been active between four and six hours. As mentioned above, the tumour demarcation ratio decreases as time passes. The position where the spectra in the surrounding skin were recorded is also of importance. In this study the tumour centre was compared to a point 5 mm outside the visible border of the tumour. However, there was not much change if the point 10 mm outside was used instead.

5. ACKNOWLEDGEMENTS

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