Increase in protoporphyrin IX after 5-aminolevulinic acid based photodynamic therapy is due to local re-synthesis

Henriëtte S. de Bruijn, Bastiaan Kruijt, Angélique van der Ploeg – van den Heuvel, Henricus J. C. M. Sterenborg and Dominic J. Robinson*

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Protoporphyrin IX (PpIX) fluorescence that is bleached during aminolevulinic acid (ALA) mediated photodynamic therapy (PDT) increases again in time after treatment. In the present study we investigated if this increase in PpIX fluorescence after illumination is the result of local re-synthesis or of systemic redistribution of PpIX. We studied the spatial distribution of PpIX after PDT with and without cooling using the skin-fold observation chamber model. We were unable to show a correlation between the local PpIX fluorescence increase and the distance from a blood vessel. The spatial distribution of PpIX fluorescence within normal tissue or tumour is not changed in response to the illumination. These observations suggest that there is no diffusion of PpIX into the treated tissue. Cooling the tissue to 12 °C, a temperature at which PpIX synthesis is inhibited, inhibited the PpIX fluorescence increase normally observed after illumination. We also found a strong correlation between local PpIX photobleaching during illumination and the fluorescence intensity 1 h after illumination similar to what we have observed in patients treated with ALA-PDT. Therefore we conclude that the increase in PpIX fluorescence after illumination is due to local cellular re-synthesis.

Introduction

The clinical response of human skin cancer to photodynamic therapy (PDT) using aminolevulinic acid (ALA) is significantly improved after illumination with two light fractions separated by a dark interval of 2 h.[1,2] The increased efficacy of a two-fold illumination scheme was first shown in studies using pre-clinical animal models.[3–6] This type of light fractionation has been developed to utilise the PpIX fluorescence that returns after illumination which is a feature of (pre-) clinical ALA-PDT.[1,7–12] While the kinetics and localisation of PpIX fluorescence after ALA administration have been investigated extensively[5,9–11,13–18] only a few studies have been performed investigating the PpIX fluorescence kinetics after illumination[5,9–11] and little is known about the spatial distribution or the source of PpIX fluorescence after illumination. In general, the increase in fluorescence after illumination could either be the result of local PpIX re-synthesis or of systemic PpIX redistribution or a combination of both. It is important to determine this since this may have important implications for possible mechanisms of action of the two-fold illumination scheme.[19]

Systemic redistribution requires transport of porphyrins through the circulation, followed by passive diffusion of PpIX from the vasculature into the tissue. In this case fluorescence images recorded in time after illumination should show a gradient of fluorescence around the blood vessels. Henderson et al[17] has shown circulating porphyrins after topical ALA application on mouse skin showing that systemic redistribution of PpIX is theoretically possible. Local re-synthesis, on the other hand, requires local enzyme activity. Several investigators have shown that the enzymatic conversion of ALA to PpIX is inhibited at a temperature below 15 °C.[20,21] In case of local re-synthesis cooling the tissue below this temperature should inhibit the fluorescence increase in time after PDT.

In the present study we determined the source of the return in PpIX fluorescence after illumination in the skin-fold observation chamber model. In the chamber we can distinguish tumour, vessels and normal tissue allowing us to investigate the spatial distribution of PpIX fluorescence after ALA-PDT in a 2-dimensional geometry. We have determined the relationship between the fluorescence kinetics at different distances from the vessel and the vascular response during and immediately after PDT. We have also determined the effect of cooling the tissue to a temperature at which the enzymatic conversion of ALA to PpIX is inhibited[20,21] on the fluorescence kinetics after illumination.

Results

Fluorescence kinetics after ALA administration

The autofluorescence of tumour, vessels and normal tissue was variable but not significant different (3368 ± 1380 counts, $n = 117$). At one hour after ALA administration the fluorescence intensity in tumour tissue was 1.5 times higher compared to normal tissue (9551 ± 4834 counts and 6451 ± 3069 counts respectively with $p = 0.004$). Vessels and normal tissue showed no significant difference in fluorescence intensity over the investigated time frame. The fluorescence kinetics for all tissues reached a plateau at approximately the same intensity 2.5 h after administration (11597 ± 739 for tumour and 10196 ± 1327 counts for normal tissue).

Center for Optical Diagnostics and Therapy, Department of Radiation Oncology, Room Wk-319, Erasmus MC, PO box 2040, 3000, CA Rotterdam, The Netherlands. E-mail: d.robinson@erasmusmc.nl

Fluorescence kinetics after PDT in tumour, vessels and normal tissue

Fig. 1 shows the increase in fluorescence after illumination using different fluences for tumour, normal tissue, arterioles and venules. Illumination with 5 J cm^{-2} resulted in photobleaching in tumour and normal tissue to 69 and 76% respectively of that present before illumination. Similar amounts of photobleaching were observed in arterioles and venules (74% of the initial fluorescence intensity for both). Thirty min after illumination the average fluorescence intensity in each tissue type increased to the initial fluorescence intensity. The fluorescence kinetics thereafter closely followed that of the ALA only control. Illumination with 50 J cm^{-2} resulted in relatively more photobleaching with 39, 51, 47 and 48% of the fluorescence intensity remaining in tumour, normal tissue, arterioles and venules respectively. The increase in fluorescence in time after PDT was less than that following illumination with 5 J cm^{-2}. Illumination with 100 J cm^{-2} showed similar levels of photobleaching and increase in fluorescence compared to that observed with 50 J cm^{-2}. Fig. 2 shows the correlation in individual tissue locations between the extent of photobleaching during the illumination and the increase 1 h after PDT in tumour and normal tissue in this model (Spearman rank correlation, $r_S = 0.56$; CI, 0.316–0.735; $p = 0.0004$). The fluorescence intensity at the start of illumination was inhomogeneous in most tumours. In more than two thirds of tumours, the difference between the highest and lowest intensity region was greater than 15% and showed no correlation with the location in the tumour, *i.e.*, the centre or border. Fig. 3 illustrates the distribution of PpIX fluorescence and shows that the variation within a single tumour is much smaller

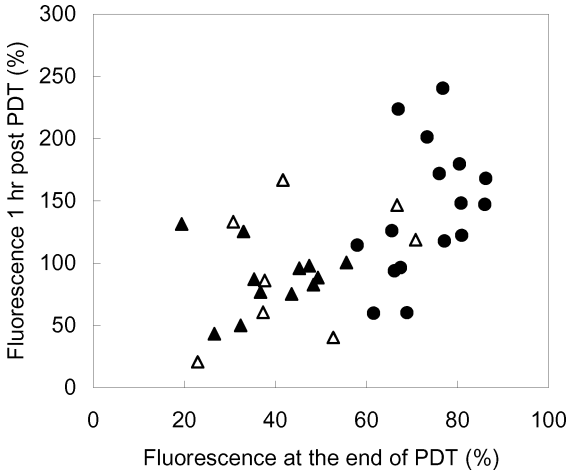

Fig. 2 The relative return in PpIX fluorescence 1 h after ALA-PDT using different fluences (● 5 J cm^{-2}, △ 50 J cm^{-2}, ▲ 100 J cm^{-2}) in relation to the relative fluorescence at the end of PDT of normal and tumour tissue.

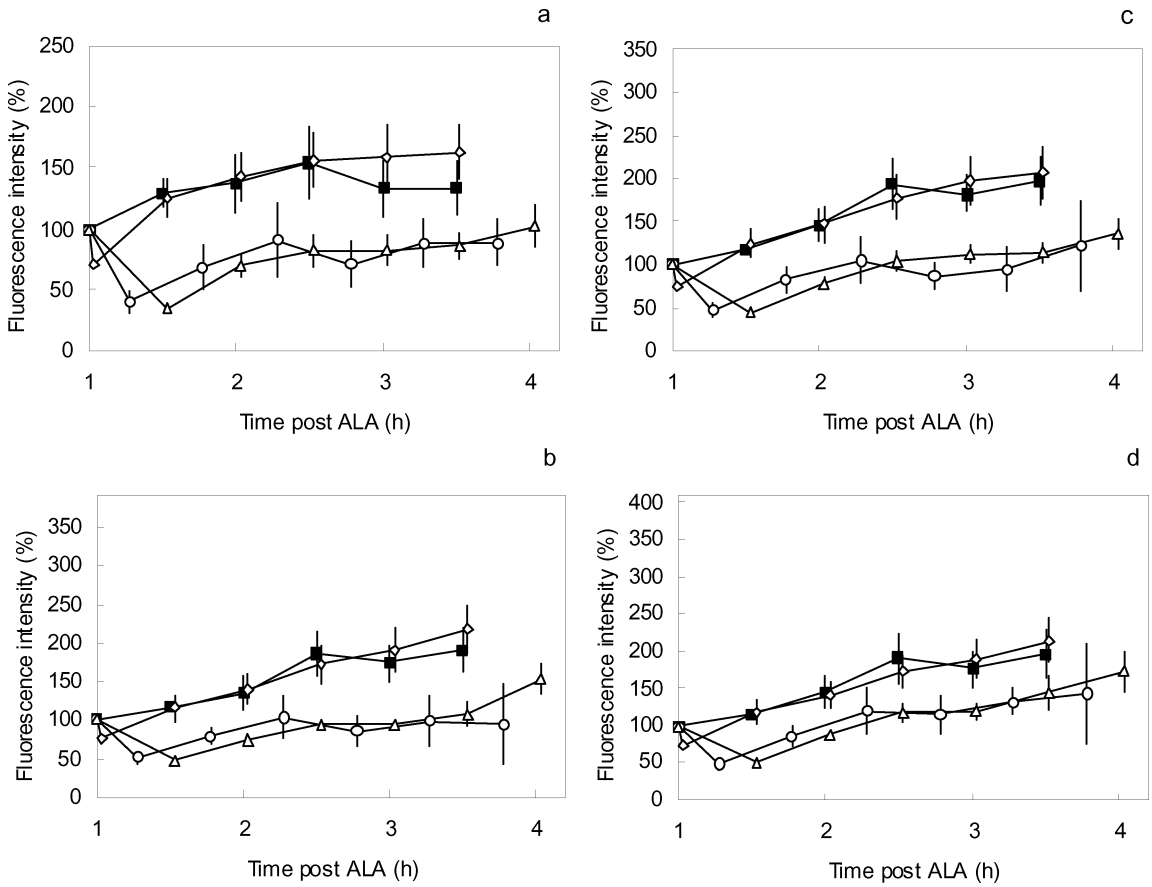

Fig. 1 Normalised PpIX fluorescence kinetics after PDT at 1 h after ALA administration using different light doses (■ no PDT, ◇ 5 J cm^{-2}, ○ 50 J cm^{-2}, △ 100 J cm^{-2}) in tumour (a), normal (b), arteriole (c) and venule (d) tissue. Data was normalised to the pre-illumination fluorescence intensity for each individual rat and each tissue type. Differences between tissue types in the pre-illumination fluorescence intensity are displayed in the relative re-scaling of the Y-axis of the kinetics graphs. Results are shown as mean ± sem.

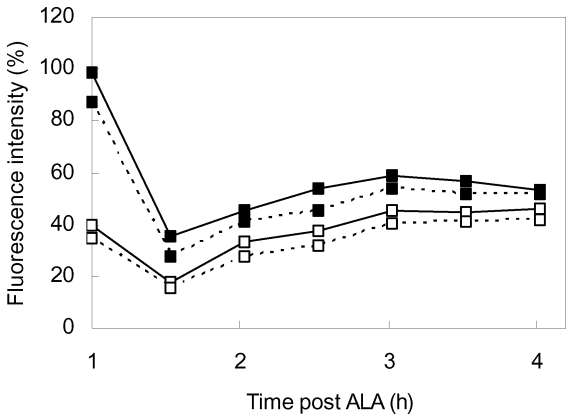

Fig. 3 The relative PpIX fluorescence kinetics in time after ALA-PDT using 100 J cm^{-2} in two representative tumours (■ and □). Data are normalised to the region with the highest fluorescence intensity measured before illumination (■), *i.e.* 1 h after ALA administration, to show the intra-animal variation. For each tumour data from the highest (solid line) and lowest (dashed line) fluorescence intensity region are displayed to show the inter-tumour variations in fluorescence kinetics.

than the variation between tumours. Overall these variations in fluorescence intensity observed at the start of illumination were not significantly different in time after PDT with any of the light doses investigated in the present study.

Fluorescence kinetics after PDT in relation to the distance from the vessels

The fluorescence increase after ALA-PDT was independent of the distance from the nearest vessel or the fluence used for illumination. Fig. 4 shows the increase in fluorescence in each region after illumination with 100 J cm^{-2}. The average diameter for arterioles was 51 ± 25 μm (n = 20). The two regions in which the fluorescence increase in relation to the distance from the arteriole was determined were at a distance of 84 ± 38 μm and 217 ± 70 μm. The average diameter for venules was 126 ± 54 μm (n = 23). The two regions in which the fluorescence increase in relation to the distance from the venule was determined were at a distance of 127 ± 25 μm and 368 ± 115 μm.

Vascular response immediately after ALA-PDT

Fig. 5 shows the vascular response of arterioles and venules in the first hours after PDT using different light fluences and Table 2 shows the number of animals that showed stasis of capillary flow in tumour and normal tissue. Immediately after illumination with 5 J cm^{-2} none of the blood vessels in the chambers showed vasoconstriction. Only 1 out of 8 animals showed mild vasoconstriction of both the arteriole and the venule 60 min after PDT. The venule returned to the normal diameter within 30 min, the arteriole was still mildly constricted at 90 min after PDT.

Illumination with 50 J cm^{-2} resulted in significantly more animals showing arteriole constriction compared to 5 J cm^{-2} (p = 0.01, 0.009 and 0.009 immediately, 60 and 90 min after illumination respectively). At the end of PDT 3 out of 4 animals showed arteriole constriction ranging from mild to complete developing in severe constriction at 60 and 90 min. Only two animals showed venule constriction, one mild and one severe in the hours after

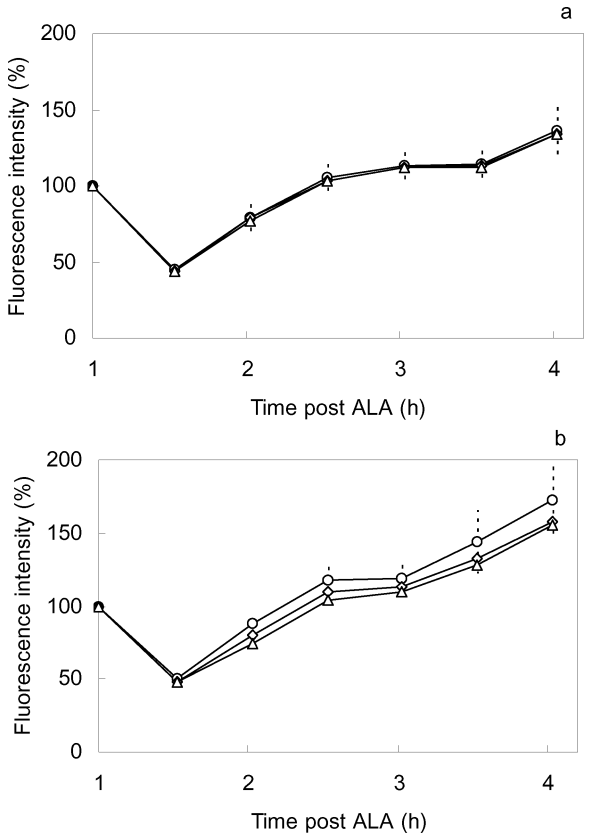

Fig. 4 The PpIX fluorescence kinetics in time after PDT at 1 h after ALA administration using 100 J cm^{-2} in relation to the distance from an arteriole (a) or a venule (b); (○) in the vessel, (◇) close to the vessel and (△) further away form the vessel. Results are shown as mean ± sem.

Table 1 The number of animals used for each tissue type

Fluence/J cm^{-2}	Normal	Tumour	Arterioles	Venules
0	3	3	3	3
5	8	6	7	8
50	4	4	4	4
100	7	6	6	7
100 cooling	10	7	6	10

Table 2 Capillary stasis in response to ALA-PDT. The number of animals showing stasis of the blood flow in tumour and normal capillaries immediately and 2 h after PDT. In normal tissue the size of the region showing stasis was determined whereas tumours were scored when all capillaries showed stasis

	Normal tissue		Tumour tissue	
Fluence/J cm^{-2}	0	2 h	0	2 h
50	0/3[a]	2 (25%)/3[a]	0/3[a]	0/3[a]
100	3 (60%)/6[a]	5 (79%)/7	2/5[a]	4/6

[a] The response of one animal could not be determined.

illumination. Immediately after PDT all animals showed normal flow in the capillaries of normal and tumour tissue. Two hours after PDT 2 out of 3 animals showed stasis in a small region in the normal tissue whereas the blood flow in the tumour was not hampered.

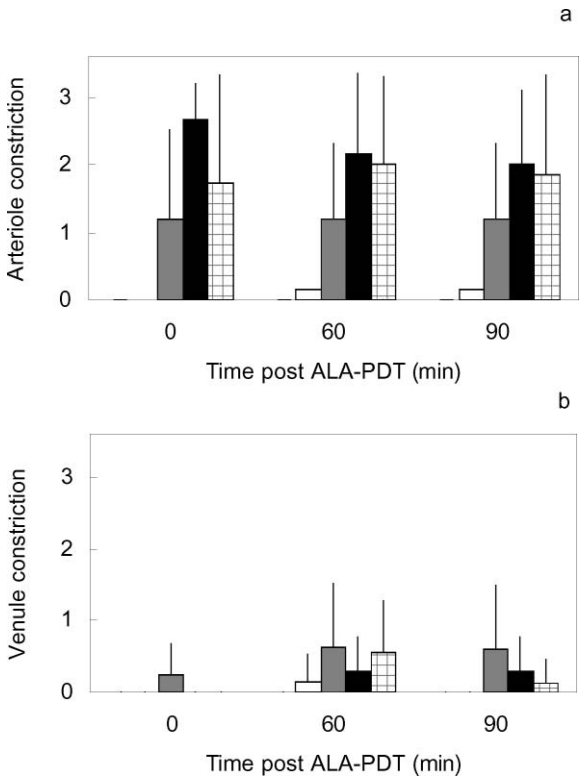

Fig. 5 Arteriole (a) and venule (b) constriction at 0, 60 and 90 min after ALA-PDT using different light doses; 5 J cm^{-2} (white bar), 50 J cm^{-2} (grey bar), 100 J cm^{-2} (black bar) and 100 J cm^{-2} followed by 1 h of cooling (square-patterned bar). Results are shown as mean ± sem.

Illumination with 100 J cm^{-2} resulted in arteriole constriction in all animals at the end of PDT; 2 animals showed severe and 4 showed complete constriction. At 60 and 90 min after PDT 3 out of 6 showed a small recovery resulting in 1 normal, 3 severely constricted and 2 completely constricted arterioles. Compared to 50 J cm^{-2} the damage was more severe although not statistically significant ($p = 0.08$, 0.38 and 0.49; immediately, 60 and 90 min after illumination). No change in diameter of the venules was observed at the end of PDT. At 60 to 90 min after PDT 2 out of 7 showed mild constriction. The constriction of venules after 100 J cm^{-2} was not statistically different from that observed after 5 and 50 J cm^{-2}. Immediately after illumination the 3 out of 6 animal showed large areas of blood stasis in the capillaries of normal tissue. The number of animals showing stasis and the region size increased in time after PDT. For tumours the result was comparable; 2 out of 5 tumours showed complete stasis in all capillaries immediately after PDT increasing to 4 out of 6 tumours 2 h after PDT.

Influence of temperature

The vascular response observed in the first hours after ALA-PDT was not influenced by the drop in local tissue temperature to 10–12 °C for 1 h after the end of illumination (Fig. 5). Microscopically, we did not observe blood stasis before or after cooling in the arterioles and venules in any of the animals. Fig. 6 shows that the fluorescence increase in time after illumination using 100 J cm^{-2} was temperature dependent. During illumination the fluorescence bleached to 35, 43, 49 and 49% of the initial fluorescence intensity in tumour, arterioles, venules and normal tissue respectively. After 1 h of cooling to 10–12 °C there was no significant increase

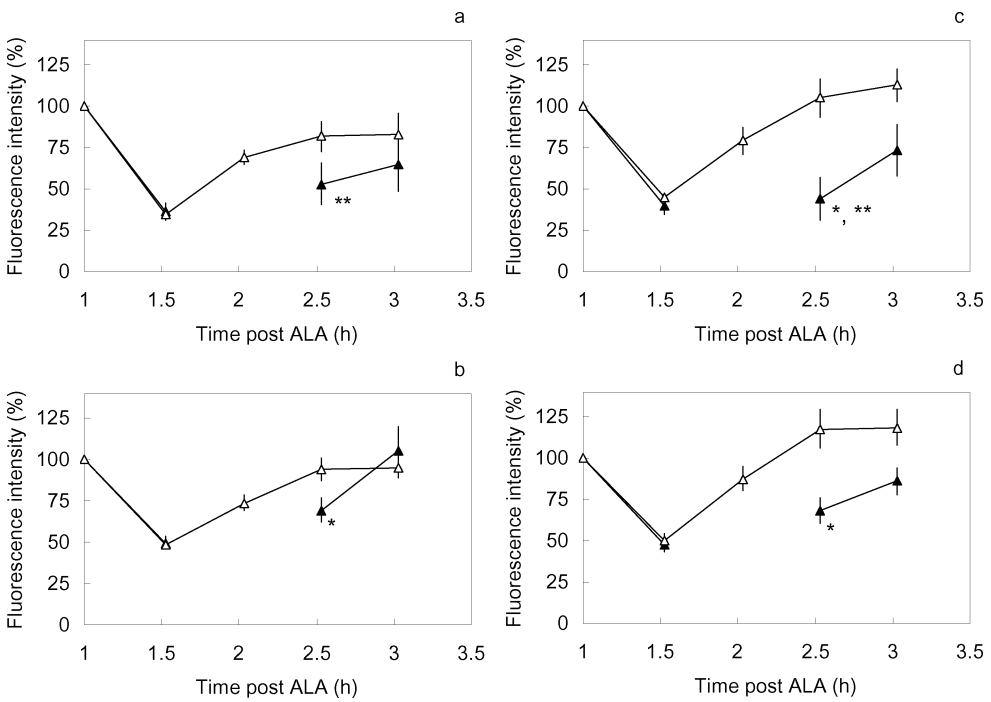

Fig. 6 Normalised PpIX fluorescence kinetics after PDT at 1 h after ALA administration using 100 J cm^{-2} either followed with 1 h of cooling the tissue to 10–12 ° C (▲) or kept at normal temperature (△) in tumour (a), normal (b), arteriole (c) and venule (d) tissue. Results are shown as mean ± sem; * significant less PpIX increase at this time point compared to normal temperature groups; ** no significant PpIX increase at this time point compared to immediately after PDT.

in fluorescence in arterioles and tumour ($p = 0.68$ and 0.28, respectively). There was a small increase in fluorescence both in venules and in normal tissue ($p = 0.04$ and 0.03 respectively) but this increase was lower compared to the increase in the normal tissue and venules of animals kept at normal temperature ($p = 0.002$ and 0.03, respectively). Subsequent warming the chambers to normal conditions after the cooling period resulted in an increase in PpIX fluorescence in all of the tissues investigated.

Discussion

The aim of the present study was to determine if the increase in PpIX fluorescence after ALA-PDT is due to local re-synthesis or systemic redistribution. We investigated this by studying the spatial distribution of PpIX after PDT with and without cooling in the skin-fold observation chamber model. By cooling the tissue to a temperature at which no PpIX is formed (10–12 °C)[20,21] we were able to inhibit the return in PpIX fluorescence after illumination (Fig. 6). This inhibition was reversible as we found that PpIX fluorescence increases after the tissue is returned to normal temperature. These observations are consistent with those of Juzenas *et al.* in the skin after topical ALA application.[20] It is important to consider that changing the temperature of tissue has been shown to affect the vessel diameter. Unthank[22] showed that cooling normal rat skin from 35 to 25 °C resulted in significant constriction of arterioles in the subcutus. ALA-PDT is also known to induce vasoconstriction and stasis.[17,23,24] The combined effects of cooling and PDT may influence our ability to detect the systemic redistribution of PpIX. We have shown that the extent of vasoconstriction following PDT is dependent on the light fluence (Fig. 5). Although 60% of the animals treated with 100 J cm^{-2} show complete arteriole constriction immediately after illumination the circulation in the chamber is not completely shut down; the venules and most capillaries are still flowing (Table 2). Cooling the tissue for 1 h after ALA-PDT with 100 J cm^{-2} did not result in significantly more vasoconstriction of both arterioles and venules. Based on these results we assume that the blood supply within the chamber after PDT is similar with or without cooling. Our results on the spatial distribution of the fluorescence kinetics in and around vessels (Fig. 4) shows there is no correlation between the distance from a blood vessel and the rate of PpIX fluorescence increase after PDT. Whether the vessel showed vasoconstriction had no influence on this result. Several investigators[12,18] have hypothesised that the reappearance of PpIX after ALA-PDT might be the result of diffusion from the surrounding tissue. Our results on the spatial distribution of fluorescence around the vessels of the present study do not support this hypothesis. Furthermore we show that the level of fluorescence in a blood vessel was as high as the surrounding tissue suggesting that the amount of circulating porphyrins, if any, is small. In Fig. 2 we demonstrate a strong correlation between the local level of photobleaching and the fluorescence increase 1 h after PDT. More photobleaching during illumination suggests more damage to the tissue resulting in a reduced capacity to convert ALA into PpIX. This result is consistent with that we have observed in our clinical study treating superficial BCC using topical ALA-PDT.[1] Also other investigators[7,12] have shown PpIX fluorescence returns after ALA-PDT in both superficial and nodular BCC. A systemic redistribution in the clinical situation would be unlikely due to the much smaller ALA dose to body mass ratio. Our present data obtained in the window chamber model using systemic ALA administration supports the conclusion that the return in PpIX fluorescence after PDT is the result of local re-synthesis. The fact that we have shown this under these circumstances implies that this is also true after topical ALA in both pre-clinical and clinical models.

The rate and magnitude of PpIX re-synthesis after illumination in the window chamber is relatively high compared to our previous studies using other pre-clinical models.[5,10,11] In normal mouse[5] or in pig skin[11] the re-synthesis kinetics were dependent on the fluence delivered but did not increase to the pre-PDT level even after illumination with a small fluence. In transplanted rhabdomyosarcoma we have also shown that re-synthesis is dependent on the fluence delivered.[10] In this model illumination with a small fluence resulted in a fluorescence intensity higher than the pre-PDT level although not to the dark control. Apparently the tissue under investigation, the main difference between these and the present study, has an influence on the fluorescence kinetics observed after PDT. In mouse and pig skin, epidermal cells dominate the fluorescence data. In the transplanted rhabdomyosarcoma model other tissues are also involved since the model also contains tumour cells, connective tissue and vessels. The subcutis of the chamber is highly vascularised and contains fat cells and transplanted tumour cells. Consistent to the observations of Roberts *et al*.[25] our results in Fig. 1 and 4 show that the vascular endothelium synthesises PpIX and contributes significantly to the fluorescence intensity. Interestingly, 5 out of 48 lesions treated in our clinical study also show a fluorescence increase to the pre-PDT value or higher 2 h after illumination.

Although the increase of PpIX after illumination was the motivation for designing a two-fold illumination scheme we have shown earlier that the mechanism of action behind the two-fold illumination in ALA-PDT is more complicated than just the utilisation of the returned PpIX during dark interval.[3–4,6,10] Recently[6] we have hypothesised that the spatial distribution of PpIX and the site of PDT response within the illuminated volume is an important factor in the mechanism underlying the two-fold illumination scheme. One could even imagine that the first light fraction influences the spatial distribution of PpIX fluorescence. In the present study we show that the PpIX fluorescence intensity at the start of PDT was very variable between animals and within each chamber as shown by a representative example (Fig. 3). Although the preparation of the chambers was standardised it was impossible to create chambers with standard tissue thickness or vascular structure density. The heterogeneity in the tumour fluorescence intensity as shown in Fig. 3 might be explained by the heterogeneity in their oxygen supply and metabolic activity. More important is the observation that the spatial distribution of PpIX (re)-synthesis in the tumour is not influenced by the illumination. A tumour area that showed little fluorescence compared to the rest of the tumour also showed a relatively lower rate of re-synthesis after illumination independent of the fluence used. Also the observed differences between tumour, vascular and normal tissue in fluorescence intensities at the start of PDT remained the same after illumination. Apparently, the relative capacity to convert ALA to PpIX is equally affected in tumour, vascular or normal tissue. It is important to bear in mind that the tissue in this model is highly vascularized which means that the

fluorescence kinetics determined in tumour and normal tissue also contain information from the vascular endothelial cells of the capillaries.

Conclusions

In summary; we have shown that cooling the tissue to 10–12 °C inhibited the PpIX fluorescence increase after illumination. We were also unable to show a gradient of fluorescence around the vessels. Therefore we conclude that the increase in PpIX fluorescence after illumination is the result of local cellular re-synthesis in rats after systemic ALA administration. Furthermore we have shown that the spatial distribution of fluorescence within normal tissue and tumour is not changed after PDT.

Experimental

Animal model

Skin-fold observation chambers were prepared using a slightly modified technique to the one that has been described previously.[24,26] Briefly, the chamber was prepared on the back of female fisher-344 rats in four operations spread over a period of two weeks. As a result of these operations a thin layer of subcutus tissue was clamped between mica and a cover slide without obstruction of the circulation (Fig. 7(a)). All operations were carried out under general Ethrane/O_2/N_2O anaesthesia. In the first operation sterile air (12 mL) was subcutaneously injected on the back of the rat to gently separate the skin from the underlying tissues. In the second operation, a few days later, a home made plastic ring and cover slide was positioned under the skin above the subcutaneous tissue containing vessels with the air below this layer of tissue. In the third operation, one week later, the skin was folded, prepared and fixed in the splint so that it can be placed under the microscope for observation. In the final operation the R3230AC tumour was transplanted in the layer of normal tissue, which could be reached by unscrewing the cover slide on top of the chamber. Within four to eight days the chamber was ready for treatment when the transplanted tumour was supported by blood vessels and had grown. Ideally the chamber contained fat cells and capillaries over an area of approximately 1 cm in diameter with some supporting arterioles and venules within. In a small number of animals one or more types of tissue was not available. Table 1 shows the actual number of animals used per tissue type for each experimental group. The animal experiments committee of the Erasmus University Medical Centre approved the experimental protocol.

Experimental design

The animals were divided over five experimental groups. The PpIX fluorescence kinetics after ALA administration was determined in the first group. In three groups the increase in PpIX fluorescence in time after ALA-PDT was investigated. PDT was performed 1 h after systemic ALA administration, the time point of the first illumination of a two-fold illumination scheme, using 514 nm light at a fluence rate of 50 mW cm^{-2} to a fluence of 5, 50 or 100 J cm^{-2}. Fluorescence and transmission images were recorded every 30 min until 2.5 h after the end of illumination. In the final group the effect of cooling on the PpIX fluorescence increase after ALA-PDT was investigated. PDT was performed 1 h after ALA administration using 514 nm light at a fluence rate of 50 mW cm^{-2} to a fluence of 100 J cm^{-2}. Immediately after completion of PDT the tissue was cooled to 10–12 °C for 1 h. Thereafter the temperature of tissue was allowed to return to normal (28–30 °C). Fluorescence and transmission images were recorded before and after PDT after cooling and 30 min after the temperature had returned to normal.

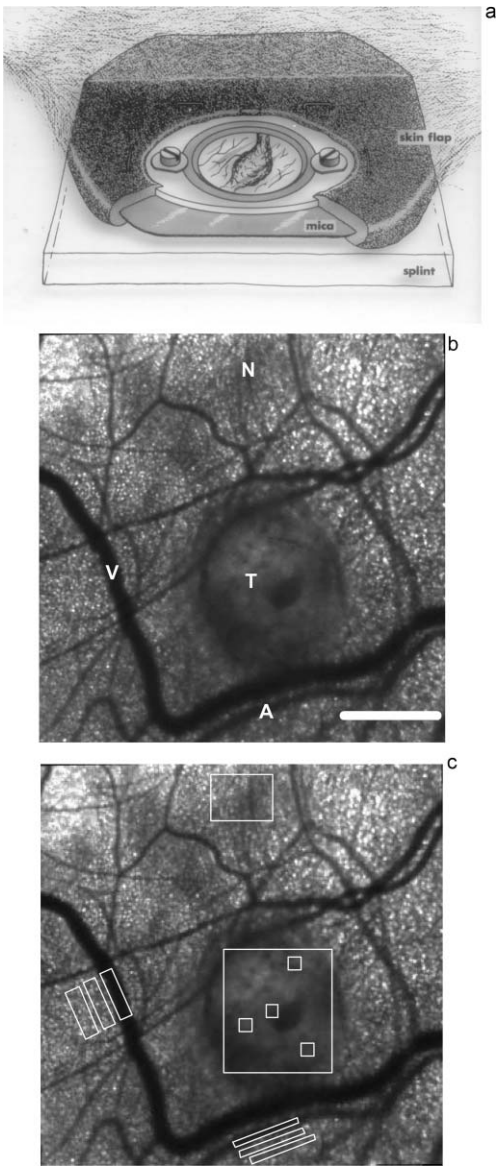

Fig. 7 (a) A schematic drawing of the chamber on the back of a rat. (b) Typical transmission image of the skin-fold observation chamber, diameter 1 cm. Ideally the chamber consists of different types of tissues and vessels; tumour cells and capillaries (T), normal cells and capillaries (N), arterioles (A), venules (V). The white bar represents 1 mm. (c) A typical example of the different regions of interest in which the fluorescence was determined. The different regions were: normal tissue, vascular tissue accompanied by the two perpendicular regions in which the correlation to the distance from the vessels was determined and tumour tissue including the four smaller areas in which the heterogeneity within the tumour was determined.

ALA preparation and administration

5-Aminolevulinic acid hydrochloride (ALA, Medac, Wedel, Germany) was dissolved in sterile 0.9% NaCl infusion solution to a concentration of 90 mg mL^{-1}. A freshly prepared ALA solution was administered i.v. to a dose of 200 mg kg^{-1} body weight under ethrane/O_2/N_2O anaesthesia. NaOH 4 M was added to the solution to obtain pH 5–6. After PDT animals were kept under subdued light conditions for the first 24 h.

Experimental set-up for PDT illumination, fluorescence and transmission imaging

PDT illumination and fluorescence/transmission imaging was accomplished using the same experimental set-up. The animal was placed on a temperature-controlled X-Y stage. For PDT illumination green, 514 nm, excitation laser light (Spectra Physics, Darmstadt, Germany) with a fluence rate of 50 mW cm^{-2} was projected on the backside of the whole chamber using a system of condensing lenses to produce a uniform fluence rate distribution.

For fluorescence and transmission imaging light transmitted through the chamber was imaged onto a Peltier-cooled 16 bit, 512 × 512, slow scan CCD camera (Princeton Instruments Inc., Princeton, USA) using a *f* 2.8/105 mm macro lens. The different detection filters were placed in a filter wheel (Oriel, Stratford, USA) between the macro lens and the CCD camera in order to obtain the fluorescence (625 ± 20 nm) and transmission (514 ± 2 nm) images. Images recorded at the start and end of illumination were collected within the treatment session using the therapeutic light for excitation. Images recorded before ALA administration and in time after illumination were recorded using the same light source but at a lower fluence rate to prevent additional PDT induced tissue damage. Before each measurement a fluorescence standard (1309, 0.25 mm, VINK, Zeist, The Netherlands) was recorded to correct for these differences in excitation light intensity. The extra delivered fluence due to these measurements was approximately 0.15 per measurement times five is 0.75 per animal. The PDT treatment and fluorescence measurements were carried out under general ethrane/O_2/N_2O anaesthesia. Between measurements animals were conscious and placed in a dark and warm environment.

Chamber cooling

In one group of animals the chamber tissue was cooled immediately after illumination with 100 J cm^{-2} (1 h after the administration of ALA) for 1 h. A copper rod was placed in iced water in direct contact with the mica on the base of the window chamber and a small reservoir of iced water on top of the chamber. The temperature of the cover slide on top of the chamber was monitored continuously during the cooling period using a thermocouple. In all cases the temperature of the cover slide on top of the chamber was <12 °C within 8 min after the end of illumination and maintained between 10–12 °C for 1 h. Warming was initiated by removing the iced water from the top and the copper rod from the bottom of the chamber. Within 2 min the temperature of the cover slide on top of the chamber had returned to normal (28–30 °C). The body of the animal was placed on a temperature-controlled stage to maintain normal body temperature. The general anaesthesia that was used during PDT was maintained during the cooling period.

PpIX fluorescence kinetics of different tissue types

Fluorescence and transmission images were recorded at several time points; before ALA administration (autofluorescence), before PDT illumination and in time after PDT illumination. Fluorescence images were corrected for intensity differences using a reference standard. The sequence of fluorescence images from each animal was registered by translation and rotation using anatomical landmarks identified in the corresponding transmission images. The registration of images enabled us to determine the fluorescence intensity of each tissue type from the same area. In the corresponding transmission image the regions of interest were chosen for each tissue type as shown in Fig. 7. Tumour and normal tissue regions of interest were chosen so that no large chamber vessels were in or close to the region. The heterogeneity of the fluorescence in tumour was determined in four smaller regions in the tumour area as observed using white light microscopy. The position of these four areas was determined in the fluorescence image collected at the start of illumination with the aim to investigate the highest and lowest fluorescing area in the centre and at the border. The relationship between the increase in PpIX fluorescence and distance from an arteriole and a venule was investigated by determining the return in fluorescence within three regions of interest associated with each vessel. A rectangular region of which the width of the short side was equal to the width of an arteriole was placed within an arteriole. A second and third rectangle was placed adjacent to the arteriole at increasing distances from it. These regions were carefully chosen so that no other vessel was close to the arteriole under investigation. A similar procedure was followed for venules.

Vascular response

We distinguish two vascular responses; the change in diameter of arterioles and venules and the disruption in flow. The change in vascular diameter due to the treatment was scored at the end of PDT, 60 and 90 min after PDT using the collected transmission images. While the original vessel size was variable between animals we scored the change in vascular diameter in percentages of constriction. No change in vessel diameter was scored 0, mild vasoconstriction (less then 50%) was scored 1, severe vasoconstriction (more than 50%) was scored 2 and complete vasoconstriction was scored 3. The status of the blood flow in tumour and normal capillaries was determined at the end of PDT and 2 h after PDT using 50 and 100 J cm^{-2} using white light microscopy. While it is our experience that capillary flow in the chamber model is not fluently we used a rough discrimination and scored flow (0) or no flow (1). In normal tissue we determined the size of the region whereas tumours were scored when all capillaries showed stasis.

Statistics

Student *t* test was used to determine significance for the fluorescence kinetics measurements and vascular damage scores. The Spearman-rank test was used to determine the significance of the relationship between the photobleaching and re-synthesis

of PpIX. Results with a p value below 0.05 were considered significant. Data are presented as mean ± sem unless reported differently.

Abbreviations

PDT, photodynamic therapy; ALA, 5-aminolevulinic acid; PpIX, protoporphyrin IX; BCC, basal cell carcinoma.

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