Antitumor Effect of 5-Aminolevulinic Acid-mediated Photodynamic Therapy Can Be Enhanced by the Use of a Low Dose of Photofrin in Human Tumor Xenografts

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ABSTRACT

Practically all of the exogenous photosensitizers used for clinical photodynamic therapy (PDT) target mainly vasculature. Although effective in tumor destruction, they also, unavoidably, induce phototoxicity of normal tissues. Porphyrins synthesized endogenously from 5-aminolevulinic acid (ALA) accumulate within cells. Tumor eradication would be more efficient if both cellular components and vascular stroma of a tumor could be targeted. Thus, PDT with a mixture of ALA and Photofrin (Pf, a vessel-targeted sensitizer) may simultaneously destroy the two elements. Using chemical extraction assays, pharmacokinetics of ALA and ALA-induced porphyrins were studied in the plasma and tumors of nude mice bearing human WiDr and KM20L2 colonic carcinomas after an i.p. injection of 250 mg/kg body weight of ALA. Subsequently, PDT efficacy of the two tumor models with ALA, Pf, or with the two drugs in combination was evaluated. The phototoxic effects on tumor cells in vitro with the combined drugs was also determined. Moreover, histological and ultrastructural alterations of the treated tumors were investigated, and tumor cell clonogenicity was assessed as a function of time after in vivo PDT using an in vitro colony formation assay. Finally, the photosensitivity of normal skin tissue treated according to various protocols was compared. The amounts of ALA peaked at 0.5 h after administration in both plasma and WiDr tumor. The rates of ALA clearance seemed to follow a one-compartment model with half-lives of ~18 and 88 min in the plasma and tumor, respectively. About 100 and 60 times higher concentrations of ALA were needed to induce a given concentration of porphyrins in the plasma and tumor, respectively, although the plasma porphyrins may not only be released from blood cells but also from other organs. Similar kinetics of distribution patterns of ALA- and ALA methylster-induced porphyrins were found in the plasma and tumors, and the elimination rates were consistent with a two-compartment model. ALA induced much more porphyrins than ALA methylster in both plasma and tumors. Tumors PDT-treated with ALA plus Pf at a low dose (1 mg/kg) grew significantly more slowly than those treated with either of the drugs in both WiDr and KM20L2 models. However, the enhanced antitumor effect was not found in the tumor cells under in vitro conditions. Morphological studies demonstrated that PDT with the combined regimen resulted in necrosis of neoplastic cells and severe disruption of tumor microvasculature. This was supported by the findings obtained from the studies of in vivo PDT and in vitro clonogenic assay that showed a progressive reduction in tumor cell viability with times following PDT. Such a combined PDT protocol did not induce any phototoxicity in normal skin tissue. These data indicate that targeting both neoplastic cells and stroma with ALA and Pf (a low dose) can potentiate antitumor PDT effect with no risk of prolonged skin photosensitivity.

INTRODUCTION

PDT2 has developed to become an important new cancer treatment (1). The treatment typically involves systemic administration of a tumor-localizing photosensitizer and its subsequent activation by light of an appropriate wavelength to create a photochemical reaction causing photodamage to the tumor (1). Pf was studied worldwide in clinical PDT during the past two decades and has been approved by governments of many countries for several medical indications. However, the major side effects associated with Pf-based PDT are the risk of skin photosensitization (2) and damage to surrounding normal tissue potentially leading to stenosis of the esophagus (3) and shrinkage of the bladder (4). To a considerable extent these side effects restrict the use of clinical Pf PDT. PDT with ALA-derived endogenous porphyrins has already shown promising clinical results in the treatment of several superficial disorders of the skin and internal hollow organs (5). The principle of this new approach is that ALA is a precursor of several porphyrin intermediates, particularly PpIX, a potent photosensitizer, in the heme biosynthetic pathway. The synthesis of heme is largely controlled by a heme-mediated feedback inhibition of the enzyme ALA synthase (6). Such an ALA/heme feedback control can, however, be bypassed by administering excess amounts of exogenous ALA. As a result, endogenous porphyrins accumulate in cells because of the limited capacity of enzymes (e.g., ferrochelatase) to convert porphyrins into heme (6). When light activated, the accumulated porphyrins produce a photodynamic reaction (6). One of the main advantages of using ALA PDT is that ALA-derived porphyrins are cleared from the body within 24–48 h after systemic ALA administration. This would reduce or avoid the risk of prolonged skin phototoxicity.

Our earlier studies have shown that porphyrins synthesized endogenously from ALA localize within neoplastic cells of CaD2 tumors. Subsequent light activation leads to inactivation of the tumor cells (7). Pf is known to distribute mainly in the vascular stroma of the tumor (8, 9), thereby destroying the vascular system of the tumor during light exposure (10, 11). Our data suggest that efficient eradication of a tumor by PDT requires destruction of both cellular components and vascular stroma of the tumor (12, 13). Therefore, we have hypothesized that the antitumor effect of ALA PDT may be enhanced by the use of vessel-localizing sensitizers like Pf, particularly at a low dose of the sensitizer that does not induce any risk of prolonged photodamage of normal tissues. In the present study, we have combined ALA with Pf in the photodynamic treatment of human colon carcinoma WiDr and KM20L2 xenografts in nude mice and demonstrate that the combined PDT regimens did not only enhance significantly PDT efficiency of the tumors but also avoided skin phototoxicity of the mice.

MATERIALS AND METHODS

Chemicals. ALA and its methylster were supplied by Photocure ASA (Oslo, Norway). The drugs were dissolved in Dulbecco’s PBS on the same day as the experiments started, and the pH was adjusted to 7.0 by addition of 5 M NaOH. Pf (porfimer sodium) was a kind gift from QLT PhotoTherapeutics Inc. (Vancouver, British Columbia, Canada). The stock solution of Pf was made up in isotonic solution containing 5% dextrose and stored at −70°C. Because the exact molecular weight of Pf is not known, the molarity units of 18 U.S.C. Section 1734 solely to indicate this fact.
Pf, ALA, and ALA methylester were not applied in the present study. All of the other chemicals used were of the highest purity commercially available.

**Animals and Tumor Line.** Approval for protocols of this study was obtained from The Norwegian National Animal Research Authority, and all experiments of animals were conducted according to The National Ethical Committee’s Guidelines on Animal Welfare. Female BALB/c athymic nude mice were bred at the nude rodent facility of The Norwegian Radium Hospital, housed five/cage, and kept under specific pathogen-free conditions. The mice were 6 weeks old and weighed 20–25 g when the experiments started.

Two established human colon adenocarcinoma lines, WiDr (14) and KM20L2 (15), were used in the present study. WiDr cells (≈5 × 10⁶) suspended in 0.04 ml of PBS were s.c. implanted in the flank of the mice. Nonneoptotic tumor material obtained by sterile dissection of large flank tumors was gently minced with a Kai sterile surgical blade (Kai Surgical Instrument Co., Ltd, Tokyo, Japan) to make a tumor-tissue suspension, 0.02 ml of which was then injected into the dorsal side of the right hind foot of each syngeneic mouse. At this site the tumor was easily accessible to treatment and to assessment of response. The tumor volume was calculated using the following formula:

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V = \frac{\pi}{6} (D_1 \times D_2 \times D_3)
\]

where \(D_1, D_2, \text{and } D_3\) are three orthogonal diameters of the tumors that were measured every 2nd day using a caliper. No spontaneous necrosis was observed in the tumors that grew to reach 130–220 mm³ on the day of treatment. The same procedure was also used for xenografts of the KM20L2 tumor.

**Determination of ALA in Plasma and Tumor.** After WiDr-bearing mice had been anesthetized by i.p. injection of 50 μl/25 g of a mixture (1:1 by volume) of Dormicium (Roche, Basel, Switzerland) and Hypnorm (Janssen, Beerse, Belgium), the blood was drawn directly from the left ventricle of the heart, and the animals were immediately killed by cervical dislocation. The total volume of the blood obtained from each mouse was ≈0.8 ml and collected in EDTA-treated tubes. The plasma was separated from blood cells by centrifugation at 5700 × g for 10 min. In the case of the tumor, the tissue was brought into suspension in saline (0.25 g/ml) by a Potter Elvehjem homogenizer, and the tissue suspension was sonicated twice (1 min for each) before being centrifuged as described previously. The resulting supernatant was collected. ALA concentrations in the plasma and supernatants were measured by a method of Tomokuni et al. (16) with slight modifications. This assay is based on derivatization of ALA with acetylacetone followed by HPLC analysis on the fluorescent derivative. The HPLC system was composed of a pump (Spectra Physics 8800, Hempstead, United Kingdom), a reverse-phase column [Hypersil H5ODS (4.6 × 250 mm); Supelco, Gland, Switzerland], a fluorescence detector (Spectra Physics FL2000) with excitation/emission wavelengths of 370/460 nm, and an integrator (Spectra Physics Data-jet) coupled to a computer. The mobile phase was methanol/water/acetic acid (430/570/10 by volume), flow rate was 0.7 ml/min, and column temperature was room temperature. The concentrations of ALA in the samples were calculated from standard curves made by addition of known amounts of ALA to respective extracts from untreated mice and expressed as μg ALA/ml plasma or g wet tumor tissue.

**Determination of ALA- or ALA Methylester-derived Porphyrins in Plasma and Tumor.** Samples of plasma and tumor tissue samples were taken from WiDr- and KM20L2-bearing mice were prepared by the same ways as described for ALA measurements except that the solvent was a mixture of acetonitrile and ethanol (1:7 by volume). The plasma or tumor tissue samples (0.1 ml) were mixed with 0.9 ml of the mixture solvent and sonicated for 1 min before centrifugation (5700 × g for 10 min). Porphyrins in the supernatants were spectrophotometrically measured using a Perkin-Elmer LS50B spectrophotometer. The samples were excited at 400 nm, and emission was scanned from 550 to 750 nm with a slit width of 15 nm on both sides. A catch filter was used on the emission side to remove scattered light of wavelength <530 nm from the light reaching the detection system. With this procedure it was possible to extract and detect porphyrins (>95%) induced by ALA or its methylester in the plasma and tumor tissue. The concentrations of porphyrins in the samples were calculated from standard curves made by addition of known amounts of PpIX to respective extracts from untreated mice and expressed as μg PpIX/ml plasma or g wet tumor tissue.

**PDT Protocol.** Mice with WiDr tumors of the appropriate size were randomly divided into groups (a minimum of 5 animals/group) as follows:

- Group 1, neither drug nor light; group 2, light only on the tumors; group 3, mice given an i.p. injection of ALA at a dose of 250 mg/kg body weight followed by light irradiation of the tumor; group 4, mice given an i.v. administration of Pf at a dose of 1 mg/kg before light exposure of the tumor; group 5, irradiated tumors after mice had been given both i.p. ALA (250 mg/kg) and i.v. Pf (1 mg/kg). Also, tumors of mice were treated with PDT using ALA plus an increased dose of Pf to see if the increased doses of Pf could additionally enhance ALA-PDT efficiency. Group 6 was PDT of tumor with Pf (2.5 mg/kg) alone; group 7, PDT of tumors with ALA (250 mg/kg) and Pf (2.5 mg/kg); group 8, PDT of tumors with Pf (5 mg/kg) alone; and group 9, PDT of tumors with ALA (250 mg/kg) plus Pf (5 mg/kg). ALA methylester was also studied in combination with Pf in PDT of the tumors. Group 10 was PDT with ALA methylester (250 mg/kg) alone, and group 11 was PDT using ALA methylester and Pf (1 mg/kg). The tumor model KM20L2 was applied in experiments parallel to those as described previously. The time intervals between drug administration and light irradiation were chosen to be 3 h for ALA and Pf and 1 h for ALA methylester. This was on the basis of the peak levels of ALA- and ALA methylester-induced porphyrins in the tumor tissues and on a previous report that showed a peak effect on the vascular system at 3 h after hematoporphyrin ester-mediated photosensitization (17). Responses of the treated tumors were evaluated by tumor regression/regrowth time and Kaplan-Meier method. The size of the tumors was measured every 2nd day, and when the treated tumors reached a volume 5 times that of the volume on the day before light irradiation, the mice were sacrificed. The data obtained from the measurements on tumor volumes in each group were pooled to represent mean tumor regrowth curves. The fraction of tumors that failed to reach 4 times the initial volume was also estimated by the Kaplan-Meier method.

The laser light irradiation of the tumors was performed by fixing unanesthetized mice in specially designed plastic holders so that the mice were immobile during illumination. The tumor area was exposed to red light (632 nm) from a 0.3 W diode laser (CeramOptec GmbH, Bonn, Germany) at a fluence rate of 150 mW/cm² for 15-min exposure in all cases. The fluence rate of the light on the tumor area was regularly checked by a calibrated integrating sphere with a photodiode coupled to a digital multimeter (Keithley Instruments, Munich, Germany) before and immediately after light illumination.

**Influence of Pf on Production of ALA-induced Porphyrins in WiDr Cells in Vitro.** Monolayer cultures of the WiDr cells were grown in RPMI 1640 supplemented with 10% FCS, penicillin (100 IU/ml), and streptomycin (0.1 mg/ml) at 37°C in an incubator flushed with 5% carbon dioxide in air. The cells were subcultured twice/week. WiDr cells (5 × 10⁵) were seeded into six-well dishes (Nunc/lon) and left for 2 days for proper attachment to the substratum. After washing with RPMI 1640, the cells had been incubated with the serum-free medium containing ALA, Pf, or ALA plus Pf at various concentrations for 4 h before they were rinsed twice with PBS and brought into a solution containing 10% methanol by scraping with a Costar cell scraper. After 5 min of incubation, the cell debris was removed by centrifugation. Practically all of the ALA-induced porphyrins and Pf could be extracted from the cells with this procedure. Furthermore, aggregated porphyrins could be dissolved into fluorescentmonomers in the extracting solution so that there was a linear relationship between the amount of porphyrin monomers and fluorescence intensity. The intensity of fluorescence of ALA-induced porphyrins, Pf, and porphyrins plus Pf in the samples, was recorded as described previously. The excitation wavelengths were 411, 407, and 411 for ALA and Pf and 1 h for ALA methylester. This was on the basis of the peak fluorescence of ALA-induced porphyrins, Pf, and porphyrins plus Pf in the samples, was recorded as described previously. The excitation wavelengths were 411, 407, and 411 for the induced porphyrins, Pf, and porphyrins plus Pf, respectively, and the emission wavelength for all of the cases was scanned from 550 to 700 nm. In this way the influence of Pf on the production of ALA-induced porphyrins in the WiDr cells can be determined.

**PDT of WiDr Cells with the Combined Regimens in Vitro.** WiDr cells (~400) were inoculated in 25 cm² of plastic tissue culture flasks. After incubation of the cells with ALA (168 μg/ml), Pf (0.02 μg/ml or 0.1 μg/ml), or ALA (168 μg/ml) plus Pf (0.02 μg/ml or 0.1 μg/ml) for 4 h as described previously, the cells were exposed to light (for 30 s) from a bank of four fluorescent tubes (Model 3026; Applied Photophysics, London, United Kingdom) emitting light mainly ~405 nm. The fluence rate of the light reaching the cell monolayer after irradiation, the medium was replaced with drug-free RPMI 1640 containing 10% FCS, and the cells were then left for 10 days to allow for the formation of colonies. The cells were finally fixed in ethanol, stained with methylene blue, and the colonies counted.
Histological and Ultrastructural Studies. Before and 1, 3, 24, and 48 h after PDT with ALA, Pf, or ALA plus Pf, the WiDr tumor tissues (three tumors/time point) were excised and fixed in 10% buffered formalin solution. The specimens were then dehydrated and embedded in paraffin. Sections were cut and stained with H&E for light microscopy. After excision, tumor specimens were also immediately fixed in a 0.1 M cacodylate-buffered mixture of 1% glutaraldehyde and 4% formoldehyde (18), followed by postfixation for 1 h in cacodylate-buffered 1% osmium tetroxide. The samples were then dehydrated in progressive ethanol-water in 10-min steps (70, 90, 96, and 100%) and in propylene oxide (3 times each for 10 min) before being embedded in an Epon/Araldite mixture. Semisections were cut with glass knives, mounted on glass slides, and stained with toluidine blue for light microscopic orientation. Ultrathin sections were cut with diamond knives, floated on 200 mesh copper grids, stained with uranyl acetate as well as lead citrate, and finally examined with a Philips CM-10 transmission electron microscope.

In Vivo PDT of WiDr Tumor and in Vitro Clonogenic Assay. Zero, 1, 3, and 24 h after PDT with ALA, Pf, or ALA plus Pf, mice were killed, and the WiDr tumors (five tumors/time point) were excised and weighed. The tumors were then minced with a scalpel, and a single-cell suspension was prepared in 5 ml of an enzyme mixture (Sigma Chemical Co.) of collagenase (0.2%), protease (0.05%), and DNase (0.02%) for 1 h at 37 °C with stirring. Single cells were then minced with a scalpel, and a single-cell suspension was prepared in RPMI 1640, and finally resuspended in 10 ml of the medium containing 10% FCS. Fifty and 100 μl of cell suspensions after appropriate dilutions (10–100 times depending on the number of cells) were seeded in six-well plates (six wells/tumor with 2 different volumes of cell suspension) and left for 2 weeks with medium changed once/week. All manipulations of the experiments were carried out in subdued light. Cell colonies were fixed with 96% ethanol for 10 min and stained with methylene blue for 15 min. The total number of macroscopic cell colonies was counted and calculated as per gram of wet tumor tissue. Data represent the survival fraction relative to the control tumors (light only) that are expressed as 100% survival.

PDT of Normal Skin of Mice. To investigate the risk of prolonged skin phototoxicity with the combined PDT regimen, normal foot response of nude mice bearing no tumor (three mice/group) to PDT was examined. The feet of the mice were treated in the same manner (including light dose) as that used for tumors. Because the antitumor efficiency of PDT with 250 mg/kg ALA plus 1 mg/kg Pf is comparable with that with 5 mg/kg Pf alone, the two PDT protocols and 4 time intervals between drug administration and light irradiation (1, 2, 4, and 10 days) were used. PDT-mediated response of the right hind foot of the mice was compared with that of the unexposed left hind foot of the same mice. The average thickness (PDT-induced edema) of the treated foot (Tt) and of the untreated foot (Tu) was measured every 2nd day for 20 days, and the response was calculated as (Tt/Tu)-1.

Statistical Analysis. Data on tumor responses to various PDT protocols were analyzed using Log-rank test and others were performed using Student’s t test.

RESULTS

Uptake and Clearance of ALA in Plasma and Tumor. The kinetics of uptake and clearance of ALA in the plasma and tumors of WiDr-bearing mice is shown in Fig. 1. ALA methylester is not included, because the method applied for ALA measurement has been found to be unsuitable for the determination of ALA methylester. ALA had a similar kinetic pattern of uptake and clearance in the plasma and tumor after i.p. administration. The amounts of ALA peaked at 0.5 h after administration in both plasma and tumor, but the level of ALA in the plasma was only one-third of that in the tumor. The rates of clearance of ALA seem to follow a one-compartment model with half-lives of ~18 and 58 min in the plasma and tumor, respectively.

Accumulation and Elimination of ALA- and ALA Methylester-derived Porphyrins in Plasma and Tumor. Figs. 2 and 3 demonstrate the kinetic patterns of accumulation and elimination of porphyrins in the plasma and tumor tissues of WiDr- and KM20L2-bearing mice after i.p. administration of ALA or its methylester. The clearance of ALA-induced porphyrins from plasma and tumor tissue of the WiDr-bearing mice appears to be consistent with a two-compartment model with an initial rapid fall (t1/2, 3 and 4 h) followed by a slower phase of decline (t1/2, 10 and 19 h) in the plasma and tumor, respectively. A similar pharmacokinetic pattern was also found after injection of ALA methylester, with half-lives of 3 and 15 h in the plasma as well as 4 and 18 h in the tumor. About 70% of the maximal amounts of ALA- and its methylester-derived porphyrins were cleared.
from plasma and tumor within the period of the first decline. As shown in Fig. 2, ALA induced more porphyrins than ALA methylester by a factor of 2.4 in the plasma and 7.3 in the tumor. Furthermore, although both ALA- and ALA methylester-derived porphyrins in the plasma peaked at 1 h after administration, ALA-induced porphyrins were eliminated from the tumor more slowly than those induced by ALA methylester. In the case of KM20L2 tumor-bearing mice there was a similar biexponential decline in concentrations of the induced porphyrins with half-lives of 4 and 13 h in the plasma as well as 5 and 14 h in the tumor after ALA administration (Fig. 3). Moreover, the first decrease in levels of porphyrins (t1/2, 3 and 4 h) was followed by a slower fall (t1/2, 16 and 14 h) in the plasma and tumor, respectively, after ALA methylester administration (Fig. 3). Again, ALA methylester produced less porphyrins than ALA in both plasma and tumor. Interestingly, ALA induced more porphyrins in the WiDr tumor than in the KM20L2 tumor.

Responses of WiDr and KM20L2 Tumors to PDT with the Combined Regimens. In Fig. 4 Kaplan-Meier (a) and regrowth (b) curves show the response of WiDr tumors to PDT with ALA, Pf, or ALA plus Pf. Control tumors that were untreated, treated with light alone, or PDT using Pf (1 mg/kg) alone had similar exponential growth rates. This was also true for the tumors treated with ALA or Pf alone without light irradiation (data not shown). None of the control tumors regressed spontaneously. Tumors of mice treated with ALA PDT grew significantly more slowly than control tumors (P = 0.0029). Moreover, tumors of mice given ALA and Pf followed by light exposure showed a significant growth delay when compared with those PDT-treated with either of the drugs (P = 0.0002 and P = 0.0001). Similar findings were also made in the model of KM20L2 tumors. In this case, PDT using ALA plus Pf produced a significantly higher KM20L2 tumor response than the treatments with ALA alone (P = 0.0038) or Pf alone (P = 0.0155). As shown in Fig. 5, PDT with Pf alone, with doses ≤5 mg/kg, resulted in a comparable antitumor efficacy of WiDr tumors as the combined protocol (1 mg/kg Pf and 250 mg/kg ALA; P = 0.5821). PDT with ALA and an increased dose (2.5 or 5 mg/kg) of Pf generated a larger tumor regrowth delay than those with Pf only (P = 0.015 and P = 0.0018; Fig. 6). Fig. 7 demonstrates a significantly stronger tumor regression with the combined regimen using 2.5 mg/kg of Pf than 1 mg/kg (P = 0.0254), but additionally increasing the dose to 5 mg/kg did not give rise to any significantly better antitumor effect (P = 0.6487). WiDr tumors of mice treated with PDT using ALA methylester plus Pf had a significantly reduced growth rate as compared with the tumors treated with ALA-methylester-PDT (P = 0.0423; Fig. 8), but the effect was not as large as that using regimen ALA plus Pf (P = 0.007). In the case of the KM20L2 tumor, the protocol with ALA methylester and Pf gave a slight but insignificant antitumor effect (P = 0.1127).

Influence of Pf on Endogenous Porphyrins Induced by ALA in WiDr Cells in Vitro. To understand the mechanism of the enhanced antitumor effect by PDT with combined regimens, the influence of Pf on the production of endogenous porphyrins induced by ALA was
studied in the WiDr cells in vitro. A number of different concentrations of Pf and ALA were used. As shown in Fig. 9, the fluorescence intensity of intracellular Pf and ALA-induced porphyrins is just a sum of the two individual fluorescence indicating no effect of Pf on biochemical metabolism of porphyrin synthesis induced by ALA in the cells.

PDT of WiDr Cells in Vitro with ALA, Pf, or ALA Plus Pf. The combined PDT protocols used in the WiDr tumor in vivo were also applied to treat WiDr cells cultured in vitro. PDT with ALA (168 µg/ml) alone resulted in ~35% killing of the cells (Fig. 10a; P = 0.001). When combined with Pf at a low concentration (0.02 µg/ml) that alone did not induce any phototoxicity, the treatment did not show any enhanced cell killing (P = 0.12). As shown in Fig. 10b, ALA PDT with an increase in the dose of Pf to 0.1 µg/ml could achieve a larger effect on the destruction of the cells, but this effect is simply attributable to photodynamic action with the increased dose of Pf.

Histological and Ultrastructural Studies. Control tumors showed no evidence of histological and ultrastructural alterations (Figs. 11a and 12a). Tumors sampled at various times after ALA PDT displayed edema, degeneration, and some necrosis of neoplastic cells (Fig. 11b) with some endothelial cells also damaged. Tumors treated with PDT using Pf alone at a concentration of 1 mg/kg demonstrated...
apparent vascular reaction such as edema and congestion, with little morphological change of the tumor cells (Fig. 11c). However, 1 and 3 h after PDT with ALA and Pf (1 mg/kg) severe congestion and some hemorrhage were observed in the tumor (Fig. 11, d and e). A number of platelets clumped against the endothelium of the vessels, and edema in the subendothelial zone was evident (Fig. 12b). Forty-eight h after treatment the vascular reaction was even stronger, together with severe necrosis of the tumor cells (Fig. 11f). Ultrastructurally, complete destruction of vascular endothelium and subendothelial zone was observed (Fig. 12c). Studies using the ApopTag Plus in situ apoptosis detection kit (Oncor, Inc., Middlesex, United Kingdom) did not show any positive apoptotic cells in the treated tumor tissues (data not shown).

**In Vitro Survival of WiDr Cells after in Vivo PDT.** The experiments of in vivo PDT and in vitro clonogenic assay were designed to assess the viability of tumor cells with the time for which the tumors remained in situ following PDT. This can provide the information on PDT-mediated destruction of the tumor vascular system. The kinetics of tumor cell death in vitro after PDT in vivo with ALA, Pf, or ALA plus Pf are presented in Fig. 13. The surviving fraction of clonogenic WiDr cells was not substantially reduced with the time up to 24 h following PDT with only ALA or Pf (1 mg/kg). In the case of PDT with ALA plus Pf (1 mg/kg), an initial slight reduction in tumor cell viability that was attributable to a direct action of killing was followed by a progressive decrease to reach a level of only 10% clonogenic cells by 24 h. This indicates that the combined PDT regimen had an effect on the vascular stroma of the tumor.

**PDT Effect on Normal Skin Tissue.** The normal skin phototoxicity of Pf (5 mg/kg) or ALA plus Pf (1 mg/kg) at various time intervals between drug(s) administration and light exposure is represented in Fig. 14. No response of feet was seen when light irradiation was given 1 day after administration of ALA and Pf (1 mg/kg), suggesting that the mice suffered no risk of skin photosensitivity from the 2nd day following the combined PDT. However, a strong phototoxicity of normal skin could be induced by light exposure 1–4 days after injection of Pf (5 mg/kg) and did not completely disappear until day 15 after PDT. Even 10 days after administration of the drug, light-induced foot response was still observed, although the response recovered by day 7 after PDT (Fig. 14).

**DISCUSSION**

The rationale of the present study is based on findings that PDT with ALA-induced endogenous porphyrins damages cellular components, whereas all of the exogenous sensitizers used for clinical PDT target in principle the vasculature of tumor tissue (1, 6), and that destruction of both neoplastic cells and vessels may lead to superior effects (12, 13). The main purpose of the study was to assess if the antitumor effect of ALA PDT could be enhanced by the use of Pf, a well known vessel-targeted sensitizer that induces long-term skin phototoxicity, the major side effect of clinical PDT (2). We wanted to test a low dose of this drug so that phototoxicity of normal tissues could be avoided, whereas some effect on the vascular system might be retained.

This is the first report of pharmacokinetics of ALA in both plasma and tumor tissue of nude mice. A similar kinetic pattern of ALA in the plasma and tumor was seen with a peak at 0.5 h after systemic administration (Fig. 1). Interestingly, the maximal amount of ALA in the plasma was only about one-third of that in the WiDr tumors. This is at least partly attributable to the fact that the large fraction of injected ALA that bound to cellular components of the blood was not included. It is also likely that ALA was degraded faster in the plasma than in the tumor. The concentration ratios of ALA to induced porphyrins at peak times are $\sim 100$ in the plasma and $60$ in the tumor (Figs. 1 and 2) suggesting that 100 and 60 times higher concentrations of ALA are needed to induce a given concentration of porphyrins in the plasma and tumor, respectively. It should, however, be pointed out that ALA-induced porphyrins in the plasma are not only released from blood cells but also from other organs. Similar patterns of accumulation and clearance of porphyrins induced by ALA and its methylster were found in the plasma of the WiDr- and KM20L2-bearing mice as...
well as in the two models of human tumor xenografts (Figs. 2 and 3). The short half-lives of ALA and ALA-induced porphyrins in the body indicate the reduced risk of prolonged skin phototoxicity. The amounts of ALA-induced porphyrins were higher in the WiDr tumors than in the KM20L2 tumors, suggesting that the capacity of ALA-induced porphyrins is tumor model-dependent. Among these induced porphyrins are PpIX (>90%) and several other porphyrin species such as heptaporphyrin, coproporphyrin, and uroporphyrin (19).

Both WiDr and KM20L2 tumors showed a significantly stronger response to PDT with ALA and Pf (at a low dose of 1 mg/kg) than the treatments with either of the drugs (Fig. 4). The antitumor effect of the combined PDT regimen was similar to that of PDT with Pf alone at a high dose of 5 mg/kg (Fig. 5). Moreover, the efficiency of ALA PDT could be additionally enhanced by the use of Pf at a higher concentration of 2.5 mg/kg (Figs. 6 and 7). However, it appeared that increasing the dose of Pf to 5 mg/kg did not continue to significantly potentiate the effect of ALA PDT, suggesting that the efficacy of ALA PDT can be enhanced by the use of Pf within a limit of certain concentrations. The antitumor efficiency of ALA methylster-based PDT was slightly increased by Pf (1 mg/kg) in both tumor models, but the effect was significantly lower than that of PDT using ALA and Pf (Fig. 8). This may be attributable to lower amounts of porphyrins induced by ALA methylster in the tumor tissues (Figs. 2 and 3). However, the efficacy of ALA methylster-PDT could be significantly potentiated by disulfonated aluminum phthalocyanine (a vessel-targeted sensitizer) in the WiDr tumor model (data not shown).

Studies in vitro showed that Pf did not interfere in production of ALA-induced porphyrins in the WiDr cells (Fig. 9). Consistently, phototoxicity of the cells with ALA was not increased by use of Pf at a low concentration. This demonstrates that no enhanced effect was achieved at a cellular level when ALA-PDT was applied in combination with Pf. Our data are in agreement with the results of Messmann et al. (20) that showed no potentiation of phototoxicity when HT-29 cells were simultaneously incubated in vitro with ALA and Photosan-3. Histological studies indicated that the combined PDT regimen led to a much earlier and stronger effect on tumor microvasculature than the treatments with ALA or Pf alone (Fig. 11). Ultrastructural investigations showed that PDT with ALA alone gave rise to a moderate damage to the endothelial cells, whereas a treatment with a mixture of ALA and Pf destroyed both the endothelium and subendothelial layer (a sensitive target; Ref. 21). This may result in a lethal collapse of the whole microvascular system of the tumor that, in
turn, additionally affects the viability of tumor cells that have already been attacked by the action of ALA-mediated PDT. This explanation is strongly supported by the results obtained from the studies of in vivo PDT and in vitro clonogenic assay. A progressive decrease in clonogenic cells was found with times following in vivo PDT using ALA and Pf (Fig. 13). This reduction is known to be a consequence of the destruction of tumor microvasculature (22). The present results are consistent with a study of Nelson et al. (23). They found that PDT with two vessel-targeted dyes (Pf II and TPPS 4) achieved a 40% higher cure rate of EMT-6 mammary tumors than PDT with either of the drugs. The increased tumor kill was attributable to an enhanced damage to tumor microvasculature. Furthermore, two recent reports have shown that PDT with antiangiogenic agents could significantly improve the antitumor efficiency (both of which used a vessel-localizing photosensitizer for PDT; Refs. 24, 25). These studies indicate that an increased PDT effect on tumor vasculature by either a vessel-targeted sensitizer or an antiangiogenic agent can potentiate tumor eradication.

Pf, like most of photosensitizers used for clinical PDT, can cause prolonged skin phototoxicity because it localizes mainly in the vasculature of the skin with a long retention. A low dose of Pf associated with only a minor skin phototoxicity will significantly improve the clinical outcome and acceptance. The present investigation shows that the effect of ALA PDT can be enhanced by the use of a low dose of Pf that does not induce any significant skin photosensitivity. Thus, this combined PDT regimen may have an important clinical implication. At present, our clinical trials with the combined protocol for the treatment of early esophageal cancer are being performed. Preliminary results indicate that PDT with the combined regimen produces a stronger response of the treated esophageal cancer than PDT with ALA alone. Although it is too early to draw any conclusion on the antitumor efficacy, none of treated patients have suffered any prolonged skin phototoxicity nor any PDT-induced perforation and stricture of the esophagus.

![Fig. 12](image1.png)

Fig. 12. Electron microphotographs of WiDr tumors taken after treatment with light alone as a control (a); 1 (b) and 48 (c) h after PDT with ALA and Pf (1 mg/kg). ×8,500 for all photographs. E, endothelium; P, platelet; R, RBCs. a, an unchanged vessel; b, congestion, aggregation of platelets against the wall of a vessel, and edema of subendothelial zone but endothelium undamaged; c, congestion, necrosis of endothelium, and considerable edema and disruption of subendothelial zone.

![Fig. 13](image2.png)

Fig. 13. WiDr tumor cell survival in vitro as a function of time for which the tumors remained in situ following in vivo PDT with various regimens as indicated. PDT treatments were the same as described in Fig. 4. Bars, ± SE based on at least five individual animals.

![Fig. 14](image3.png)

Fig. 14. Normal mouse skin was treated with PDT (as indicated) in the same manner as that for PDT of tumors. The photo-induced skin edema was evaluated as described in “Materials and Methods.” SE was <10% of given values for all points.
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Antitumor Effect of 5-Aminolevulinic Acid-mediated Photodynamic Therapy Can Be Enhanced by the Use of a Low Dose of Photofrin in Human Tumor Xenografts

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