

Evidence for an Important Role of Neutrophils in the Efficacy of Photodynamic Therapy *in Vivo*¹

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Abstract

To investigate the role of neutrophils in the efficacy of photodynamic therapy (PDT) in rhabdomyosarcoma-bearing rats, the number of these circulating phagocytes was decreased or increased before interstitial PDT by use of rabbit anti-rat neutrophil serum or granulocyte colony-stimulating factor, respectively.

After administration of the antiserum, the number of circulating neutrophils decreased by 99.9%. However, the number of monocytes, lymphocytes, and platelets decreased as well (by 100%, 80%, and 25%, respectively). Under these conditions, PDT did not retard tumor growth at all. However, after cessation of the antiserum treatment 5 days after PDT, a striking decrease in the growth rate occurred subsequent to an increase above the normal range of the number of circulating neutrophils.

Administration of the granulocyte colony-stimulating factor led to a specific 4-fold increase in the number of circulating neutrophils. In these rats, the tumor growth at day 2 after PDT was retarded as compared with PDT-treated rats that received saline only.

Statistical evaluation of both experimental conditions showed that the efficacy of PDT, expressed as the percentage of change in tumor volume at day 2 after treatment, was dependent on the number of circulating neutrophils present at the day of PDT ($P = 0.001$; $r^2 = 0.482$). Apparently, neutrophils are indispensable for successful PDT *in vivo*.

Introduction

The mechanisms that play a role in tumor eradication after Photofrin-based PDT³ are subject to extensive study. The first event occurring after illumination of a photosensitizer is the formation of very reactive oxygen species such as singlet oxygen (1–3). These reactive oxygen species are involved in direct tumor cell death by oxidation of plasma membranes, mitochondria, and lysosomes (4, 5). However, tumor cells are killed mainly indirectly by the effect of Photofrin-based PDT on the normal and tumor vasculature. Henderson and colleagues (6–8) clearly showed that the kinetics of the loss in tumor cell clonogenicity *in vivo* coincided with the late blood flow stasis, leading to deprivation of the tumor from oxygen and nutrients. The mechanisms underlying the effect of PDT on the (tumor) vasculature are not fully elucidated as yet. It is well established that endothelial cells are very sensitive to PDT (9, 10). These cells not only rapidly lose their ability to respond to acetylcholine stimulation after PDT (11), which leads to vasoconstriction, but also contract themselves, as has been observed *in vivo* (12) and *in vitro* (13), exposing the thrombogenic subendothelium. This will stimulate edema formation, platelet aggregation, thromboxane release, and thrombus formation,

finally leading to blood flow stasis with subsequent regression of the tumor (10, 14–17).

There is growing evidence that an inflammatory reaction by immunocompetent leukocytes might play a role in tumor destruction after PDT as well. Neutrophils adhere to the vascular wall (18) and infiltrate the tumor area after PDT (19). Moreover, several immunotherapeutic approaches have been reported to potentiate the effects of PDT (20–23). In this study, we investigated the relationship between the number of neutrophils in the circulation and the efficacy of PDT as determined by the effect on growth rate of the rat rhabdomyosarcoma R-1 *in vivo*. To this end, the number of blood neutrophils was decreased by use of rabbit anti-rat granulocyte serum or increased by G-CSF before the start of PDT.

Materials and Methods

Animals and Tumor Model. Female WAG/Rij rats, ages 12–20 weeks, were obtained from Harlan (Zeist, the Netherlands). Small pieces of a well-characterized isologous rhabdomyosarcoma, designated as R-1 (24), were implanted s.c. into both thighs. Tumor growth was assessed by caliper measurements on three orthogonal diameters, at least three times a week. Tumors were treated when the volume was between 1200 and 2000 mm³. Blood samples were obtained from a tail vein with EDTA as the anticoagulant. Total leukocyte counts were determined with a microcell counter, and differential counts were carried out on May-Grünwald and Giemsa-stained blood smears.

Photosensitizer and Drugs. The photosensitizer Photofrin II (PII) was obtained from Quadra Logic Technologies, Inc. (Vancouver, British Columbia, Canada) and was reconstituted in 5% glucose before use. The photosensitizer was administered i.v. into a tail vein at 10 mg PII/kg 24 h prior to light delivery.

Adsorbed polyclonal rabbit anti-rat granulocyte antiserum was purchased from Accurate (Westbury, NY), and lyophilized recombinant human G-CSF was purchased from Amersham International (Buckinghamshire, England) and dissolved in saline (0.9%) containing 1% NRS (Central Laboratory for Blood Transfusion, Amsterdam, the Netherlands).

Light Delivery. The light source was a dye laser pumped by an argon ion laser (Spectra Physics model 375B and 2040E, respectively). A birefringent filter and monochromator were used to tune the dye laser to emit light at 625 ± 1 nm wavelength using 4-dicyanomethylene-2-methyl-6(*P*-dimethylaminostyryl)-4*H*-pyran) as a dye. The light was directed via a beam splitter to three cylindrical diffusers of 15 mm in length (Rare Earth Medical, Dennis, MA).

Treatment Protocol. In the first series of experiments, rats were administered 2 ml/kg granulocyte antiserum by i.p. injection at 2 days before PDT treatment, followed by daily i.p. injections of 0.5 ml/kg antiserum for 6 consecutive days. Control rats were given injections of equal volumes of saline. In the next series of experiments, 7.5 μ g/kg G-CSF containing 1% NRS was injected i.v. at 9 h before and 9 h and 27 h after PDT treatment. Control rats were given injections of equal volumes of saline containing 1% NRS.

Interstitial PDT treatment was performed as described previously (25), with minor adaptations. In brief, rats were anesthetized by i.m. injection of 1 ml/kg Hypnorm (fluanisil/fentanyl mixture; Janssen Pharmaceutica, Beerse, Belgium). Animals were placed on a heated support during treatment to control body temperature. In one of two tumors, a diffusor was inserted into the central axis of the tumor parallel to the body axis. In the contralateral tumor a

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³ The abbreviations used are: PDT, photodynamic therapy; G-CSF, granulocyte colony-stimulating factor; NRS, normal rat serum.

dummy was placed. The output of the diffuser was kept below 50 mW/cm of diffuser length to avoid hyperthermic effects. Tumor core temperature was measured during PDT using a thermocouple probe and never exceeded 40°C. Total applied radiant energy was 270 J/cm of diffuser length. This treatment modality resulted in a delay of tumor growth, but not in tumor regression. At the end of the observation period, rats were sacrificed, and sections of tumor tissue were H&E-stained according to standard histological procedures.

Statistical Analysis. Data were analyzed using the paired or unpaired Student's *t* test and Pearson correlation analysis where appropriate and considered significant when *P* < 0.05.

Results

Effect of Antigranulocyte Antiserum on Tumor Growth after PDT. To evaluate the effect of antigranulocyte antiserum on tumor growth after PDT, the number of neutrophils, monocytes, lymphocytes, and platelets was determined first. We found that 1 day after the start of antiserum administration, the neutrophils were depleted by 99.9% (Table 1). The number of neutrophils remained at that low level until the administration of antiserum was stopped. The antiserum was not specific for neutrophils because the number of blood monocytes, lymphocytes, and, to a lesser extent, platelets was decreased as well, *i.e.*, by 100%, 80%, and 25%, respectively (Table 1).

To investigate whether the condition induced by the administration of the antiserum interferes with tumor growth or the efficacy of PDT, the tumor volumes of control and PDT-treated tumors were determined at various time points (Fig. 1). As shown, the growth rate of the control tumors of the antiserum-treated rats was not significantly different from the control tumors of the saline-treated rats. The current PDT treatment resulted in a delay of tumor growth for 5 days in saline-treated rats (Fig. 1). However, in the antiserum-treated rats, PDT did not influence tumor growth significantly. Statistical evaluation showed irrespective of antiserum treatment an inverse association between the percentage of increase of tumor volume at day 5 after PDT and the number of neutrophils (*P* = 0.004; *r*² = 0.663) and lymphocytes (*P* = 0.011; *r*² = 0.573) present in the circulation at the day of PDT.

Strikingly, after the administration of antiserum was stopped (at day 5 after PDT treatment), a delay in tumor growth was observed. This was preceded by an increase to about the normal level of circulating monocytes (70%), lymphocytes (112%), and platelets (116%), whereas the number of neutrophils increased even further to approximately a factor 9.5 above the normal range. Statistical analysis showed that the percentage of increase in tumor volume during that time for all of the PDT-treated tumors inversely correlated with the increase in the number of neutrophils (*P* = 0.015; *r*² = 0.593), but not with the increase in the number of monocytes, lymphocytes, or platelets.

Effect of G-CSF on Tumor Growth after PDT. If the growth rate of PDT-treated tumors indeed depends on the number of neutrophils

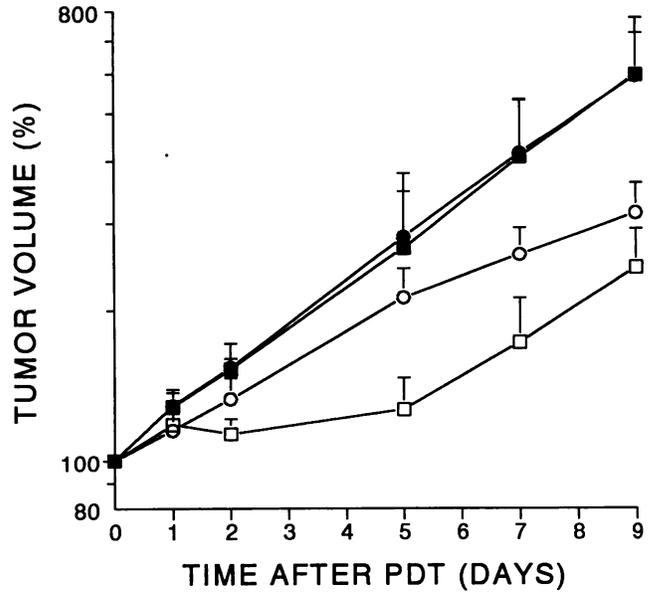


Fig. 1. Effect of antigranulocyte antiserum on tumor growth after PDT. Volumes of PDT-treated (○) or control (●) tumors of rats given injections of antiserum and of PDT-treated (□) or control (■) tumors of rats given injections of saline are expressed as a percentage of the tumor volume at the day of treatment. Data are the means of five rats. Bars, SD.

in the circulation, increasing the number of blood neutrophils before PDT should amplify the efficacy of tumor treatment. To study this, G-CSF was administered *i.v.* 9 h before and 9 h and 27 h after PDT. This led to a 4-fold increase in the number of circulating neutrophils at the start of PDT, whereas the number of monocytes, lymphocytes, and platelets stayed within the normal range (Table 2).

Under this condition, the growth rate of control tumors was not changed, but PDT was more effective as compared to saline controls up to and including day 2 after PDT (Fig. 2). Thereafter, the PDT-treated tumors of both the G-CSF- and saline-treated animals resumed growth at a similar rate, which did not differ from that of the control tumors (Fig. 2). Statistical analysis showed an inverse relationship between the percentage of increase in tumor volume at day 1 and the number of neutrophils (*P* = 0.001; *r*² = 0.792) and lymphocytes (*P* = 0.005; *r*² = 0.699) at the day of treatment.

Evaluation of the data from both experiments (Figs. 1 and 2) using Pearson correlation analysis confirmed that the effect of this PDT treatment modality on the growth rate of the tumors at day 2 after therapy depended on the number of neutrophils (*P* = 0.001; *r*² = 0.482) present on the day of PDT treatment, whereas there was no significant relationship with the number of monocytes, lymphocytes, or platelets.

Histological Examination of Tumor Tissue. At the end of the observation period, rats were sacrificed, and tumor tissue was examined. The analysis of the PDT-treated tumors of all rats showed vital rhabdomyosarcoma tissue next to necrotic tumor tissue. On the border of these vital and necrotic areas, large infiltrates of leukocytes were observed which mainly consisted of neutrophils (Fig. 3).

Discussion

The major finding of this study was that the efficacy of PDT *in vivo* is dependent on the number of neutrophils in the circulation. In the absence of neutrophils, a condition achieved by the administration of antigranulocyte antiserum, PDT had no effect on tumor growth, whereas an increase in the number of neutrophils upon G-CSF

Table 1 Effect of administration of antigranulocyte antiserum on the number of blood cells

Type of cell	Antigranulocyte serum		Saline	
	Start	1 Day later	Start	1 Day later
Neutrophils (×10 ⁹ /liter)	1.49 ± 0.4 ^a	0.001 ± 0.003 ^b	1.18 ± 0.4	1.35 ± 0.22
Monocytes (×10 ⁹ /liter)	0.12 ± 0.08	ND ^c	0.16 ± 0.07	0.14 ± 0.09
Lymphocytes (×10 ⁹ /liter)	10.2 ± 2.2	2.0 ± 0.7 ^b	9.5 ± 1.6	8.1 ± 1.2
Platelets (×10 ¹² /liter)	877 ± 93	656 ± 77 ^d	746 ± 189	891 ± 58

^a Data are the means ± SD of five rats.
^b Data are significantly different (*P* < 0.005) from the normal number at the start of administration.
^c ND, not detectable.
^d Data are significantly different (*P* < 0.05) from the normal number at the start of administration.

Table 2 Effect of administration of G-CSF on the number of blood cells

Type of cell	G-CSF		Saline	
	Start	9 h later	Start	9 h later
Neutrophils ($\times 10^9$ /liter)	1.3 ± 0.3^a	6.1 ± 0.9^b	1.7 ± 0.6	2.0 ± 0.8
Monocytes ($\times 10^9$ /liter)	0.16 ± 0.08	0.2 ± 0.08	0.18 ± 0.04	0.14 ± 0.09
Lymphocytes ($\times 10^9$ /liter)	8.3 ± 0.9	7.6 ± 0.9	8.2 ± 1.5	6.7 ± 1.9
Platelets ($\times 10^{12}$ /liter)	853 ± 175	898 ± 148	901 ± 85	844 ± 85

^a Data are the means \pm SD of five rats.

^b Data are significantly different ($P < 0.001$) from the normal number at the start of G-CSF injection.

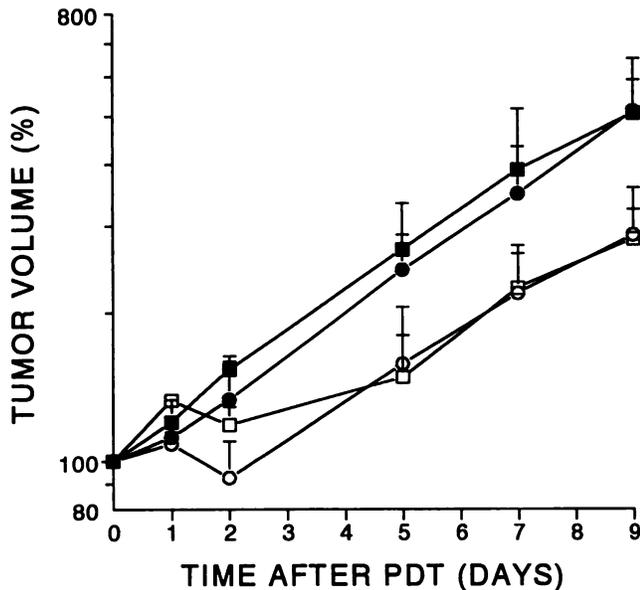


Fig. 2. Effect of G-CSF on tumor growth after PDT. Volumes of PDT-treated (\circ) or control (\bullet) tumors of rats given injections of G-CSF and of PDT-treated (\square) or control (\blacksquare) tumors of rats given injections of saline are expressed as a percentage of the tumor volume at the day of treatment. Data are the means of five rats. Bars, SD.

administration before PDT led to a temporary increase in the efficacy of PDT.

In the literature, there are several reports that point at a role for neutrophils in combination with PDT. The adhesion of granulocytes to the vascular wall is one of the first events to occur after PDT (18). This observation was confirmed by our group, and we proposed a mechanism that may underly the adherence of granulocytes to the vascular wall after PDT (13). Recently, neutrophils were also reported to rapidly infiltrate squamous cell carcinoma after treatment by PDT (19). Moreover, up-regulation of the activation status of the immune system as reflected by the number of Mac-1-positive cells by the immunostimulant schizophyllan before PDT led to a 3-fold decrease in colony-forming tumor cells *in vitro* as compared to PDT only (20). From the data presented here, we conclude that apparently the neutrophils determine the outcome of tumor regrowth after PDT *in vivo*.

We postulate that neutrophils might adhere via β_2 -integrins to stretches in the vascular wall where endothelium as a result of PDT has contracted (13), and where the subendothelial matrix is exposed as has been reported previously (12). Neutrophils, most likely attracted by chemotactic factors, could infiltrate the tumor area, releasing proteolytic enzymes that degrade attenuated tumor cells, which otherwise may continue to proliferate.

Direct tumor cell eradication by oxidative stress is limited to a short time frame, since very soon after the start of PDT vasoconstriction (11), platelet aggregation and blood flow stasis has been observed (15), inhibiting further oxygen transport to the tumor area. Although this in fact prevents an efficient PDT, it turns into good because vessel

occlusion deprives the tumor from its essential supply of oxygen and nutrients. In this manner, tumor cells that have escaped the direct kill by oxidative processes will be attenuated as yet. It is generally accepted that this effect on the vasculature is indispensable for tumor regression after Photofrin-based PDT and that platelets play an important role therein (3). Our study does not argue against this concept, since we suppose here that the attenuation of the tumor cells is a prerequisite before neutrophils are able to destroy them. However, how this condition is achieved, either directly during PDT or later as a result of vessel occlusion, possibly does not matter and depends on the type of photosensitizer. This is substantiated by our finding that neutrophils are not able to retard the growth rate of control tumors, while these phagocytes were increased in number by the administration of G-CSF (Fig. 2, \bullet). In seeming contradiction to this idea is the finding of Fingar *et al.* (26) that inhibition of the release of thromboxane by indomethacin not only prevents PDT-induced vascular stasis but also destruction of the (chondrosarcoma) tumor. Even though under those conditions the direct killing potential of PDT would be fully utilized because of the uninterrupted supply of oxygen, the failing PDT treatment apparently stresses the sole importance of the vascular effect. However, neutrophils as the final effector cells need to become fully activated by factors like G-CSF that can be produced by, *e.g.*, endothelial cells, monocytes/macrophages, lymphocytes, and platelets (27, 28). And indomethacin, like other non-steroidal anti-inflammatory drugs, not only inhibits the release of thromboxane (26) but also inhibits the activation of the neutrophil (29). Therefore, these phagocytes were not able to eradicate the directly injured tumor cells. In keeping with this idea is the striking finding of the present study that PDT-treated tumors under neutropenia still remain vulnerable to eradication by neutrophils for a long time after treatment. Namely, when the administration of antiserum was stopped five days later, leading to a 10-fold increase of neutrophil numbers, tumor growth was subsequently retarded (Fig. 1, \circ).

Recently, Dellian *et al.* (30) showed that the number of leukocytes that adhered to the tumor microvessel wall of the amelanotic melanoma A-Mel-3 within 3 h after PDT was small. Also, Wu *et al.* (31) found a diminished leukocyte-endothelium interaction in tumor microvessels. These findings may seem in contrast to the present study. However, we found large infiltrates mainly consisting of neutrophils present in all PDT-treated rhabdomyosarcoma tumors at the end of the observation period. Taken together, we conclude that neutrophils are indispensable for efficient PDT *in vivo*.

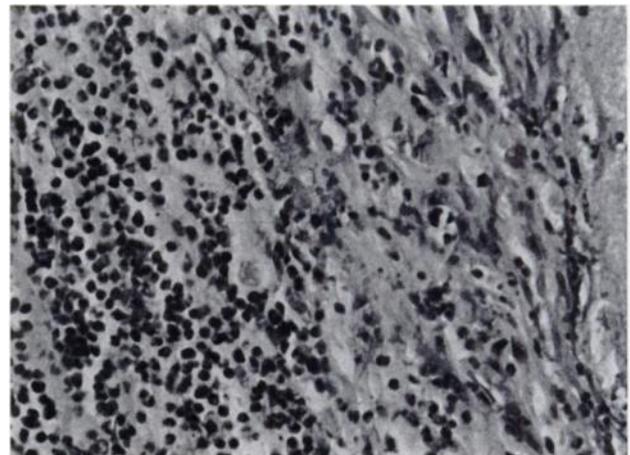


Fig. 3. Histological examination of tumor tissue. Tissue sections of PDT-treated tumors of all rats were H&E stained. Shown is a typical infiltrate at the border of vital and necrotic tumor tissue, mainly consisting of neutrophils. $\times 200$.

The use of G-CSF could be a promising accessory modality to improve the efficacy of PDT. G-CSF is a hematopoietic growth factor which principally stimulates the proliferation and differentiation of specifically neutrophil progenitor cells by binding to high-affinity G-CSF-specific receptors on their cell membranes (32). This is confirmed by our findings as well (cf. Table 2). It also increases the activities of mature neutrophils, including chemotaxis, phagocytosis, and oxidative metabolism (28). Furthermore, recombinant human G-CSF is now commercially available and approved (Filgrastim) or is in Phase IV clinical trial (Lenograstim) (33, 34). On this basis, additional studies on the optimal dose and duration of G-CSF administration as an accessory treatment to improve PDT are warranted.

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