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Identification of neutrophils as important effector cells in photodynamic therapy

Identificatie van neutrofielen als belangrijke
effector cellen bij fotodynamische therapie

Proefschrift

Ter verkrijging van de graad van doctor
aan de Erasmus Universiteit te Rotterdam
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Ter nagedachtenis aan Jeannette.

Voor Marleen en Bram.

Contents

CHAPTER 1

General introduction

Introduction to photodynamic therapy

Photodynamic therapy (PDT) is a treatment modality, which is at present widely used on an experimental basis for the treatment of cancer patients. It is based on the light induced excitation of light sensitive chemical compounds localized in malignant tissue. These so-called photosensitizers are capable of absorbing photons due to their extended conjugated ring systems. As a result the compound is excited to the singlet state. By decay of the excited electrons to the ground state electromagnetic energy is released in the form of fluorescence, but some singlet state molecules will undergo intersystem crossover to the triplet state. The energy of these triplet state molecules is conveyed in either of two ways. 1) In the absence of oxygen electrons are transferred to other molecules of the photosensitizer or to biological substrates which will lead to negatively or positively charged radicals (Type I reaction). 2) In the presence of oxygen the energy of the photoexcited sensitizer is directly transferred to oxygen, leading to the formation of singlet oxygen (Type II reaction). As a result of these reactions the excited photosensitizer will return to its ground state ready to absorb photons again.

Selectivity and application of PDT

Over the past decade several photosensitizers have been developed differing in physical/chemical properties that determine the cellular localization and intensity of the above-described cytotoxic reactions. The first sensitizer to be used clinically was hematoporphyrin derivative (HPD). This sensitizer consists of a mixture of mono-, di-, and oligomers of hematoporphyrin. Photofrin (PII) is a refined form of HPD enriched in the photoactive moiety. Although widely used in clinical settings, both sensitizers have the disadvantage of aselectivity, that is these photosensitizers are retained for a long period of time in normal tissue like skin. In search for compounds that are more selective for the intended target tissue and show a reduced clearance time from normal tissue the so-called second generation photosensitizers have been developed. Examples of these second generation photosensitizers are phtalocyanines, (bacterio)chlorins, purpurins and benzopor-

phyrin derivatives. Photochemical activity in target tissue can be achieved in either of two ways: 1) by systemic administration of the above-described exogenous photosensitizers or 2) by the generation of endogenous photosensitizers in target tissue. The latter is achieved by the (topical) administration of 5-amino-levulinic-acid (ALA). This compound has no photochemical activity on its own, but bypasses the rate limiting step of haem synthesis leading to an excess of endogenous protoporphyrin IX in target tissue, which does have photochemical activity.

After systemic administration the lipophilic photosensitizers tend to bind to lipoproteins in the blood. Since fast growing cells like tumor cells need more cholesterol then resting cells, photosensitizers accumulate to a higher extent in these cells. Therefore PDT in principle can be used for treatment of cancerous conditions, under the condition that light can be directed to the treatment site only in order to spare the non-malignant tissue. At first PDT was applied as a palliative treatment for various cancers. Nowadays PII-based PDT is registered in various countries for treatment of (early stage) cancers of the lung, esophagus, stomach, cervix and bladder. Preclinical studies have shown that PDT might be applicable for other malignancies as well. In addition to treatment of cancers specific photosensitizers can be used for early diagnosis for malignancies. This is based on the above-described preferential accumulation of lipophilic photosensitizers in tumor cells and the fluorescence emitted by the photosensitizer upon illumination.

PDT is also used in non-malignant applications like cosmetic removal of age related macular degeneration and for purging donor blood from HIV or other viral infected blood cells. Recent and promising applications of PDT concern non-cancer related diseases like psoriasis, intimal hyperplasia/restenosis, and rheumatoid arthritis.

To activate the photosensitizers present in target tissue local illumination is needed. In PDT this is achieved mainly by use of argon pumped dye or diode lasers. These devices are capable of producing light of a single wavelength matching a specific absorption maximum of the applied photosensitizer. Normally, red or infrared light (wavelengths of 610 nm and higher) is used to activate photosensitizers. Although absorption by the photosensitizers HPD and PII is not maximal in this region of the visible spectrum light of longer wavelengths penetrates deeper in tissue and thus will reach more likely the photosensitizers in target tissue. Laser light is usually directed to the target tissue via optical fibers and dispersed via light diffusers shaped in a form matching the form of the tissue or organ to be treated. Due to recent developments in endoscopic and surgical

techniques, virtually every organ or tissue can be reached for treatment.

Primary effects of PDT

The generated singlet oxygen has been identified as extremely oxidative (1). Due to this strong reactivity its lifetime in biologic systems is short (less then 40 ns), and therefore singlet oxygen will exert its oxidative potency only in the immediate vicinity of the site in which it was formed (2). In this respect it is important to consider the physical and chemical properties of the used photosensitizers, since these properties determine the cellular site of oxidation. Lipophilic photosensitizers are incorporated in lipid(bi)layers as found in the cell membrane, mitochondria, and the nucleus. The PDT-induced singlet oxygen-mediated oxidation of the cell membrane leads to leakage of electrolytes, membrane depolarization, inhibition of uptake of nutrients, and alterations of (membrane associated) proteins and enzymes. A visual sign of oxidative damage to mitochondrial membranes is the formation of swollen mitochondria. This results in the inhibition of the enzymes present in the mitochondrial membrane participating in the oxidative phosphorylation and respiratory chain. Consequently, the generation of for example ATP is diminished (3). Furthermore, an enhanced expression of heat shock proteins (4) and early response genes (5) have been observed. Oxidation and disruption of the nuclear envelope exposes the genomic DNA to oxidative molecules. Consequently, random DNA single strand breaks can occur and DNA-DNA and DNA-protein cross-links may be induced. In contrast to lipophilic photosensitizers, hydrophilic photosensitizers are preferentially accumulated into lysosomes and endosomes after pino- or endocytosis. As a consequence mainly these organelles are disrupted upon illumination. This leads to the release of protein degrading enzymes into the cytoplasm resulting in necrotic cell death (6).

Recent studies have focussed on the phenomenon of programmed cell death or apoptosis of various types of tumor cells as a result of the application of PDT. Soon after PDT the cell membrane folds into small buds which eventually pinch off from the cell surface. This so-called blebbing of the cell is considered to be one of the symptoms associated with apoptosis. Another symptom is DNA fragmentation, which is apparent within hours after the start of illumination of various photosensitizers like aluminium phthalocyanine (7), photofrin (PII), both

in vitro (8) and *in vivo* (9), and 5-aminolaevulinic acid (10). The important role of serine/threonine and tyrosine protein kinases and phosphatases in the signal transduction pathways leading to the activation of DNA-se under PDT, have been outlined recently (11,12). Activated DNA-se can split DNA at sites unprotected by nucleosomes, thereby generating strands of discrete length (the so-called laddering). On the other hand, Dougherty *et al.* (13) proposed that PDT might initiate direct apoptotic responses, like the afore-mentioned DNA laddering, without the need for intermediate signal transduction pathways that may be missing in certain neoplastic cells. Nevertheless, the study on how exactly PDT activates signal transduction routes (involvement of cytochromes, caspases) leading eventually to apoptosis of the cell is continued.

Secondary effects of PDT
(1) Vascular damage

Depending on the photosensitizer not only tumor cells are directly affected by PDT but also the endothelial cells of the tumor vasculature which are the most proximate cells to the blood circulation. Endothelial cells are extremely sensitive to PDT (14,15). Direct cytotoxicity as a result of uptake and illumination of photosensitizers causes the retraction of endothelial cells (16). Subsequently, blood plasma and proteins will leak into the treated tissue causing the (temporary) swelling of the tumor. The exposed subendothelial matrix is highly thrombogenic. Upon their adherence thrombocytes are activated and release potent vasoactive eicosanoids like thromboxane (17). This leads to a (temporary) constriction of primarily arterioles (18). In the presence of clotting factors released by endothelial cells upon PDT (19), thrombi are formed that occlude the blood vessels. Both the occlusion and constriction of blood vessels eventually lead to a complete stasis of the blood flow. It was postulated that this secondary effect of PDT could contribute to indirect tumor cell kill by the deprivation of oxygen and nutrients (20). Indeed, it was found that the clonogenicity of tumor cells is not directly affected after *in vivo* PII-based PDT, but deteriorates with the gradual reduction in oxygen and nutrients supply in time (21). The necessity of vascular damage for tumor regression seems further supported by the fact that indomethacine (22) or indomethacine-related drugs (23) and aspirin (24,25), which all inhibit the release

of thromboxane by activated thrombocytes, decreased the efficacy of PDT and the vascular responses after PDT. Nowadays it is widely accepted that the damaging effect of PDT on the tumor vasculature is indispensable for tumor cell death.

Secondary effects of PDT
(2) Activation of the immune system

Photooxidation of the lipids and proteins of plasma membranes of tumor cells also induces the rapid activation of membrane-borne phospholipases (26). This results not only in the accelerated degradation of oxidized phospholipids but also induces a massive release of lipid fragments and metabolites of arachidonic acid (27). These substances are highly chemotactic, enabling a massive recruitment of immune cells to the damaged site. Other humoral immunological mediators like histamine, components of the complement cascade, and proteins of the clotting system have been implicated in the PDT-induced accumulation of immune cells (28).

Cytokines are important messenger proteins, regulating the inflammatory and immunological responses of the host towards invading bacteria, viruses and so on. Interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) are so-called pro-inflammatory cytokines, that is they are capable of inducing or enhancing an inflammatory response directed to the tumor site. *In vitro* IL-6 expression was induced at both the mRNA and protein level of Hela epithelial cells (29) and murine macrophages expressed TNF-α upon stimulation by PDT (30). PDT can affect the expression of IL-6 and IL-10 of tumor and normal tissues *in vivo* (31). In the urine of patients undergoing PDT of the bladder enhanced levels of IL-1β, IL-2, and TNF-α were found as compared to healthy control subjects (32) Apparently, PDT is capable of inducing the expression or enhancing the levels of several cytokines.

Induction of cellular responses after PDT has been documented as well. Within 5 min after the start of PII-based PDT of rat cremaster muscle adhesion of granulocytes to the vessel wall has been observed (17). In murine squamous carcinoma tumors this is followed by a rapid and massive accumulation of neutrophils after the start of PII-based PDT (33). It is likely that these phagocytes are present at the inflammatory site to remove tumor cell debris caused by PDT. Whether this is the only role of neutrophils in the PDT-induced tumor cell death

remains unknown. Later, other immune effector cells like lymphocytes and monocytes/macrophages are recruited to the lesion. The latter cells are tumoricidal by themselves. Both *in vitro* (34) and *in vivo* (33,35) experiments have shown that PDT can potentiate this function.

Macrophages were reported to preferentially recognize PDT treated cancer cells as their targets (36). After phagocytosis and processing of tumor cell debris these macrophages function as so-called antigenpresentingcells (APCs). Upon presentation of tumor associated proteins in the context of MHC II molecules on the membranes of APCs, $CD4^+$ T-lymphocytes may recognize the antigens, become activated and start to proliferate. These lymphocytes in turn will enhance the proliferation of activated $CD8^+$ T-lymphocytes. This is of course a desirable situation since systemic immunity may lead to the eradication of untreated tumors as well. Severe combined immunodeficient (SCID) or nude mice showed no complete cure from sarcoma tumors by PDT alone. However, after the transplantation of bone marrow from immunocompetent BALB/c mice cured by PDT a complete remission occurred after PDT (37). This was also the case when spleen cells from BALB/c mice cured from sarcoma tumors were adoptively transferred to SCID mice (27). Spleen cells from BALB/c mice cured from other tumors or virgin spleen cells did not have that ability. These experiments suggest the involvement of memory (T) cells sensitized to the tumor treated by PDT. Evidence for the involvement of other specific immune cells like B-lymphocytes or natural killer cells is still scantly at the moment.

On the basis of the acquired knowledge about the mechanisms underlying the indirect cell kill of PDT the question was studied if the efficacy of PDT could be improved by enhancing the tumor directed immune response. These studies include the stimulation of the non-specific immune response after hematoporphyrin derivative-sensitized-phototherapy of murine transitional cell carcinomas by injection of *Corynebacterium parvum* (38), or *Bacillus Calmette-Guérin* (39). Mycobacterium cell-wall extract was used to increase the curative effect of PDT on murine EMT6 tumors (40), and the non-specific immunostimulator schizophyllan to improve the PDT treatment of squamous cell carcinomas (41).

All these strategies appear to improve the efficacy of PDT, but do have the disadvantage of being not specific. A more specific approach to potentiate the effect of PDT was directed to the activation of tumoricidal macrophages. This was achieved by the administration of granulocyte-macrophage colony-stimulating factor (GM-CSF; 42). This growth factor is a key regulator controlling maturation

and function of both granulocytes and monocytes/macrophages. Macrophages can also be activated by a specific macrophage-activating factor derived from vitamin D_3-binding protein. The systemic injection of this protein potentiated the curative effect of PDT on murine squamous cell carcinoma (43). Reports on cytokine use to stimulate the inflammatory reaction induced by PDT are scarce. So far only the administration of the pro-inflammatory recombinant human TNF-α has been reported to stimulate PDT (44).

Aim of this study

The finding that PDT evokes an inflammatory reaction in the tumor lesion provides the opportunity to increase the potency of this treatment modality by modulating the inflammatory response. In the past, this was achieved by stimulating the overall immune reaction by heat-killed bacteria or bacterial products, which indeed successfully enhanced the tumor cure rates of PDT. Obviously, tumor associated macrophages are the chief and final executioners of tumor cell death under those conditions. The significance of the observed adhesion of granulocytes and the consequences of this phenomenon for the efficacy of PDT however are less clear. The aim of this study was therefore to elucidate the mechanisms underlying the interaction between endothelial cells and granulocytes and to investigate the relevance of this interaction for the effect of PDT. This may lead to new ways for a specific stimulation of the potency of PDT.

Outline of this thesis

Chapter 2 describes the *in vitro* model used to study the interaction of endothelial cells and granulocytes. In this model, human umbilical vein endothelial cells grown to confluency on plastic culture dishes were treated by PDT before incubation with freshly isolated human granulocytes. The adhesion of granulocytes was studied both quantitatively and qualitatively. The contribution of various cellular adhesion molecules on both the endothelium and granulocytes to the observed adhesion was determined using inhibitive compounds. Furthermore, we questioned whether the activation status of the granulocytes used was altered and

was of relevance to the adhesion.

In chapter 3 the findings of the PDT-induced interaction of endothelial cells and granulocytes are extended in the context of the suitability of PDT to prevent restenosis after coronary angioplasty. In this chapter we studied if a decrease in the anti-adhesive ability of PDT treated endothelial cells underlied the increased adherence of neutrophils. To this end we determined the release of the anti-adhesive factors nitric oxide and prostacyclin. Furthermore, the expression of the neutrophil adhesion molecule P-selectin on endothelial cells and the release of the neutrophil adhesive protein von Willebrand factor by endothelial cells upon PDT treatment was investigated.

In chapter 4 the therapeutic significance of the granulocyte adherence after PDT was studied in an *in vivo* model using rats transplanted with rhabdomyosarcoma tumors. To this end two approaches were used: 1) a decrease in the number of circulating neutrophils in the blood of the tumor-bearing rats prior to PDT by the administration of anti-granulocyte antiserum, and 2) an increase of the number of circulating granulocytes prior PDT by the administration of granulocyte-colony stimulating factor (G-CSF). The effect of these adjunctive treatments on the tumor growth is described.

Chapter 5 extends the investigations in the *in vivo* model. It was observed that very early after the start of PDT granulocytosis occured. The mechanism underlying this increase in the number of granulocytes was studied by determining the time courses of the levels of the pro-inflammatory cytokines IL-1β and TNF-α in the circulation. Furthermore the role of G-CSF in the granulocytosis and tumor growth was investigated by the use of anti-G-CSF monoclonal antibodies.

Chapter 6 deals with the significance of the vascular response after PDT. Blood flow stasis appears indispensable for indirect tumor cell death upon PII-based PDT, since direct cell kill by the generation of singlet oxygen is limited because of the transient vessel constriction. In order to leave the blood vessels open during PDT, rats transplanted with rhabdomyosarcoma tumors were treated with anti thrombocyte antiserum prior or post PDT. The effect of this treatment on tumor response at two illumination protocols and the possible mechanism are described.

In chapter 7 the results of the present studies are summarized and discussed in the context of current concepts in the literature.

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CHAPTER 2

Photodynamic treatment of human endothelial cells promotes the adherence of neutrophils *in vitro*

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(British Journal of Cancer 73: 1335-1340, 1996)

Abstract

The effects of photodynamic treatment (PDT) on venules include vascular leakage accompanied by edema formation, vasoconstriction and blood flow stasis. The goal of this study was to gain insight into the mechanism underlying these vascular events by studying one of the earliest observations after PDT, that is granulocyte adhesion, in an *in vitro* model.

For this purpose human umbilical vein endothelial cells (HUVEC) preincubated with Photofrin II (PII) were illuminated with red light and incubated with neutrophils. PDT led to a dramatic change in the morphology of the endothelial cells. Clearly, neutrophils adhered to the subendothelial matrix and their adherence coincided with an increase in the percentage of exposed subendothelial matrix by the gradual contraction of endothelial cells. Furthermore, the increase in adherence was dependent on drug dose, illumination time and the time delay after PDT.

The neutrophil adherence could be inhibited by anti-β_2 integrin antibodies, which suggests that the α_L-, α_M- or α_X-β_2 receptors of the neutrophil mediated this phenomenon. At 4°C or by preincubation of the neutrophils with staurosporin their adherence to the subendothelial matrix exposed by PDT of endothelial cells could be prevented. Apparently, activation of the β_2-integrin receptor by interaction with the subendothelial matrix is necessary for the increased binding of neutrophils.

Taken together, these *in vitro* findings suggest that the PDT-induced contraction of the endothelial cells permits neutrophil adherence to the subendothelial matrix. It is conceivable that a similar mechanism contributes to the initial adherence of granulocytes to the vessel wall as observed after PDT *in vivo*.

Introduction

Photodynamic treatment is a relative new therapy for the treatment of various forms of cancer (1). The therapy involves the systemic administration of a photosensitizer followed, after some hours to days necessary for the relative accumulation of the sensitizer in the tumor, by the illumination of the tumor area with light of appropriate wavelength. At present Photofrin II® (PII), a mixture of haematoporphyrins is the only photosensitizer used with limited approval for use in cancer patients. Its illumination leads to the formation of highly reactive oxygen

species such as singlet oxygen (2,3). Singlet oxygen is involved in direct cell cytotoxicity by oxidation of the plasma membranes, mitochondria and lysosomes (4,5).

Besides this direct cell kill, PDT also is reported to mediate vasoconstriction and blood flow stasis (6). These events appear to be indispensable in the destruction of tumor tissue (7,8). One of the earliest events after PDT observed in rat cremaster muscle vessels (9) and rat skinfold vessels (own unpublished observation) is the adhesion of granulocytes to the vessel wall. Granulocytes play a key role in inflammatory reactions and these phagocytes therefore may also contribute to tumor destruction after PDT. Although the effect of PDT on endothelium has been the subject of many studies (10-14) the mechanism underlying the adherence of granulocytes to the endothelial lining is not known.

In this study we investigated in an *in vitro* model the adherence of neutrophils after PDT of endothelial cells in order to elucidate this phenomenon.

Materials and methods

Photosensitizer and drugs

The photosensitizer Photofrin II® (PII) was obtained from Quadra Logic Technologies Inc. (Vancouver, BC, Canada) and was reconstituted in 5% glucose before use. Mepacrine was from Sigma Chemical Co. (St. Louis, MO, USA). WEB 2086 was kindly provided by Boehringer Ingelheim (Ingelheim, Germany).

Monoclonal antibodies (MAb)

MAb to the β_1- (CD29), β_2- (CD18), and β_3- (CD 61) integrins and a mouse control IgG_1 MAb were purchased from Becton-Dickinson (San Jose, CA, USA).

Isolation and culture of endothelial cells

Endothelial cells were isolated and cultured according to previously described methods (15) with minor adaptations. In short, the cells were isolated from umbilical cords which were kept in cord buffer (140 mM NaCl, 4 mM KCl, 11 mM D-glucose, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.3). A vein was

cannulated and rinsed with cord buffer before endothelial cells were detached by 20 min incubation at 37°C in 0.1% collagenase (Sigma Chemical Co., St. Louis, MO, USA) in M199 medium (Flow Laboratories, Irvine, Scotland). Cells were collected by perfusion with M199 and centrifugation (400 x g, 10 min) and next resuspended in culture medium: M199 medium supplemented with 10% pooled human serum (Red Cross Bloodbank, Rotterdam, The Netherlands), 10% fetal calf serum (Boehringer Mannheim, Mannheim, Germany), 175 μg/ml endothelial cell growth factor isolated as previously described (16), 840 μg/ml $NaHCO_3$, 15 U/ml heparin (Leo Pharmaceutical Products, Weesp, The Netherlands) and 50 U/ml penicillin and 50 μg/ml streptomycin (Boehringer Mannheim, Mannheim, Germany) in 25 cm^2 culture flasks precoated for 30 min at room temperature with 10 μg/ml fibronectin isolated as previously described (17). Endothelial cells were identified by well-accepted methods (15). When grown to confluence the cells were detached with trypsin/EDTA (Gibco, Breda, The Netherlands) and subcultured in fibronectin-precoated 96- or 24-wells culture plates or were stored in liquid nitrogen until use. For experiments, only confluent monolayers between passage 1 to 6 at least three days after subculture were used.

Isolation of neutrophils

Neutrophils were isolated from fresh citrated human blood (kindly provided by the Red Cross Bloodbank, Rotterdam, The Netherlands). In short, blood cells were diluted two times in PBS and separated by density gradient centrifugation (800 x g, 20 min at room temperature) over isotonic Lymphoprep (9.6% sodium metrizoate and 5.6% Ficoll; density 1.077 g/ml; Nycomed, Oslo, Norway). The pellet fraction, containing erythrocytes and granulocytes was treated twice with ice-cold isotonic ammonium-solution (155 mM NH_4Cl, 10 mM $NaHCO_3$ and 0.1 mM EDTA) to lyse the erythrocytes. The remaining granulocytes were washed with PBS and resuspended in PBS. In general this fraction contained approximately 95 to 100% granulocytes, of which the majority (94%) were neutrophils.

PDT protocol

Endothelial cells in 24- or 96-wells culture plates were incubated with PII at a concentration of 25 μg/ml (unless stated otherwise) in culture medium for 20 h at 37°C, 5% CO_2 and 100% humidity. This concentration equals the initial plasma level of PII in patients after injection of 2 mg/kg. Next, cells were washed three times and

suspended in Krebs-Ringer bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 1.0 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, and 5.5 mM glucose; pH 7.3). For light treatment culture plates were illuminated in a mirror box for 15 min (unless stated otherwise). Red light was delivered by a slide projector with a 250-W lamp (type 7748S EHJ, Philips, Eindhoven, The Netherlands) and a cut-off filter (< 610 nm, #59512, Oriel Co., Stratford, CT, USA). Average fluence rate was measured in the culture wells with an isotropic light detector and amounted to 4 mW/cm^2, which leads to 3.6 J/cm^2 after 15 min of illumination. After illumination the endothelial cells were incubated at 37°C, 5% CO_2 and 100% humidity for 30 min (unless stated otherwise) and then freshly isolated neutrophils were added. After 10 min of incubation (unless stated otherwise) the culture plates were washed three times with ice-cold PBS to remove the non-adherent cells.

Determination of neutrophil adherence

Myelo-peroxidase (MPO) was used as an enzymatic marker to quantify the number of adherent neutrophils according to previously described methods (18). Briefly, the adherent neutrophils were lysed during 15 min at 4°C with 0.5% hexadecyltrimethylammonium-bromide (HTAB) in PBS (pH 6.0). The amount of MPO activity in the lysate, which reflects the number of adherent neutrophils was determined by a colorimetric assay using o-dianisidine dihydrochloride (0.2 mg/ml) and H_2O_2 (2 mM) in PBS (pH 6.0). The change in absorbance at 450 nm was followed during 15 min at 37°C in a Thermomax microplate reader (Sopar Biochem, Nieuwegein, The Netherlands). The number of adherent neutrophils was determined from the maximal velocity by interpolating from a standard concentration curve and the adherence expressed as the percentage of the total number of added neutrophils.

Determination of lactate dehydrogenase (LDH)

Homogenates of HUVEC were obtained by sonification during 5 min on ice of the endothelial monolayer in phosphate buffer (100 mM KH_2PO_4 and 100 mM Na_2HPO_4; pH 7.0). In both homogenates and culture supernatants LDH-activity was determined by a colorimetric assay with pyruvate (5.75 mg/ml) and NADH (4.7 mg/ml) as substrates. Absorbance was read at 340 nm with a Thermomax microplate reader.

Measurements of porphyrin levels

Porphyrin levels in endothelial cells were determined as previously described for animal tissues (6). Endothelial cells were lysed with 0.1 M NaOH. Porphyrins in the lysate were hydrolysed and extracted by adding 2% sodium dodecyl sulphate (SDS) followed by heating (100°C) during 15 min. After centrifugation (1500 x g, 15 min) fluorescence intensity in the supernatant was measured in a fluorescence spectrophotometer (MPF-3, Perkin Elmer Corporation, Norwalk, CN, USA) at an excitation wavelength of 404 nm and an emission wavelength of 627 nm. Fluorescence peaks were compared with standards of known concentrations of PII in 2% SDS and 0.1 M NaOH to calculate the amount of porphyrins retained in the endothelial cells.

Determination of the exposed surface area

To determine the size of the exposed area of the subendothelial matrix time-lapse pictures after PDT of the endothelium were analyzed by measuring the surface area of the endothelial cells in relation to the total area. This was performed with the software-drafting package Autosketch 2.0 obtained from Autodesk Inc. (Sausalito, CA, USA).

Statistical Analysis

Data are presented as means ± s.d. of triplicate experiments (unless otherwise stated) and were analysed using multiple regression analysis, Student's t-test or analysis of variance (ANOVA) with Bonferroni's correction where appropriate. Differences between group-means were considered significant when $P<0.05$.

Results

Effect of PDT of endothelial cells on the adhesion of neutrophils

To elucidate the mechanism underlying the increased adherence of granulocytes after PDT we investigated the effect of PDT of endothelial cells on the adherence of blood neutrophils *in vitro*. For this purpose neutrophils were added for 10 min at

various time-delays after illumination (15 min of red light) of PII-treated (25 μg/ml for 20 h) HUVEC. The results show that the adherence of neutrophils increased linearly (R^2=0.850, P<0.0001) with time to a maximum at a time delay of 30 min before neutrophil addition and then remained at that increased level up to the end of the observation period (Figure 1). Pretreatment of endothelial cells with PII or light only had no significant effect on the adherence of neutrophils as compared to untreated HUVEC.

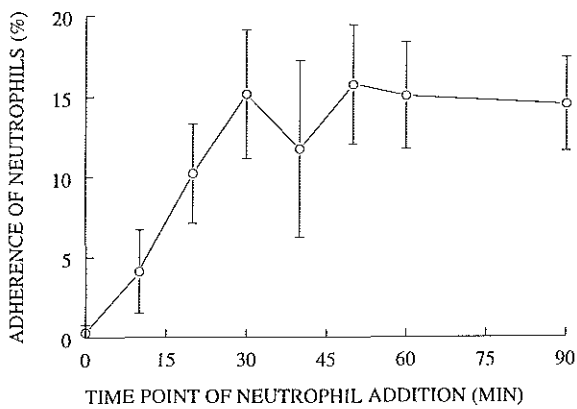

Figure 1. Relationship between the adherence of neutrophils and the time delay of their addition after PDT of HUVEC. After their addition neutrophils were allowed to adhere for 10 min. Next, non-adherent cells were removed. The adherence is expressed as the percentage bound neutrophils corrected for the adherence after treatment of HUVEC by red light only. The data represent the mean ± s.d. of three separate experiments with determinations in triplicate.

To study if this increased adherence was drug dose-dependent, HUVEC were preincubated with various concentrations of PII for 20 h and thereafter illuminated for 15 min. Neutrophils were added after a time delay of 30 min, which is sufficient for maximal adherence (cf. Figure 1). As shown in Figure 2 there was a linear relationship between PII-dose and neutrophil adhesion (R^2=0.962, P<0.0001). To investigate whether this drug dose-dependent effect of PDT on the neutrophil adherence was directly associated with the amount of PII in HUVEC the porphyrin

concentration retained after 20 h of PII incubation in serum-supplemented M199 was determined (Figure 2). A linear relationship between the incubation dose and the cellular porphyrin content was found (R^2=0.94, P<0.0001). Approximately 0.3 to 0.6% percent of the amount of administered PII was retained by the cells.

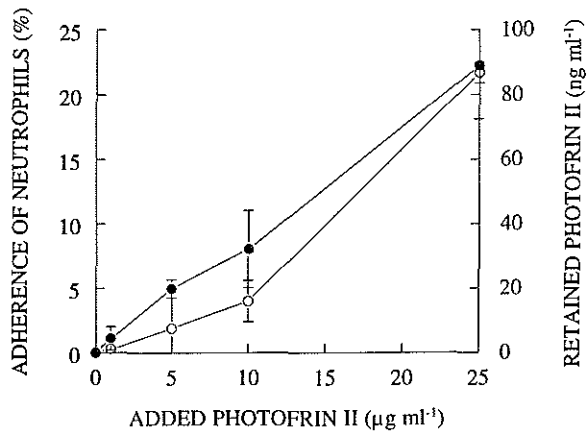

Figure 2. Relationship between the amount of PII in the endothelial culture medium (abscissa), the amount of PII retained by the endothelial cells (closed circle; right ordinate) and the adherence of neutrophils after PDT of the endothelium (open circle; left ordinate). The adherence of neutrophils was corrected for the adherence after treatment of HUVEC by red light only. The data represent the mean ± s.d. of three separate experiments with determinations in triplicate.

To evaluate if the effect of PDT was dependent on the light-energy dose HUVEC were illuminated for various times after treatment for 20 h with a fixed dose of PII (25 μg/ml). Neutrophils were added after a delay of 30 min after the end of illumination. Upto 20 min of illumination, which is equivalent to 4.8 J/cm^2, we found a linear relationship between the illumination time and the adherence of neutrophils (Figure 3; R^2=0.662, P<0.0001). The adherence reached a maximum at 20 min of illumination. Longer illumination times did not lead to further increment of neutrophil adherence.

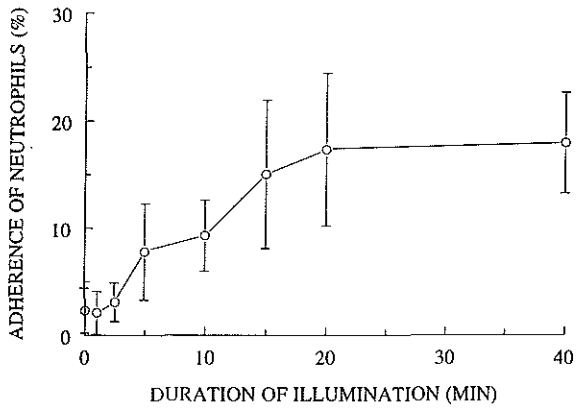

Figure 3. Relationship between the adherence of neutrophils and the red light dose (illumination time) used for PDT of HUVEC. After various periods of illumination the neutrophils were added 30 min later and allowed to adhere for 10 min. The adherence of neutrophils was corrected for the adherence after treatment of HUVEC by red light only. The data represent the mean ± SD of three separate experiments with determinations in triplicate.

Mechanism of adhesion

Light microscopic study of the endothelial cells at 30 min after PDT (cf. Figure 1) showed that the morphology of the endothelial cell was dramatically altered as compared to control HUVEC (Figure 4a,b). Retraction of the endothelial cells and formation of large membrane vesicles was observed resulting in the exposure of a large area of the subendothelial matrix. The adherent neutrophils were mainly associated with this exposed matrix rather than the contracted endothelial cells (Figure 4c). To examine whether a decrease in membrane integrity of HUVEC occurred after this PDT protocol, the release of LDH was determined 30 min post PDT (Table 1). We found no significant increase in LDH-release which indicates that the membrane integrity of the endothelial cells was not severly affected at that time.

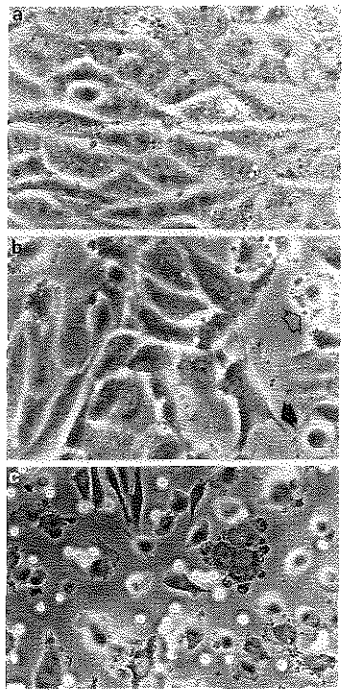

Figure 4. Effect of PDT on the morphology of endothelial cells and the adherence of neutrophils. Endothelial cells were photographed at 30 min after red light treatment only (A) or 30 min after PDT (B) or 30 min after PDT followed by addition of neutrophils for 10 min (C). Note the presence of large membrane vesicles (open arrow) and "pseudopodia" (closed arrow) after PDT of HUVEC. Magnification A,B,C: x 200.

Table 1. Effect of photodynamic treatment (PDT) of human umbilical vein endothelial cells (HUVEC) on the release of lactate dehydrogenase (LDH).

Condition	*% LDH in HUVEC*	*% LDH in supernatant*
Untreated	91.8 ± 2.7	8.2 ± 2.7
PII only	89.3 ± 4.5	10.7 ± 4.5
Red light only	88.0 ± 4.8	12.0 ± 4.8
PDT	85.1 ± 7.1	14.9 ± 7.1

Data are the means ± SD of three experiments. The percentage of LDH was calculated from the total amount in supernatant and cells. Measurements were performed in triplicate.

To determine whether the extent of exposure of the subendothelial matrix was related to the increased adherence of neutrophils pictures were taken at various time delays after PDT to measure the exposed matrix. As shown in Figure 5 there was an increase in the percentage exposed area from 20% at 10 min to approximately 65% at 40 min after PDT.

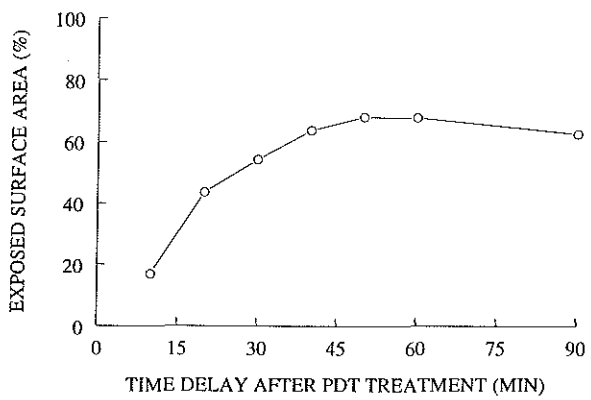

Figure 5. Time course of exposure of the subendothelial matrix after PDT of HUVEC. The percentage exposed surface was determined as described under material and methods.

To study whether the adhesiveness of the matrix for neutrophils was dependent on a direct effect of PDT, the contraction of the endothelial cells was induced by calcium-free buffer (PBS) instead of PDT. The results show that the increase in neutrophil adherence to this matrix was similar in magnitude as to the matrix exposed by PDT-induced contraction of the endothelial cells (Figure 6), showing that the adhesiveness of the subendothelial matrix per se does not depend on PDT. Furthermore, we found that PDT of fibronectin, which we used to coat the endothelial culture wells, did not lead to an increase in its adhesive properties for neutrophils (Figure 6). To investigate which type of membrane receptor is involved in the increased adherence neutrophils were preincubated for 30 min with blocking MAb to members of the β_1,- β_2- or β_3-integrin adhesion receptor family (Figure 7). Preincubation with anti-β_1 (CD29) or anti-β_3 (CD61) MAb did not influence neutrophil adherence as compared to control MAb. However, incubation of neutrophils with Mab to β_2-integrin (CD18) blocked their adherence substantially.

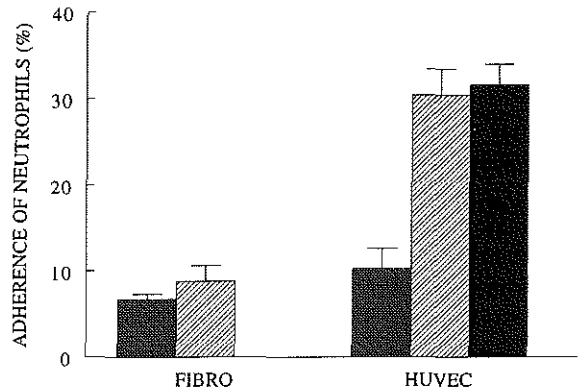

Figure 6. Adherence of neutrophils to the subendothelial matrix. Neutrophils were added after red light only (dark-grey bar) or PDT (light-grey bar) treatment of fibronectin (FIBRO) or endothelial monolayer (HUVEC) or treatment of the monolayer with calcium-free PBS (black bar). Each bar represents the mean ± SD of three determinations.

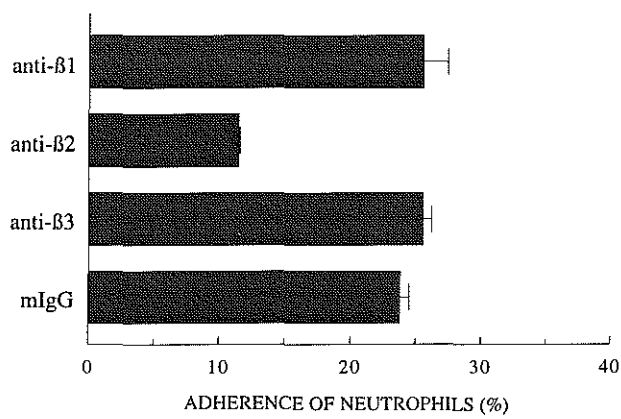

Figure 7. Effect of monoclonal antibodies (MAb) to isotypes of the integrin receptor on the adherence of neutrophils after PDT of HUVEC. Neutrophils were preincubated for 30 min at 4°C with 10 µg/ml of the MAb under investigation and then added to HUVEC 30 min post PDT for 10 min at 37°C in the presence of that MAb. Each bar represents the mean ± SD of three determinations.

Since it is known that efficient binding of a leucocyte to its ligand depends on a protein kinase C-dependent phosphorylation of the β_2-receptor (19), we studied whether intracellular signaling via protein kinases was involved in the increased adherence of neutrophils under the present conditions as well. We found that at 4°C neutrophils failed to adhere (0% ± 0%, not shown). Furthermore, preincubation of neutrophils with staurosporin, a protein kinase inhibitor, prevented their adherence in a concentration-dependent fashion (Figure 8). At the highest concentration of staurosporin, which almost completely prevented the neutrophil adherence, the viability of neutrophils during the experiment was not affected as monitored by trypan blue exclusion.

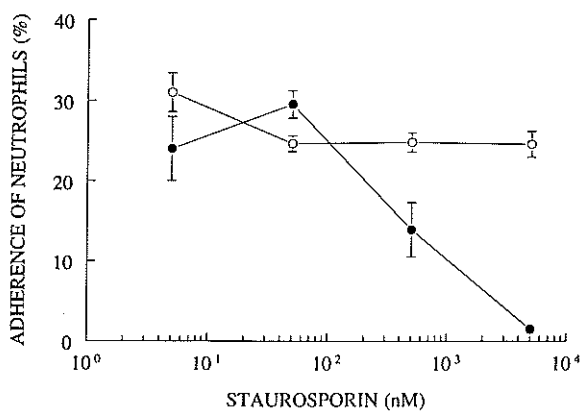

Figure 8. Effect of staurosporin on the adherence of neutrophils. Neutrophils were preincubated for 10 min at 4°C with various concentrations of staurosporin (closed circle) or solvent (DMSO) only (open circle) and then added to HUVEC 30 min post PDT for 10 min at 37°C in the presence of staurosporin or DMSO. The data represent the mean ± SD of three determinations.

Discussion

The major finding of this study was that PDT of endothelial cells *in vitro* led to an increased adherence of neutrophils to the subendothelial matrix. This adherence was dependent on the PII dose, the illumination time and timecourse after PDT. The gradual increase in neutrophil adherence coincided with a gradual exposure of the subendothelial matrix (ECM) due to contraction of the endothelial cells.

The endothelium after PDT showed a striking resemblance with the morphology of endothelial cells treated by tert-butylhydroperoxide (t-BuOOH), a lipophilic reactive oxygen species, namely extensively contracted cells with large membrane vesicles (20). Patel *et al.* (20) showed that the vesicles eventually pinched off and contained PAF-like molecules that stimulated the adherence of granulocytes to gelatin. PII is a photosensitizer which is also lipophilic and therefore accumulates in the cell membrane. As a result of illumination of the photosensitizer highly reactive oxygen species are formed. These may generate PAF or PAF-like molecules that could be responsible for the increased adherence of neutrophils to the exposed subendothelial matrix. However, we found that preincubation of neutrophils for 10 min with 10^{-4} to 10^{-8} M WEB 2086, a synthetic PAF antagonist, did not inhibit their adherence 30 min after PDT of HUVEC (not shown). Preincubation of HUVEC for 10 min with 10^{-5} to 10^{-8} M mepacrine, a phospholipase A2 inhibitor that prevents the synthesis of PAF, did not inhibit neutrophil adherence either (not shown). This indicates that the adherence of neutrophils under the present conditions was not mediated by membrane-bound PAF or PAF-like molecules.

The use of blocking antibodies to three isotypes of the integrin family of adhesion receptors show that β_2-integrins on the neutrophil membrane are involved in their adherence. Others have shown that a PKC-dependent phosphorylation of the cytoplasmic domain of the β_2-integrin receptors of leukocytes is necessary for binding to their ligands (19). We found that at 4°C and after preincubation of the neutrophils with staurosporin the adherence to the exposed subendothelial matrix was reduced. This indicates that a protein kinase-dependent activation of the β_2-receptor is also necessary for binding of neutrophils to the subendothelial matrix exposed as a result of PDT of endothelial cells. *In vivo* also, granulocytes were found to adhere to spaces between the endothelial cells after PDT of rat cremaster muscle vessels (9). Whether activated β_2-integrins are involved here as well, remains to be established.

The ECM produced by endothelial cells in culture at the baso-lateral side consists of various matrix proteins like collagens (types I, III and IV), proteoglycans (mostly heparan and dermatan sulphate proteoglycans), laminin, fibronectin, and elastin. Several of these ECM-proteins have been shown to be involved in the adherence of neutrophils (21). Which type of ECM-protein is involved here is not known as yet.

Taken together, we found evidence as to the mechanism of the increased adherence of neutrophils after PDT of endothelial cells. Upon this treatment endothelial cells contract exposing the ECM. Neutrophils adhere to the ECM by their β_2-integrin receptors which possibly become activated. It is conceivable that the

contraction of endothelial cells induced by PDT contributes to the granulocyte adherence as found *in vivo* as well. Whether those phagocytes play a role in further vascular collapse and tumour regression after PDT remains to be established.

Acknowledgments

The authors thank W. Star and H. Marijnissen (Dr. Daniel den Hoed Cancer Centre, Rotterdam) for their advice on light fluency measurements. This investigation was supported by the Dutch Cancer Society Grant EUR 91-01 and a donation from Mrs. E.C. Bakker-Grieszmayer.

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CHAPTER 3

Prevention of late lumen loss after coronary angioplasty by photodynamic therapy: Role of activated neutrophils

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(Molecular and Cellular Biochemistry 157: 233-238, 1996)

Abstract

Restenosis after coronary angioplasty arises from fibrocellular intimal hyperplasia and possibly failure of the artery to enlarge adequately. Which mechanisms underlie this process is only partly understood. No drugs have been clinically effective in reducing the incidence of restenosis. Since recently, photodynamic therapy (PDT) is being investigated as a possible treatment for intimal hyperplasia. PDT involves the systemic administration of a light-excitable photosensitizer that is accumulated at a high rate by rapidly proliferating cells. During laser irradiation light energy is transferred from the photosensitizer to oxygen generating the highly reactive singlet oxygen. This potent oxidizer can cause severe cellular damage. After PDT of a balloon-injured artery from the rat and rabbit the media remained acellular for several weeks to months, and intimal hyperplasia did not occur. The endothelial lining regenerated by two weeks, but why smooth muscle cells did not repopulated the media is not known.

Neutrophils seem to play an important role in the prevention of restenosis after coronary angioplasty, since the activation status of this type of phagocyte is directly related to vessel diameter at late follow-up. Furthermore, it has been observed that neutrophils adhere to the microvascular wall upon PDT *in vivo*. *In vitro* findings suggest that the increased neutrophil adherence was not dependent on a decreased release of the anti-adhesive factors NO and prostacyclin by the PDT-treated endothelial cells. Furthermore, PDT did not stimulate the expression of P-selectin by the endothelial cells, one of the adhesion receptors for neutrophils. The endothelial cells only retract upon PDT allowing the adherence of neutrophils by their β_2-integrin adhesion receptors to the subendothelial matrix. On the basis of these findings, we presume that the successful prevention of intimal hyperplasia by PDT partly depends on the presence of the neutrophil at the site of the lesion.

Introduction

Percutaneous transluminal coronary angioplasty (PTCA) was first applied clinically in the late 1970s by Gruentzig (1) to alleviate the symptoms of coronary atherosclerosis. This technique has developed into a major therapeutic tool in the treatment of single and multiple vessel coronary artery disease. PTCA involves the

placement and inflation of a balloon within a very localized segment of a coronary artery. Because PTCA is always performed in the presence of pre-existing disease, successful recanalization of the obstructed vessel is accompanied by plaque fissure, and frequently results in longitudinal intimal tears and tangential splitting of the tunica media (2).

This form of vascular injury is followed by a healing process that is characterized by intimal and medial remodelling, endothelial cell regrowth, medial and intimal cell proliferation and increased synthesis of extracellular matrix (2,3). Excessive response results in recurrent stenosis that compromised this treatment modality in about 30% of the cases (4). This has prompted a large array of new devices to improve final outcome, e.g., atherectomy devices, laser angioplasty, mechanical scaffolding devices, and local drug-delivery systems (5). However, while the acute gain defined as the increment in minimal lumen diameter achieved at the end of the procedure increased, the interest in the net gain after mechanical and subsequent biologic remodelling at follow-up angiography was only marginally better. This indicates that the large acute gain obtained by those new devices evokes a very strong wound healing response that leads to a great late lumen loss.

Numerous pharmacological agents have already been used effectively to inhibit this process of intimal hyperplasia in preclinical animal models (6). Based on the intended mode of action, these agents can be categorized into drugs that reduce recoil and spasm, inhibit platelet activation and thrombus formation, antagonize growth factors, inhibit (smooth muscle) cell proliferation, and prevent inflammation. However, in man no agent was found unequivocally effective as yet, although fish oil, and trapidil, a platelet-derived growth factor antagonist, and molsidomine, an inducer of nitric oxide release by endothelial cells, may have some favourable influence (7-9). The lack of efficient agents raises the question as to an explanation for all those failures.

Unfortunately, we can only speculate why clinical trials were unsuccessful as yet. It could be relevant that the drug dose used in a number of trials was smaller than in the respective animal model (9). However, more importantly, it is questionable whether the animal models for restenosis really meet the human situation. It is noteworthy in this respect that, except for the Watanabe hypercholesterolemic rabbit (10), atherosclerosis in animals does not occur (9) and intimal hyperplasia is evoked by mechanical injury of an otherwise healthy artery.

In the human situation other processes than wound healing may also account for restenosis considering established clinical risk factors, e.g., family history of coronary artery disease, unstable angina, current smoking and diabetes, that

determine if clinical restenosis occurs or not. One such process is the occurrence of compensatory reactions under pathological conditions. Recently, it became clear that in animal models (11) and in man (12) the diseased blood vessel increased in diameter in response to (partial) obstruction. However, if the artery fails to enlarge adequately in diameter to compensate for the intimal hyperplasia, the vessel will occlude. Which mechanisms underlie the process of vascular remodelling is not known. It could be that the adaptive enlargement is initiated by the increased wall shear stress that results from the initial stenosis (13). Alternatively, the support structure of the arterial wall is involuted by the developing plaque (11,12) or the release of proteolytic enzymes from inflammatory cells that accumulate in the lesion in response to injury (11). Therefore, an alternative strategy to prevent restenosis after angioplasty may be to promote the compensatory processes. Until this approach is successful, this leaves us with the option to knock-out the responsible (medial) smooth muscle cells at the site of the lesion directly.

Photodynamic therapy of intimal hyperplasia

The finding of Spears et al. (14) that porphyrins accumulate in atheromatous plaques and the observation that the photosensitizers are mainly taken up by rapidly proliferating and activated cells (15) focused the attention on photodynamic therapy (PDT) as a possible (adjunct) treatment modality for atherosclerosis (16-18) and restenosis (19-23). PDT has been developed as a treatment modality for malignancies and involves the cytocidal activity of a photosensitive dye after its activation by visible light (24). If the photosensitizer is excited, most commonly its energy is transferred via the photochemical type II reaction to oxygen which generates highly reactive singlet oxygen (25). Singlet oxygen is electrophilic and reacts with electron-rich regions of biomolecules causing photodegradation of lipids, proteins, and nucleic acids, respectively, depending on the localization of the photosensitizer in the cell (26,27). The first photosensitizer with limited approval for clinical use is porfimer sodium (Photofrin®), a mixture of oligomeric esters and ethers of hematoporphyrin and enriched in the photodynamically active fraction (24). Impressive complete response rates of various types of cancer have been obtained in Phase III trials including early stages of cancer of the lung, stomach, bladder and cervix (24). An inconvenient drawback of Photofrin is that it induces a transient skin photosensitivity. To avoid this problem and to improve treatment depth of PDT,

alternative so called second-generation photosensitizers have been developed such as phthalocyanines, chlorins, purpurins, bacteriochlorins, verdins and protoporphyrin IX endogenously produced from exogenously administered 5-aminolevulinic acid, which currently enter clinical trials (28,29).

The suitability of PDT for the prevention of restenosis has been studied so far in the balloon-injured rat carotid artery model using chloroaluminium sulphonated phthalocyanine (19,22) and 5-aminolevulinic acid induced protoporphyrin IX (23) as the photosensitizer. In the balloon-injured rabbit carotid (20) and iliac (21) artery model the photosensitizers Photofrin and hematoporphyrin derivative have been used, respectively. The consistent finding from these studies is that PDT (almost) completely inhibited intimal hyperplasia. The endothelial lining regenerated by two weeks after treatment, but the absence of smooth muscle cells in the treated media was striking. Despite the acellular media, the arterial segment remained patent (but non-contractile; 22), possibly due to the (singlet oxygen-mediated) crosslinking of collagen fibers of the matrix (30). Interestingly, recent evidence suggests that the addition of fluoride to chloroaluminium phthalocyanine makes PDT with this potent photosensitizer highly selective for smooth muscle cells without affecting endothelial cells (31).

One of the earliest events after PDT treatment *in vivo* is the adhesion of granulocytes to the vessel wall (32). The mechanism underlying the adherence of granulocytes to the endothelial lining is not known. We found in an *in vitro* study that endothelial cells retract after PDT exposing the subendothelial matrix to which neutrophils readily adhere (33). The endothelial cells remain viable at least during the observation period of 30 min, and we show here that the constitutive release of the anti-adhesive factors NO and prostacyclin is not significantly decreased (Table 1).

This latter finding seems in contrast with the study of Gilissen et al (34). They found that the endothelium-dependent relaxation of PDT-treated blood vessels in response to vasodilators such as acetylcholine decreased, while the endothelial lining of the vessel wall remained intact. The smooth muscle cells responded normally to administration of the exogenous NO donor nitroprusside. Apparently, PDT did decrease the release of NO by impairment of the muscarinic receptor for acetylcholine in the endothelial plasma membrane leaving the NO synthase itself intact.

Table 1. Effect of Photofrin-PDT of Human Umbilical Vein Endothelial Cells on the Release of Nitric Oxide and Prostacyclin[1]

Condition[2]	NO (μM)	PGI_2 (pg/ml)
Untreated	4.1 ± 0.5	49.3 ± 5.3
Photofrin only	4.0 ± 0.4	32.7 ± 1.3
Red light only	4.3 ± 0.5	50.7 ± 11.7
Photofrin-PDT	4.2 ± 0.4	32.5 ± 3.9

[1] Data are the means ± SD of five (NO) or three (PGI_2) experiments. Assay of the supernatant was performed in triplicate (NO) or duplicate (PGI_2).

[2] Endothelial cells were incubated for 20 hr with 25 μg/ml Photofrin (Photofrin only; Photofrin-PDT) and/or illuminated in a mirror box during 15 min with red light, which equals to 3.6 J/cm^2, delivered by a slide projector with a 250-W lamp (type 7748S EHJ, Philips, Eindhoven, The Netherlands) and a cut-off filter (<610 nm, #59512, Oriel Co., Stratford, CT) (Photofrin-PDT; red light only), or left untreated. Next, the endothelial cells were incubated for 30 min, after which the NO levels in the supernatant were assayed from the amount of nitrite using Griesz reagent, and the PGI_2 levels from the amount of 6-keto prostaglandin $F_{1\alpha}$, the stable hydrolysis product of PGI_2, which was determined with an enzyme immunoassay kit (Cayman Chemical Company, Ann Harbor, MI).

It was observed that the neutrophils adhere to the subendothelial matrix exposed by PDT-treated endothelial cells via their β_2-integrin receptors, which were subsequently activated by a direct interaction with matrix proteins (33). The endothelial cells probably do not mediate this activation, since we show here that P-selectin, the most likely candidate of the endothelial membrane receptors that can activate the β_2-integrin receptor of the neutrophil (35), was not upregulated by PDT-treatment (Figure 1). A photosensitized release of von Willebrand factor from human umbilical vein endothelial cells has previously been shown to occur after 1 hr (36) and this is accompanied by a rapid distribution (without de novo synthesis) to the cell membrane of P-selectin (37). However, von Willebrand factor was not detected within 30 min after PDT either (Figure 1), while histamine stimulated both the release of von Willebrand factor and the expression of P-selectin considerably after 30 min (Figure 1). PDT also did not stimulate the expression E-selectin and members of the immunoglobulin superfamily, including ICAM-1 and -2, PECAM-1 and VCAM-1 (not shown). Since an increased expression of these cellular adhesion molecules is dependent on de novo synthesis,

this finding is not surprising with respect to the limited time frame of our study.

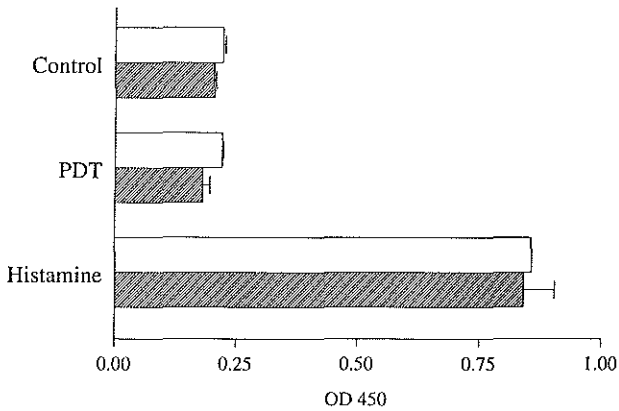

Figure 1. Effect of Photofrin-PDT (25 μg/ml Photofrin for 20 hr, followed by illumination in a mirror box during 15 min with red light, which equals to 3.6 J/cm^2, delivered by a slide projector with a 250-W lamp (type 7748S EHJ, Philips, Eindhoven, The Netherlands) and a cut-off filter (<610 nm, #59512, Oriel Co., Stratford, CT), and histamine (10^{-5} M) on the release of von Willebrand factor (white bar) and the expression of P-selectin (grey bar) assayed by ELISA after 30 min by human umbilical vein endothelial cells. Each bar represents the mean ± SD of three determinations.

Taken together, this indicates that PDT-treated endothelial cells only play a passive role in the increased adherence of neutrophils to the subendothelial matrix. What is the relevance of this finding for the PDT treatment of balloon-injured coronary arteries? In the next section we will show that neutrophils may have an important role in tissue healing after coronary angioplasty.

Role of neutrophils in decreasing late lumen loss

Some years ago, Libby et al (38) proposed an important role of blood monocytes in the cascade of events that lead to smooth muscle migration and proliferation into the intima. By the early acute release of cytokines those phagocytes evoke a secondary cytokine and growth factor response from other types of cell in the lesion.

This might establish a positive, self-stimulatory autocrine and paracrine feedback loop amplifying and sustaining the proliferative response of the smooth muscle cells.

Neutrophils are first-line defenders against invading pathogens. Since the early 1980s it became known that neutrophils also mediate tissue destruction in inflammatory diseases such as ischemic heart disease (39). Considering this, it was anticipated that neutrophils have a potential bearing in the vascular lesion after angioplasty. That prompted us to study the relationship between the late lumen loss and the activation state of both types of phagocyte before angioplasty.

The results showed that the amount of interleukin(IL)-1β that was synthesized by the patients' monocytes upon stimulation *in vitro* is positively associated with the extent of late lumen loss (40). This characteristic is genetically determined (41) and its magnitude depends also on the primed state of the monocyte (42). Surprisingly, the activation state of neutrophils as reflected by an increased expression of the membrane receptors CD64 and CD66, was inversely associated with late lumen loss (40). This indicated that activated neutrophils in fact could serve a beneficial role in tissue healing after PTCA. We can only speculate on the relevant factors in this process. CD64 is the high affinity receptor for immunoglobulin G. It was found recently that the CD66 receptor serves as a presenter molecule of the sialylated Lewis(x) antigen, which binds to the endothelial leucocyte adhesion molecule-1 on cytokine-activated endothelial cells (43). Therefore, an increased expression of the CD66 receptor may be of particular interest for the accumulation of the neutrophils at the site of the lesion. Neutrophils can generate 6-keto-prostaglandin-E_1 and 13-hydroxyoctadecadienoic acid, which are inhibitors of platelet aggregation and platelet adhesion, respectively (44). By the release of these inhibitors local thrombosis after PTCA may be prevented, which is considered as a key event in the initiation of intimal hyperplasia (38). Furthermore, neutrophils may also contribute to hydrolysis of extracellular matrix components and subsequent compensatory vascular remodelling by the release of (metallo)proteinases.

Conclusion

An important clue why PDT so effectively inhibits intimal hyperplasia may come from the observation that activated neutrophils prevent late lumen loss after coronary angioplasty (40). Clearly, after PDT of the diseased vessel all smooth muscle cells in the media were killed, but why the media did not become repopulated is a matter of

speculation. We found that after PDT neutrophils rapidly adhere to the subendothelial matrix of the vessel wall (33). These phagocytes can generate potent inhibitors of thrombosis thereby preventing the release of chemotactic factors for smooth muscle cells from activated platelets (44). On the other hand (metallo)proteinases released by these neutrophils can hydrolyse extracellular matrix components of crucial importance for the migration of smooth muscle cells into the media.

A seeming contradiction for an important role of neutrophils is the observation that inflammatory cells were absent from day 7 after PDT-treatment of the balloon-injured artery (22). However, since the acute inflammatory reaction in response to vascular injury lasts only for 2-4 days (3), it is not surprising that no inflammatory cells were seen at day 7.

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CHAPTER 4

Evidence for an important role of neutrophils in the efficacy of photodynamic therapy *in vivo*

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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 (G-CSF), respectively. After administration of the antiserum the number of circulating neutrophils decreased by 99.9%. However, the numbers 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 growth rate occurred subsequent to an increase above the normal range of the number of circulating neutrophils.

Administration of G-CSF led to a specific four-fold increase in the numbers of circulating neutrophils. In these rats the tumor growth at day two 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 percentual 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 succesfull PDT *in vivo*.

Introduction

The mechanisms that play a role in tumor eradication after Photofrin-based photodynamic therapy (PDT) are subject to extensive study. The first event occuring after illumination of a photosensitiser is the formation of very reactive oxygen species (ROS) such as singlet oxygen (1-3). These ROS 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 *et al.* (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 loose their ability to relax upon 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 immuno-competent leucocytes 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 numbers of blood neutrophils were decreased by use of rabbit anti-rat granulocyte serum or increased by granulocyte-colony stimulating factor (G-CSF) before the start of PDT.

Materials and Methods

Animals and tumor model

Female WAG/Rij rats, aged 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 subcutaneously 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 mm^3. Blood samples were obtained from a tail vein with EDTA as the anticoagulant. Total leucocyte 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, BC, 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 hr prior to light delivery.

Polyclonal rabbit anti-rat granulocyte antiserum was purchased from Accurate (Westbury, NY, USA) and lyophilized recombinant human G-CSF was purchased from Amersham International (Buckinghamshire, England) and dissolved in saline (0.9%) containing 1% normal rat serum (NRS; Central Laboratory for Bloodtransfusion, 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 DCM (4-dicyanomethylene-2-methyl-6(P-dimethylaminostytyl)-4H-pyran) as a dye. The light was directed via a beam splitter to three cylindrical diffusers of 15 mm length (Rare Earth Medical, Dennis, MA, USA).

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 injected with equal volumes of saline. In the next series of experiments, 7.5 μg/kg G-CSF containing 1% NRS was injected i.v. at 9 hr before and 9 hr and 27 hr after PDT treatment. Control rats were injected with equal volumes of saline containing 1% NRS.

Interstitial PDT treatment was performed as previously described (25) with minor adaptations. In brief: rats were anaesthetized by i.m. injection of 1 ml/kg Hypnorm (fluanisol/fentanyl mixture; Janssen Pharmaceutica, Beerse, Belgium). Animals were placed on a heated support during treatment to control body temperature. In one of both tumors a diffussor was inserted into the central axis of the tumor parallel to the body axis. In the contralateral tumor a dummy was placed. The output of the diffuser was kept below 50 mW per 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 per cm of diffussor length. This treatment modality resulted in delay of tumor growth, but not in tumor regression. At the end of the observation period rats were sacrified and sections of tumor tissue were hematoxylin-eosin stained according to standard histologic procedures.

Statistical analysis

Data were analysed by paired or unpaired Student's t-test and Pearson correlation analysis where appropriate and considered significant when $P < 0.05$.

Results

Effect of anti-granulocyte antiserum on tumor growth after photodynamic therapy

To evaluate the effect of anti-granulocyte antiserum on tumor growth after PDT, the numbers of neutrophils, monocytes, lymphocytes, and platelets were determined first. We found that one day after the start of antiserum administration the neutrophils were depleted by 99.9% (see 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 extend, the platelets were decreased as well, i.e., by 100%, 80% and 25%, respectively (Table 1).

To investigate if 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 (Figure 1). As shown, the growth rate of the control tumors of the antiserum treated rats were not significantly different from the control tumors of the saline treated rats. The current PDT treatment resulted in a delay of tumor growth for five days in saline treated rats (Figure 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 percentual increase of tumorvolume 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.

Table 1. Effect of administration of anti-granulocyte antiserum on the number of blood cells.

Type of cell	Antigranulocyte antiserum		Saline	
(X 10^9/liter)	Start	1 Day later	Start	1 Day later
Neutrophils	1.49 ± 0.4[a]	0.001 ± 0.003[b]	1.18 ± 0.4	1.35 ± 0.22
Monocytes	0.12 ± 0.08	ND[c]	0.16 ± 0.07	0.14 ± 0.09
Lymphocytes	10.2 ± 2.2	2.0 ± 0.7[b]	9.5 ± 1.6	8.1 ± 1.2
Platelets (X 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.

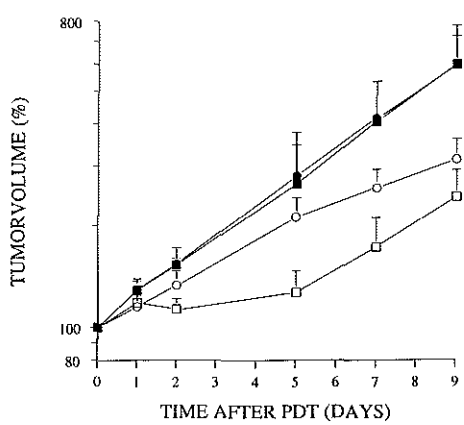

Figure 1. Effect of anti-granulocyte antiserum on tumor growth after PDT. Volumes of PDT-treated (open circle) or control (closed circle) tumors of rats injected with antiserum and of PDT-treated (open square) or control (closed square) tumors of rats injected with saline are expressed as percentage of the tumor volume at the day of treatment. Data are the means ± SD of five rats.

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%), while the number of neutrophils increased even further to approximately a factor 9.5 above the normal range. Statistical analysis showed that the percentual increase in tumorvolume during that time for all 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 photodynamic therapy

If the growth rate of PDT treated tumors indeed depends on the number of neutrophils 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 fourfold increase in the number of circulating neutrophils at the start of PDT, while the numbers of monocytes, lymphocytes and platelets stayed within the normal range (Table 2).

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

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

[a] Data are the means ± SD of five rats.
[b] Data are significantly different ($P < 0.001$) from the normal number at the start of G-CSF injection.

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 (Figure 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 the control tumors (Figure 2). Statistical analysis showed an inverse relationship between the percentual 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.

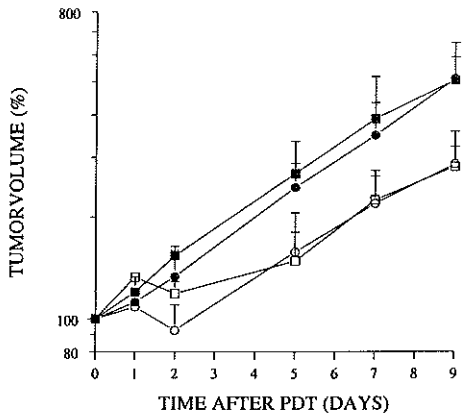

Figure 2. Effect of G-CSF on tumor growth after PDT. Volumes of PDT-treated (open circle) or control (closed circle) tumors of rats injected with G-CSF and of PDT-treated (open square) or control (closed square) tumors of rats injected with saline are expressed as percentage of the tumor volume at the day of treatment. Data are the means ± SD of five rats.

Evaluation of the data from both experiments (Figure 1 and 2) by 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), while there was no significant relationship with the number of monocytes, lymphocytes or platelets.

Histologic examination of tumor tissue

At the end of the observation period rats were sacrified 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 leucocytes were observed which mainly consisted of neutrophils (Figure3).

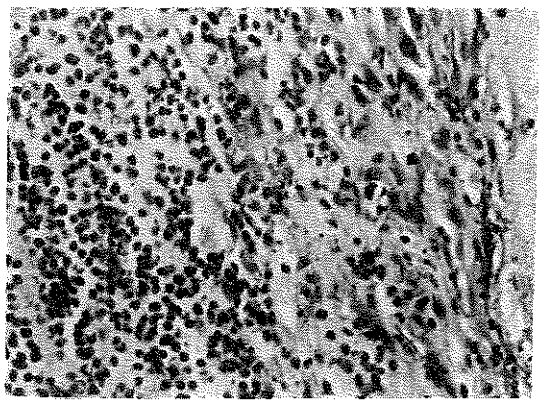

Figure 3. Histologic examination of tumor tissue. Tissue sections of PDT treated tumors of all rats were hematoxylin-eosin stained. Shown is a typical infiltrate at the border of vital and necrotic tumor tissue, mainly consisting of neutrophils. Magnification: 200X.

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 anti-granulocyte antiserum, PDT had no effect on tumor growth, while an increase in the number of neutrophils upon G-CSF administration before PDT led to a (temporary) increase in the efficacy of PDT.

In 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, upregulation 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 three-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 still. 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 (Figure 2, closed circle). 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. While 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 nonsteroidal 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 erradicate 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 five days later the administration of antiserum was stopped, which subsequently led to a tenfold increase of neutrophil numbers, tumor growth still retarded (Figure 1, open circle).

Recently, Dellian *et al.* (30) showed that the number of leucocytes 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 leucocyte-endothelium interaction in tumor microvessels. These findings may seem in contrast with 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*.

The use of G-CSF could be a promising accessory modality to improve the efficacy of PDT. G-CSF is a haematopoietic 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 in phase IV clinical trial (Lenograstim) (33,34). On this basis further study on the optimal dose and duration of G-CSF administration as an accessory treatment to improve PDT is warranted.

Acknowledgements

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CHAPTER 5

Role of interleukin 1 and granulocyte colony-stimulating factor in photofrin-based photodynamic therapy of rat rhabdomyosarcoma tumors

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(Cancer Research 57: 2555-2558, 1997)

Abstract

Neutrophils play an important role in the efficacy of photodynamic therapy (PDT). Those leukocytes rapidly accumulate into the tumor lesion after PDT and most likely eradicate the remaining attenuated tumor cells. The underlying mechanism of the accumulation of neutrophils upon PDT is not known. Therefore, we determined the effect of PDT on the course of mature and immature neutrophils in the circulation of rhabdomyosarcoma-bearing rats, and studied the changes in the level of interleukin(IL)-1β as an important stimulator of the proliferation of precursor cells of the granulocyte lineage in the bone marrow.

We found that the effect of PDT on tumor growth was preceded by a rapid and specific increase of the number of mature neutrophils in the peripheral blood as early as 4 h after the start of PDT treatment and reaching maximum values after 8 h. At 24 h the neutrophil numbers in the PDT-treated rats were still elevated as compared to sham-treated rats. In sham-treated rats the numbers of blood monocytes and lymphocytes decreased by about 50% after 2 h and returned to their normal levels as soon as 2 h later. In PDT-treated rats the course of monocyte numbers showed a similar pattern. However, lymphocyte numbers did not reach the normal range until 24 h.

The specific increment of neutrophils was preceded by an increase of band neutrophil numbers and elevated serum levels of IL-1β being maximal at 2 h after the start of PDT. Pearson correlation analysis showed a significant association between the serum levels of IL-1β at this time point and the number of band neutrophils at 4 h (R^2=0.58, p=0.03) and the number of mature neutrophils at 8 h (R^2=0.54, p=0.04). This suggests that PDT evoked an IL-1-dependent increased production rate of neutrophils in the bone marrow. Further investigation showed that the injection of anti granulocyte-colony-stimulating-factor(G-CSF) antibodies not only attenuated the increase in neutrophil numbers, but also greatly decreased the efficacy of PDT. On this basis we suppose that an IL-1-induced release of G-CSF by PDT underlies this nonspecific immune reaction to the tumor. Apparently, G-CSF not only stimulates the production rate of neutrophils in the bone marrow, but also increases the functional activity of those leukocytes to become the indispensable tumor cell killers.

Introduction

Increasing evidence arises that besides direct tumor cell kill due to formation of singlet oxygen (1) and indirect tumor cell kill as a result of deprivation of oxygen and nutrients due to vaso-occlusion (2) PDT also elicits a nonspecific (3,4) and a specific immune response directed to the tumor (5). A central role in this immune response might be performed by the endothelial cells which contract upon PDT as has been observed *in vivo* (6) and studied *in vitro* (7), exposing the thrombogenic subendothelium. Besides the fact that this endothelial contraction stimulates edema formation, platelet aggregation, thromboxane release and thrombus formation which eventually leads to blood flow stasis (8,9), it also will facilitate the extravasation of blood leukocytes into the underlying tumor tissue.

Very soon after the start of PDT neutrophilic granulocytes adhere to the vascular wall (10) and infiltrate the tumor area (11) where they may kill the attenuated tumor cells directly (12) or via a complex interaction with other cells (13). The concept that leukocytes may play an important role in the PDT-induced inflammatory reaction has led to several immuno-therapeutic approaches in order to enhance the efficacy of PDT (4, 14-16). These strategies indeed potentiated the effect of PDT, stressing the important role of a cellular inflammatory reaction in tumor kill.

Since we have shown recently that an effective interstitial PDT of rhabdomyosarcoma tumors is dependent on the presence of neutrophils in the circulation (3), the question was raised whether PDT by itself influences the number of blood neutrophils. In the present study we therefore established the effect of PDT on the course of neutrophils in the circulation of rats bearing rhabdomyosarcoma R-1 tumors and examined the underlying regulatory mechanism.

Materials and Methods

Animals and tumor model

Female WAG/Rij rats, aged 10-15 weeks, were obtained from Harlan (Zeist, The Netherlands). Small pieces of a well characterized isologous rhabdomyosarco-

ma, designated as R-1 (17) were implanted subcutaneously into the thighs. Tumor growth was assessed by calliper measurements on three orthogonal diameters, once a day. Tumors were treated when the volume was between 750 and 1500 mm^3. Blood samples were obtained by tail bleeding with EDTA as the anticoagulant. Total numbers of leukocytes were determined with a microcell counter. Differential counts were performed on May-Grünwald and Giemsa-stained blood smears in quadruplicate.

Photosensitizer and light delivery

The photosensitizer Photofrin II® (PII) was obtained from Quadra Logic Technologies Inc. (Vancouver, BC, Canada) and was reconstituted in 5% glucose before i.v. administration into a tail vein at 10 mg PII/kg 24 h prior to 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 DCM (4-dicyanomethylene-2-methyl-6(P-dimethylaminostytyl)-4H-pyran) as a dye. The light was directed via a beam splitter to three cylindrical diffusers of 15 mm length (Rare Earth Medical, Dennis, MA, USA).

Treatment protocol

Interstitial PDT treatment was performed as previously described (3) with minor adaptations. In brief: rats were anaesthetized by i.m. injection of 1 ml/kg Hypnorm (fluanisol/fentanyl mixture; Janssen Pharmaceutica, Beerse, Belgium). Animals were placed on a heated support during treatment to control body temperature. A diffusor was inserted into the central axis of the tumor parallel to the body axis. The output of the diffuser was kept below 50 mW per cm of diffuser length to avoid hyperthermia. Tumor core temperature was measured during PDT using a thermocouple probe and never exceeded 40°C. Total applied radiant energy was 270 J per cm of diffusor length. Control rats were treated in the same way as above apart from the illumination protocol (i.e. PII injection only) or PII injection (i.e. illumination only).

For studies on the involvement of growth factors, rats were injected with 500

mg of sheep polyclonal antibody against recombinant human (rh) G-CSF (Biodesign International, Kennebunk, ME, USA) i.v. 1 h prior and i.p. 8 h after the start of PDT treatment.

Cytokine ELISA

IL1-β and TNF-α levels in sera were determined by rat specific ELISA kits obtained from Biosource (Biosource Europe S.A., Fleurus, Belgium).

Statistical analysis

Data were analysed by paired or unpaired Student's t-test and by Pearson correlation analysis where appropriate and considered significant when $P < 0.05$.

Results

Effect of PDT on tumor growth

To study the effect of PDT on the number of neutrophils in the circulation, we evaluated the efficacy of the applied PDT treatment on the growth of Rhabdomyosarcoma tumors first. As shown in Figure 1 tumor growth of rats treated with PII or illumination only was not affected whereas PDT treatment did affect tumor growth. Strikingly, one day after PDT treatment the tumor volumes were significantly increased as compared to illumination ($P=0.025$) or PII ($P=0.016$) only. However, at two days after PDT treatment the tumor volumes were decreased to approximately the original volume at the start of treatment. At this point tumor growth after PDT treatment was significantly retarded as compared to the control tumors. The growth delay (i.e. the time that is needed for a tumor to reach its original treatment volume) was approximately 4.5 days. Thereafter tumor growth resumed at the same rate as before the start of PDT.

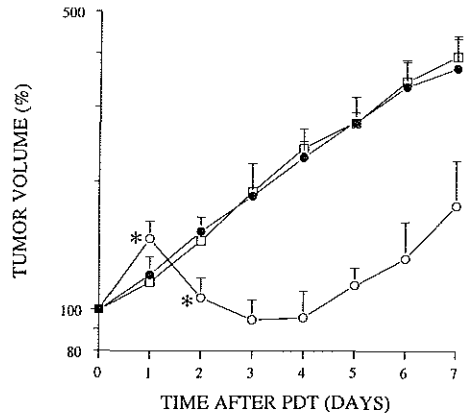

Figure 1. Tumor growth of Rhabdomyosarcoma after PDT. Volumes of PDT-treated tumors (open circle), of unilluminated tumors of rats injected with PII (closed circle), and of illuminated tumors of rats without PII injection (open square) are expressed as percentage of the tumor volume at the day of treatment. Data are the means of five rats; *bars*, SD.

Effect of PDT on the numerical course of blood leukocytes

After PDT or control treatment the number of circulating lymphocytes (Figure 2a) decreased at 2 h to about 50% of the original number at the start of treatment. Thereafter their numbers increased again to approximately the normal level after 8 h for the control groups and after 24 h for the PDT treated group. The number of monocytes decreased also transiently to about 50% of the original value at 2 h after PDT or control treatment, reaching the pre-treatment level 2 h later irrespective of the type of treatment (Figure 2b). The number of neutrophils changed dramatically upon treatment (Figure 2c). From 4 h after treatment onward their number increased four-fold in both control groups to a maximum at 8 h after treatment, while PDT treatment even led to a five-fold increase which was significantly higher than the control groups ($P<0.02$). At 24 h neutrophil numbers were decreased to the normal level in the control rats, while being still elevated in the PDT treated rats. Strikingly, immature band neutrophils appeared in the circulation as early as 2 h upon PDT treatment, reached a maximal number of $0.081 \pm 0.08 \times 10^9$/L at 4 h post PDT, and subsided thereafter to below the

detection limit (not shown)

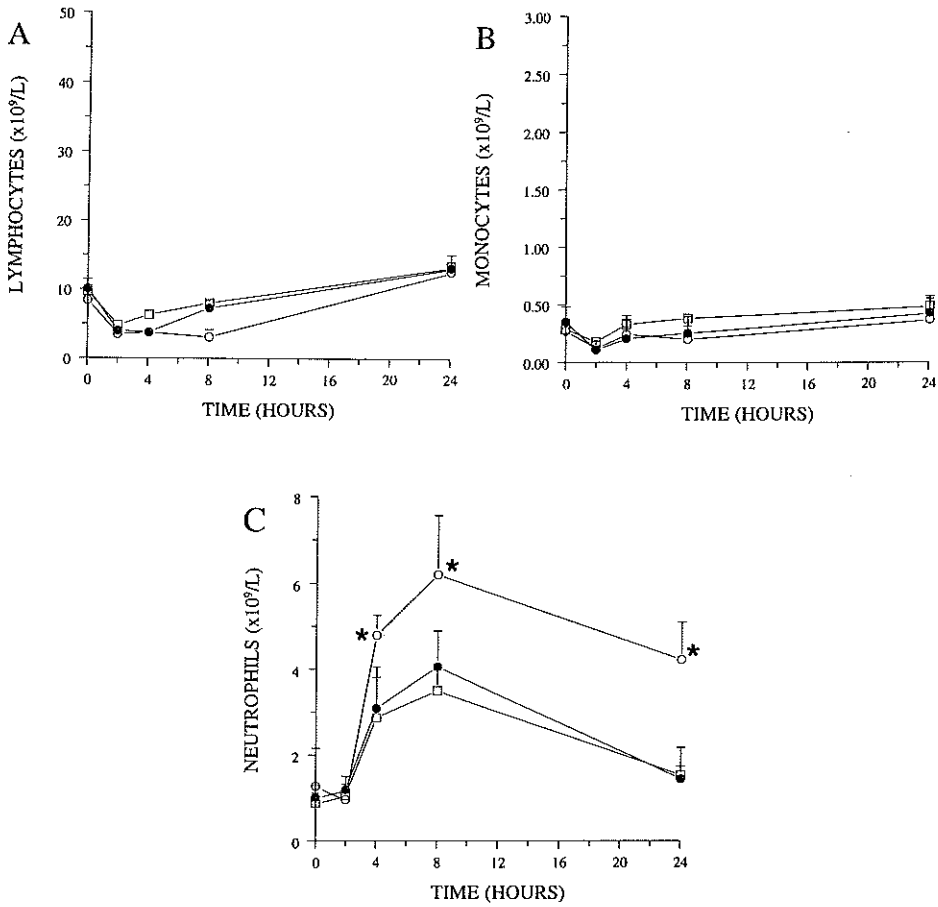

Figure 2. Effect of treatment on the numbers of peripheral blood leukocytes. Shown are the numerical courses of lymphocytes (A), monocytes (B), and neutrophils (C) after treatment of rat rhabdomyosarcoma tumors by PDT (open circle), or injection with PII (closed circle) or illumination only (open square). Data are the means of five rats; *bars*, SD.

Effect of PDT on serum levels of IL-1β and TNF-α

To investigate the regulatory mechanism of the PDT-induced increase in the number of blood neutrophils, we determined the occurrence of the proinflammatory cytokines IL-1β and TNF-α in the sera of control and PDT

treated rats (Figure 3). We found a transient, but significant increase in IL-1β levels with a maximum at 2 h after PDT as compared to the level in control treated animals ($p<0.05$). Pearson correlation analysis showed a treatment-independent relationship between the serum level of IL-1 at 2 h and the number of band neutrophils at 4 h ($R^2=0.58$, $p=0.03$) and the number of mature neutrophils at 8 h ($R^2=0.54$, $p=0.04$). The levels of TNF-α were below the detection limit and did not change upon PDT or control treatment.

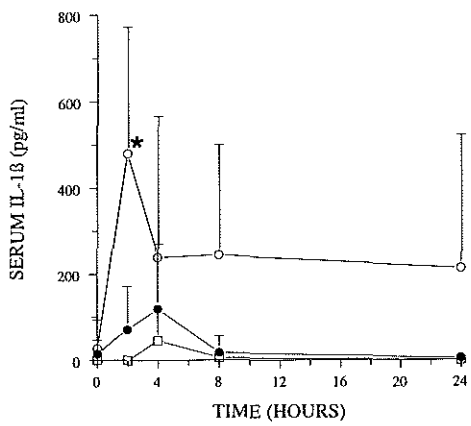

Figure 3. Effect of PDT on serum IL-1β levels. Shown are the serum levels of IL-1β of rats treated by PDT (open circle), or injection with PII (closed circle) or illumination only (open square). Data are the means of five rats; *bars*, SD.

Involvement of G-CSF

To examine whether the increase in the level of IL-1β directly underlies the observed neutrophilia, we injected polyclonal anti-G-CSF antibody at time zero and at 8 h after the start of PDT. As shown in Figure 4a this antibody treatment attenuated the PDT-induced numerical increase in blood neutrophils. If expressed as the area-under-the-curve of blood neutrophils (dimension: $10^9 x L^{-1} x 24h$) during the observation period of 24 h, these two consecutive injections of anti-G-CSF antibodies led to a decrease of about 25%, i.e., from 118.6 to 87.1. In contrast to the moderate effect on the numerical course of neutrophils in the circulation, this anti-G-CSF treatment led to a dramatic decrease in the efficacy of PDT amounting

to an estimated delay of tumor growth of about one day as compared to 4.5 days under the normal condition (Figure 4b).

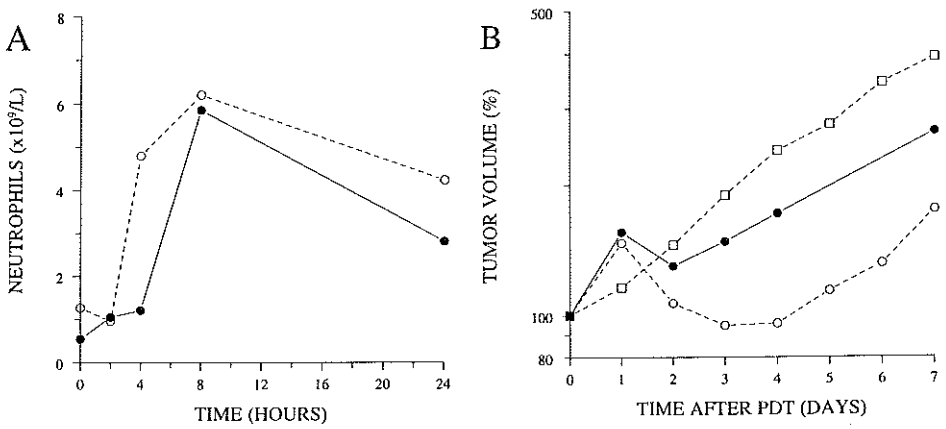

Figure 4. Involvement of G-CSF in the PDT induced neutrophilia. Shown are the courses of neutrophils in the circulation (A) and tumor volumes (B) of rats after PDT treatment (open circle; n=5) or after PDT treatment in the presence of polyclonal anti-G-CSF antibody (closed circle; n=2). For comparison (B), the tumor volumes of rats after PDT treatment (open circle) and of rats injected with PII only (open square;data of Figure 1) are shown.

Discussion

The major finding of this study was that interstitial PDT elicited a severe inflammatory reaction characterized by a profound neutrophilia that appeared dependent on an IL-1-induced release of G-CSF. Sham-treatment of the tumor-bearing rats induced a (moderate) increase in the number of blood neutrophils as well, which was possibly evoked by tissue damage upon insertion of the optic fiber. However, this did not lead to retardation of tumor growth. Apparently, neutrophils are not able to kill those vital tumor cells directly. This confirms the results of a previous study showing that a direct effect of PDT on the tumor is necessary for neutrophils to become effective in tumor cell eradication, while in the absence of neutrophils PDT on its turn is also not effective (3).

It is noteworthy that the increase in the number of blood neutrophils after PDT most likely is an underestimation of the magnitude of the inflammatory reaction.

Due to the migration and accumulation of neutrophils upon PDT into the tumor area (3,11) the half-time of neutrophils in the circulation will decrease accordingly. Furthermore, by inducing the contraction of the endothelial cells lining the vessel wall PDT treatment may also facilitate the accumulation of the neutrophils in the tumor. Indeed, exposure of the subendothelial matrix by PDT promotes the adhesion of neutrophils (7), and may also lead to edema formation explaining the temporary increase of the tumor volumes at one day after PDT treatment (Figure 1).

We found here that the peak in the number of mature blood neutrophils was preceded by a peak in the appearance of immature band neutrophils in the circulation indicating that the production rate of neutrophils in the bone marrow is increased as well. This could be brought about by IL-1 that occurred in the circulation during the early phase after PDT. IL-1 is able to induce a granulocytosis by the mobilization of neutrophils from their storage pool in the bone marrow (18,19). However, the results of the present study show that IL-1 does not act alone. The administration of anti-G-CSF antibodies revealed that the numerical increase of blood neutrophils is at least partially dependent on the endogenous production of the granulocyte-specific growth factor G-CSF. This growth factor stimulates the proliferation, and maturation of neutrophils in the bone marrow, and can also up regulate the activation status of those leukocytes (20). It is conceivable that the release of G-CSF is triggered by IL-1β upon PDT. G-CSF can be produced by many celltypes including fibroblasts and endothelial cells after IL-1 stimulation both *in vitro* (21-23) and *in vivo* (24). In this respect it is noteworthy that TNF-α is also capable of inducing G-CSF production as shown *in vitro* by endothelial cells (25) and *in vivo* (26). Macrophages release TNF-a following PDT treatment *in vitro* (27), but we were not able to demonstrate its occurrence in the circulation of the PDT-treated rhabdomyosarcoma rats.

Activated inflammatory cells make an essential contribution to the anti-tumor effect of PDT (3,4,11). Although G-CSF at least partially contributes to the neutrophilia, administration of anti-G-CSF antibodies has a much greater impact on the efficacy of PDT than could be deduced from its effect on the numerical course of blood neutrophils after PDT. This suggests that functional activation of neutrophils by the granulocyte-selective growth factor G-CSF is of great importance for the tumoricidal effect of PDT. It also supports the view that the local availability of this and other cytokines is essential to establish granulocyte-T-lymphocyte cross-talk to overcome the immunosuppressive activity of the tumor (13). Neutrophils that can kill tumor cells upon activation by G-CSF (28), may also

facilitate the establishment of a specific immune reaction against the tumor. Korbelik et al. (5) clearly showed that the activity of host lymphoid populations is essential for preventing the recurrence of EMT6 tumors in mice after PDT. In accordance with that study we found a sustained decrease of the number of lymphocytes in the circulation during the first 24 h after PDT suggesting their recruitment to the treated site.

Taken together, we suppose that the profound neutrophilia upon PDT of rhabdomyosarcoma-bearing rats is the result of the action of IL-1. However, IL-1 most likely did not act alone, but conceivably stimulated the subsequent release of the granulocyte-lineage specific growth factor G-CSF. G-CSF on its turn can increase the proliferation rate of myeloid precursors in the bone marrow, but more importantly elevates the functional activity of neutrophils at the site of the tumor lesion. In the absence of G-CSF neutrophils lack to the ability to kill the attenuated tumor cells making PDT much less effective. It is obvious that neutrophils are crucial for an effective PDT. Contra-indications for PDT should therefore regard patients that are treated with severe immunosuppressive (cytostatic) agents unless recovered from granulocytopenia (by G-CSF). Future efforts in optimalisation of PDT should concentrate on the leukocytes recognized to be important in the inflammatory reaction and the cytokines/growth factors important for their proliferation and activation.

Acknowledgements

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CHAPTER 6

The effect of thrombocytopenia on the efficacy of photofrin-based photodynamic therapy *in vivo*

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(Submitted for publication)

Abstract

In the past it was postulated that obstruction of the blood flow is beneficial in respect to tumor cure after PDT since tumor cells are deprived of oxygen and nutrients under these conditions. On the other hand, a decreased tumor oxygenation *during* PDT may inhibit the generation of toxic oxygen species and therefore may impair the direct efficacy of the therapy. Thrombocytes are involved in thrombus formation and vasoconstriction observed during and after photodynamic therapy (PDT) of tumors resulting in blood flow obstruction. To investigate whether preventing this obstruction during PDT will improve the efficacy of the therapy, the number of thrombocytes in the blood circulation of rhabdomyosarcoma-bearing rats was decreased before the start of PDT by the administration of rabbit anti-rat thrombocyte antiserum. This treatment led within 30 min to a reduction of at least 96% in the number of circulating thrombocytes which remained at that level throughout at least 48 h. Growth of tumors treated interstitially on the thigh of the animals was significantly retarded under thrombocytopenia induced prior to PDT as compared to control PDT conditions at a light energy dose of 90 J, suggesting a prominent role for tumor oxygenation in the enhanced efficacy. However, treatment of animals with the anti thrombocyte antiserum after PDT led to the same increased delay of tumor growth as compared to antiserum treatment prior to PDT, thereby outruling this possibility.

Previously we showed a prominent role for neutrophils in the effectuation of the PDT induced damage to the tumor under normal conditions. We therefore questioned whether the contribution of these phagocytes to the tumor growth retardation could be increased under thrombocytopenia. Upon PDT the amount of myeloperoxidase (MPO) activity increased in the tumors of normal and thrombocytopenic rats reflecting the accumulation of neutrophils at the site of the lesion. In normal rats the tumor MPO content reached a maximum at 24 hr after PDT, and rapidly subsided thereafter, while in thrombocytopenic rats the MPO accumulation rate was similar, but it did not slow down before 48 hr after PDT and then slowly decreased. From the areas-under-the-curves it can be estimated that at least 1.5 times more neutrophils than normal were present in the PDT-treated tumors under thrombocytopenia. Furthermore, when the number of granulocytes was decreased by the administration of anti-granulocyte antiserum prior PDT followed by anti-thrombocyte antiserum post PDT, this treatment regimen led to a considerable loss of the thrombocytopenia-dependent gain in the efficacy of PDT.

Apparently neutrophils underlie the observed enhanced efficacy of PDT under thrombocytopenia. We hypothesize that in the absence of vasoconstriction and/or local clotting either prevented during PDT or abolished after PDT the attenuated tumor cells will be better accessible to these activated tumor cell killers. This would imply that under normal clinical conditions the full granulocyte-dependent kill potential of PDT is not utilised due to the presence of thrombocytes that cause blood flow stasis.

Introduction

The mechanism underlying tumor eradication after photodynamic therapy (PDT) involves direct and indirect events affecting the viability of individual tumor cells. Direct cytotoxicity occurs as a result of the generation of highly reactive oxygen species such as singlet oxygen (1,2) upon illumination of the photosensitizer, which induces oxidation of plasma membranes, mitochondria and lysosomes (3,4). *In vivo*, not only tumor cells are affected directly by PDT but also the endothelial cells lining the blood vessels that provide the tumor with oxygen and nutrients. Direct cytotoxicity causes the retraction of the endothelial cells with the subsequent exposure of the subendothelial matrix as has been observed *in vitro* (5) and *in vivo* (6). Neutrophils adhere to the exposed subendothelium (7) and thereafter infiltrate the tumor area (8), where the attenuated tumor cells are killed directly (9) or via interaction with other leukocytes (10). The importance of this non-specific cellular immune reaction to occur is stressed by the fact that several immuno-potentiating strategies have successfully enhanced the efficacy of PDT (8, 10-15). On the other hand, depletion of neutrophils using anti-GR1 antibody or blocking the β chain of integrins by anti-CD18 antibody decreased the PDT-mediated tumor cure (13). Furthermore, inhibiting neutrophil action by anti granulocyte antiserum almost completely abrogated the effect of PDT on tumor growth (16). Cytokines and growth factors are likely to be involved in the immune response elicited by PDT as well (17-20). Interleukin 1β serum levels for example rapidly increase after the start of PDT. This pro-inflammatory cytokine may induce the release of granulocyte colony stimulating factor (G-CSF), which in turn can stimulate the proliferation and activation of neutrophils, leading to a peak in neutrophil numbers in the circulation at 8 h post PDT (20).

The exposed subendothelium is highly thrombogenic. Indeed next to the

adherence of granulocytes, thrombocytes adhere leading to the formation of thrombi and to vasoconstriction due to the release of potent vasoactive eicosanoids, e.g., thromboxane (21, 22). Eventually these events lead to complete stasis of the blood flow. It was postulated that this contributes to indirect tumor cell kill due to the deprivation of oxygen and nutrients, because the clonogenicity of tumor cells decreased with time after PDT *in vivo* (23). The necessity of vascular shutdown for tumor regression was supported by the fact that indomethacine, which inhibits the release of thromboxane by activated thrombocytes, decreased the efficacy of PDT (22). However, this could be misleading evidence since indomethacine also suppresses the activity of neutrophils, which are indispensable for the killing of attenuated tumor cells (16). Recently it has been observed that the administration of anti thrombocyte antiserum prevents the vasoconstriction and occlusion of the microvasculature upon PDT (24). Since this antiserum treatment does not directly affect neutrophil function we investigated the effect of thrombocytopenia on the efficacy of PDT with special emphasis on the role of neutrophils therein.

Materials and Methods

Animals and tumor models

Female WAG/Rij rats, aged 12-20 weeks, were obtained from Harlan (Zeist, The Netherlands). In order to perform interstitial PDT small pieces of a well-characterized isologous rhabdomyosarcoma, designated as R-1 (25) were implanted subcutaneously into the thigh. Tumor growth was assessed every day by calliper measurements on three orthogonal diameters. Tumors were treated when the volume was between 1000 and 1500 mm^3. Blood samples were obtained from a tail vein with EDTA as the anticoagulant. Total leukocyte and thrombocyte counts were determined with a microcell counter, and differential counts were carried out on May-Grünwald and Giemsa-stained blood smears.

Antisera and Photosensitizer

Adsorbed polyclonal rabbit anti-rat thrombocyte and polyclonal rabbit anti-rat granulocyte antisera were purchased from Accurate (Westbury, NY, USA), diluted

1 to 10 in 0.9% NaCl solution and sterilized by 0.2 • m filtration before use.

The photosensitizer Photofrin II® (PII) was obtained from Quadra Logic Technologies Inc. (Vancouver, BC, 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 hr prior to light delivery.

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 DCM (4-dicyanomethylene-2-methyl-6(P-dimethylaminostytyl)-4H-pyran) as a dye. The light was directed via a beam splitter to three cylindrical diffusers of 15 mm length (Rare Earth Medical, Dennis, MA, USA).

Treatment protocol

In the first series of experiments, rats were rendered thrombocytopenic by a single i.v. injection of 0.5 ml of diluted anti thrombocyte antiserum (containing approx. 1 mg Ig/ml) at 30 min prior or 5 min post PDT. In the next series of experiments, rats were rendered neutropenic by a single i.v. injection of 0.2 ml of diluted anti granulocyte antiserum at 30 min prior PDT treatment, followed by a single injection of 0.5 ml of diluted anti thrombocyte antiserum at 5 min post PDT to render the rats thrombocytopenic as well. Thereafter rats were kept neutropenic by i.v. injections of 0.1 ml of diluted anti granulocyte antiserum at 2 and 4 days after PDT. Control rats were injected with equal volumes of saline.

Interstitial PDT treatment was performed as previously described (20) with minor adaptations. In brief: rats were anaesthetized by i.m. injection of 1 ml/kg Hypnorm (fluanisol/fentanyl mixture; Janssen Pharmaceutica, Beerse, Belgium) and Valium. Animals were placed on a heated support during treatment to control body temperature. A diffuser was inserted into the central axis of the tumor parallel to the body axis. The output of the diffuser was kept below 50 mW per cm of diffuser length to avoid hyperthermic effects. Tumor core temperatures were measured during PDT using a thermocouple probe and never exceeded 40°C. Total applied radiant energy was 90 J (low dose) or 270 J (high dose) per cm of diffuser length. Both treatment modalities normally cause delay of tumor growth but not tumor regression.

Assessment of MPO activity

As an index of the number of neutrophils the tumor myeloperoxidase (MPO) content was assessed. We adapted the biochemical assay described by Grisham et al. (26), which was optimized by Graff et al. (27), for tumor tissue to overcome the interference of endogenous (enzymic and nonenzymic) reductants by two wash steps in the presence of *N*-ethylmaleimide (NEM) after homogenization, and by the addition of hexadecyltrimethylammonium bromide (HTAB) to the assay mixture to inhibit the pseudoperoxidase activity of remaining hemoglobin derived from the increasing numbers of tumor associated erythrocytes upon PDT. In short, the tumor was excised, weighed and homogenized in 3 volumes (v/w) of ice-cold modified RIPA buffer (containing 1% NP40, 0.25% sodiumdeoxycholate, 1 μg/ml aprotinin, leupeptin and pepstatin, and in mM: 50 Tris HCl, 150 NaCl, 1 EDTA, 1 PMSF, 1 $NaVO_4$, 1 NaF;pH 7.4) using a Polytron homogenizer on setting 5. The homogenate was centrifuged at 12,000 *g* for 30 min at 4°C. The pellet was resuspended in ice-cold 50 mM sodium phosphate buffer (pH 7.4) containing 10 mM NEM, centrifuged again, and if the number of erythrocytes was high, resuspended in ammoniumchloride for 20 min at 4°C and centrifuged. The pellet was sonicated in 50 mM sodium phosphate buffer (pH 6.0) containing 0.5% HTAB (PB-HTAB) by three bursts of 10 sec on setting 20, subjected to three cycles of freezing and thawing, and centrifuged (12,000 *g*, 30 min, 4°C). The MPO activity was determined by its ability to catalyze the hydrogen peroxide-dependent oxidation of o-dianisidine (o-DA). The reaction was started by the addition of 100-μl pre-warmed substrate solution (hydrogen peroxide and o-DA) to 50 μl aliquots of the serially diluted supernatant in PB mixed with 50 μl PB-HTAB (final concentrations: 3.43 mM HTAB, 0.35 mM hydrogen peroxide, and 0.63 mM o-DA). We found that under those conditions optimal reaction rates were attained, while the pseudoenzymic activity of hemo- and myoglobin was inhibited by a factor of > 100. The change in absorbance at 450 nm was measured during 2 min at 37°C in a thermostatted microplatereader (Thermomax, Sopachem, Driebergen, The Netherlands). Initial rates of enzyme activity were determined from the linear part of the curve, and converted to enzyme units using the molar extinction coefficient of of o-DA of $10{,}062 x M^{-1} x cm^{-1}$ (27). We defined one unit of MPO as the amount of enzyme that oxidizes o-DA at a rate of 1 μmol/min under the present assay conditions. Under those assay conditions 1 U of bovine MPO (Sigma) catalyzes the oxidation of o-DA at a rate of 0.15 μmol/min. In eight experiments, the amount of MPO recovered from rat blood neutrophils was 5.70 (± 0.87) $x10^{-7}$

U MPO/cell, which was nearly equal to the amount of 5.04×10^{-7} U reported by Bradley et al. (28). Enzymatic MPO activity was routinely confirmed by its complete inhibition with 1 mM sodium cyanide or 10 mM sodium azide.

Statistical analysis

Data were analysed by unpaired Student's t-test and considered significant when $P < 0.05$.

Results

Effect of PDT on thrombocyte and leukocyte numbers after anti-thrombocyte antiserum-treatment.

To study the effect of PDT on the numerical courses of platelets and leukocytes in the circulation of anti-thrombocyte antiserum-treated rats, blood samples were drawn at various time points during the observation period. PDT alone did not influence the number of thrombocytes markedly. The administration of antiserum prior to PDT, however, led to a 96 % reduction in the number of thrombocytes from 687 ± 27 to 26 ± 25 x 10^9/l within 30 min (not shown). Upon PDT that number did not change and remained at that low level throughout at least 48 h (not shown). Likewise, if the antiserum was administered directly *after* PDT the number of thrombocytes dropped to almost zero levels after 30 min and did not return to the normal level within 48 h.

The number of circulating leukocytes slowly increased after PDT from 6.9 ± 3.9 to 14.7 ± 2.6 x 10^9/liter. Administration of anti-thrombocyte antiserum prior to or post PDT initially led to a maximal decrease of 50-70% of the normal numbers within 30 min (not shown). This effect lasted for about 8 h, after which the number of leukocytes increased reaching similar elevated levels at 24 h as rats treated with PDT only.

Effect of thrombocytopenia on tumor growth after PDT.

To investigate whether by preventing blood flow stasis thrombocytopenia indeed enhances the efficacy of PDT, rats with rhabdomyosarcoma tumors

transplanted on the thigh were used. The normal tumor doubling time (here defined as the time needed to reach a doubling in tumor volume as compared to day zero) is 3.4 days (Figure 1).

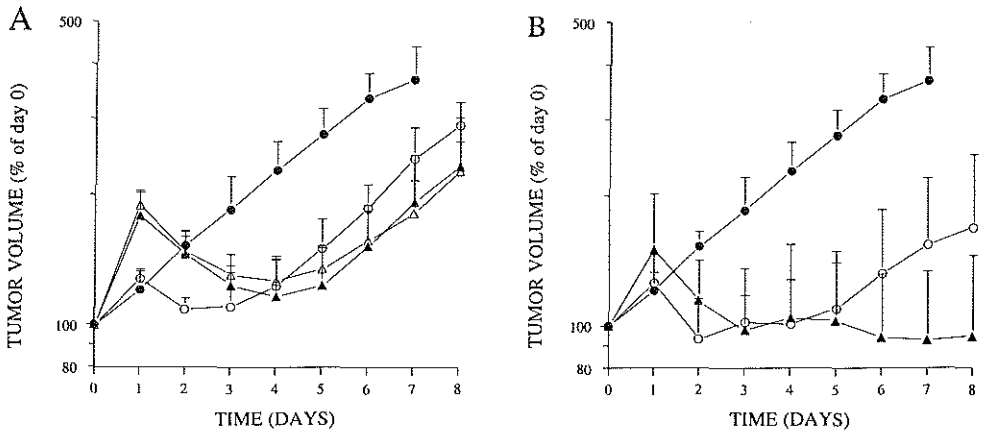

Figure 1. Effect of thrombocytopenia on tumor growth after PDT. Tumor volumes of rats treated with saline (open circle) or antiserum prior to (open triangle) or post PDT (closed triangle) and of untreated rats (closed circle) are expressed as a percentage of the tumor volume at the day of treatment. PDT was applied at 90 J (Figure 1a) and at 270 J (Figure 1b) per cm of diffuser length. Data are the mean of six rats. *Bars*, SD.

In normal animals PDT treatment at 90 J per cm of diffuser length resulted in a prolonged tumor doubling time of 6.26 ± 0.56 days (Figure 1a). Under thrombocytopenia induced prior to illumination, PDT led to a rapid and transient increase in tumor volume at one day after treatment which is possibly due to oedema formation. A striking feature of the tumors from these rats at that time-point was the clearly visible extensive and intense necrotic areas (Figure 2a) which were not observed in the PDT treated control rats (Figure 2b). Eventually under these conditions the tumor doubling time was 7.48 ± 0.84 days, which was significantly longer than under the PDT alone condition ($P = 0.014$). There was no difference in growth rates of untreated tumors between normal (Figure 1) and thrombocytopenic rats (not shown).

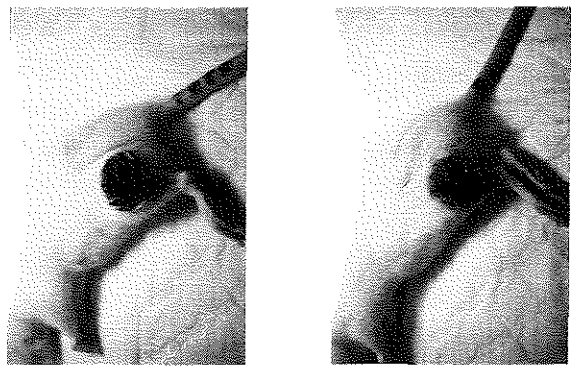

Figure 2. Macroscopic appearance of PDT-treated tumors. Shown is a tumor of an antiserum (Left) or saline (Right) treated rat 24 h after PDT treatment.

The length of the thrombocytopenic period induced by the anti thrombocyte antiserum prior to PDT is not restricted to the phase of light treatment, which makes it impossible to attribute the beneficial effect of this accessory treatment only to an improved supply of oxygen during PDT. We therefore decided to study the effect of thrombocytopenia induced *after* PDT. Strikingly the administration of the antiserum post PDT led to a comparably longer tumor doubling time (7.56 ± 1.25 days) as in the rats treated with antiserum prior the PDT treatment which was statistically different from PDT only rats (P = 0.043; Figure 1a). At the higher light energy dose of 270 J per cm of diffuser length PDT only led to an estimated tumor doubling time of 9.65 ± 2.19 days, which is significantly longer than at 90 J cm (P = 0.004; Figure 1a and 1b). Strikingly, tumors treated with this high energy light dose in combination with the antiserum treatment post PDT on average did not resume to grow at all within the observation period (Figure 1b).

The involvement of neutrophils in the enhanced efficacy of PDT under thrombocytopenia.

Since there was no difference in tumor response to PDT in rats treated with anti-thrombocyte antiserum prior versus post PDT it is unlikely that under thrombocytopenic conditions an improved supply of oxygen underlies the observed enhanced efficacy of PDT. Other mechanisms may therefore account for

this phenomenon. In a previous study we showed evidence for an important role for neutrophils in the effectuation of the PDT induced damage (16). We therefore questioned whether the activity of these phagocytes underlies the observed enhanced PDT effect under thrombocytopenia as well. In order to investigate this, tumors from each treatment group were excised at several time-points after PDT (270 J/cm) and assayed for MPO content, which is an indicator for the presence of neutrophils (Figure 3).

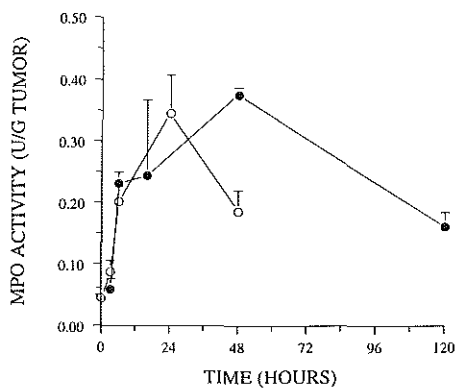

Figure 3. Effect of treatment on tumor MPO content. Shown is the activity of MPO per g tumor wet wt after treatment by PDT at 270 J/cm only (open circle), and in combination with the administration of anti thrombocyte antiserum post PDT (closed circle). Data are the mean of two to three rats. *Bars*, SD.

As shown the activity of MPO in the tumor of normal rats rapidly increased upon PDT to a maximum of 0.34 (± 0.06) U MPO/g tumor (equivalent to 6.04 x 10^5 neutrophils) at 24 hr and declined thereafter to 50% within the next 24 hr. However, under thrombocytopenia the increase in MPO activity did not diminish earlier than after 48 hr (maximum: 0.37 ± 0.01 U MPO/g tumor, equivalent to 6.56 x 10^5 neutrophils) , and only slowly subsided thereafter. From the areas-under-the-curves it can be estimated that about a factor of 1.5 more neutrophils than normal accumulated into the PDT-treated tumors under thrombocytopenia. However, the increase in the accumulation of neutrophils at the tumor lesion under thrombocytopenia does not necessarily prove their contribution to the enhanced efficacy of PDT. In order to investigate this, anti-granulocyte antiserum was

administered prior to PDT followed by anti-thrombocyte and anti-granulocyte antiserum post PDT. This treatment regimen led to a significantly lesser effect of PDT on tumor growth as compared to thrombocytopenia only (tumor doubling time of 5.44 ± 1.38 days, P = 0.021; Figure 4).

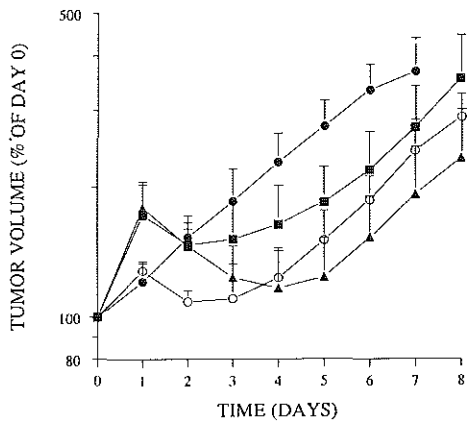

Figure 4. Effect of anti-granulocyte antiserum on the thrombocytopenia induced enhancement of PDT efficacy. Shown are the tumor volumes of rats pretreated with anti-granulocyte antiserum prior PDT, followed by treatment with anti-thrombocyte and anti-granulocyte antiserum post PDT (closed square). For comparison, the tumor volumes of rats treated with saline (open circle) or anti-thrombocyte antiserum post PDT (closed triangle) and of untreated rats (closed circle; data of Figure 1) are shown. Data are the mean of six rats. *Bars*, SD.

Discussion

This study shows that the efficacy of PDT is affected by thrombocytes, which are involved in vascular events occurring as a result of treatment. Recently it was shown that depletion of circulating thrombocytes by use of anti thrombocyte antiserum administered prior to PDT led to an inhibition of vascular constriction and occlusion by thrombi (24). In the present study we demonstrated that under these conditions the damage inflicted upon tumors by PDT is enhanced. This was expected since previous studies showed that PDT induced damage is dependent on the generation of singlet oxygen (1,2). Under thrombocytopenia Fingar et al. (24)

showed that the blood flow and thus the supply of oxygen, is uninterrupted during PDT due to the absence of vascular events that may lead to an enhanced generation of singlet oxygen. Surprisingly however we found exactly the same enhancing effect of PDT on tumors when the antiserum was administered after the end of treatment. Obviously, the latter condition did not allow for an improved generation of singlet oxygen. Apparently, another mechanism underlies the observed enhanced efficacy. Presently, we can only speculate at this point. In thrombocytopenic animals the number of neutrophils that accumulated in the PDT-treated tumor area was increased. Furthermore, the enhanced efficacy of PDT on tumors could be abrogated by pre-treatment of animals with anti-granulocyte antiserum. We therefore conclude that the enhanced killing of tumor cells is caused by an increase in the number of tumor-associated neutrophils under thrombocytopenia. Henderson *et al.* have found that the clonogenicity of tumor cells deteriorates with time after PDT, despite the fact that during PII-based PDT blood vessels occlude, thereby hampering the direct tumor cell kill (23). It was postulated that this secondary killing effect of PDT was due to the deprivation of oxygen and nutrients as a result of the blood flow stasis. The result of the present study indicate that another factor, i.e., the activity of tumor-associated granulocytes, has to be taken into account as well. These phagocytes need time to accumulate into the area of photodamage of the tumor. Fingar *et al.* (7) showed that upon PDT granulocytes adhere to the blood vessel wall within 5 min. However thereafter, a time consuming process of diapedesis of the subendothelial matrix, migration to the attenuated tumor cells and their destruction will follow. If indeed these granulocytes play a major role in the efficacy of PDT, the blood flow stasis will not promote, but inhibit the secondary PDT-dependent tumor cell kill by hindering their entry into the lesion. We found evidence that under thrombocytopenia induced *after* PDT the blood vessels apparently reopen facilitating the accumulation of neutrophils into the damaged tumor, because between 16 and 48 hr later the hemoglobin content of the tumors in thrombocytopenic rats was a factor of 3-4 times higher than in PDT-treated normal rats (not shown). This hypothesis is supported by the evidence found by others (29,30) that inhibition of nitric oxide (NO) synthase or scavenging NO may increase the efficacy of PDT through relieving the NO mediated suppression of the extravasation and tumoricidal activity of neutrophils. Indeed, NO can inhibit the adherence of neutrophils to the blood vessel wall (31) and inactivate the superoxide anion radical produced by activated granulocytes (32).

In conclusion, we found that under thrombocytopenic conditions, the efficacy

of PII-based PDT of the rhabdomyosarcoma R-1 increased. It seems likely that an increased accumulation of neutrophils in the PDT-treated tumor underlies this phenomenon.

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CHAPTER 7

General discussion

General discussion

The research described in this thesis was aimed at improving the efficacy of PDT. The vast majority of studies on PDT report about improving "conditional" aspects; i.e. the development of photosensitizers with absorption maxima at longer wavelengths, improved illumination techniques and protocols, the use of diverse routes of administration and vehicle of the sensitizer and so on. These are all important factors determining the primary tumor cell kill mediated by singlet oxygen. However, primary cell kill may not be sufficient for total tumor control. Secondary cell killing mechanisms are needed for complete cures as well. Therefore, a total understanding of the "basics" on how PDT works is essential. The results of our studies described in the previous chapters show the indispensable contribution of activated neutrophils for successful PDT. We found that the number of neutrophils in the circulation and in the tumor lesion increased upon PDT (Chapter 5,6). Definitive proof however was the finding that their depletion led to a considerable reduction in the treatment success rate (Chapter 4). An open question remains how these phagocytes improve the effect of PDT. Despite that, it is likely that this role of neutrophils in establishing the (long-term) treatment success is not restricted to PII-based PDT in rat rhabdomyosarcomas, but can also be valid for PII or other sensitizers in other experimental (tumor) models as well. For example, in murine squamous cell carcinoma tumors (1) or in EMT6 tumors (2) a rapid and massive accumulation of neutrophils after the start of PII-based PDT was found. Similar results were obtained when meta-TetraHydroxyPhenylChlorin (mTHPC) was used as a photosensitizer in the EMT6 tumor model (3). Also the illumination of mTHPC in normal trachea of pigs led to an intense inflammatory reaction with edema and infiltrating PMN cells (4). Moreover, one day after mTHPC-based interstitial PDT of the peritoneum of rats a shift in the differential counts of peripheral leucocytes from lymphocytes towards predominantly granulocytes was found (5). Clearly, these observations are circumstantial, but recently our conclusion about the role of neutrophils in the secondary effect of PDT has been confirmed in a murine tumor model. The anti-GR1 antibody which depleted neutrophils diminished the PDT induced cure rate in tumor-bearing mice (6). However, using the anti-CD 18 antibody which blocks the function of the β integrin chain of neutrophils the outcome was dependent on the tumor type. While the curative effect of PDT on the EMT6 tumor was completely abolished, surprisingly, the effect on the SCCVII tumor was markedly potentiated

(7). This latter result may indicate that other cell types than neutrophils are involved in the resistance to this squamous cell carcinoma.

The finding that granulocytes play an important role in the PDT induced damage to tumor cells (chapter 4) was very surprising. The general concept is that immune responses directed to the tumor normally consist of mainly tumor-infiltrating (cytotoxic) lymphocytes or macrophages rather than granulocytes. Evidence exists that (T)-lymphocytes indeed are important in the subsequent tumor control upon PDT. This evidence is based on the curative effect on PDT treated tumors of adoptively transferred bone marrow (8) or spleen cells (9) from mice cured by PDT to immunodeficient scid mice with a syngeneic tumor. More recently the use of specific antibodies against the T-cytotoxic lymphocyte determinant CD8 intervened with an effective PDT response (10). It seems not very likely that T-lymphocytes are involved in direct tumor cell kill since the notion that breast cancer and Karposi sarcoma patients with acquired immunodeficiency syndrome (AIDS) responded well to tin etiopurpurin-based PDT in terms of tumor cure (11). This is confirmed in an experimental mouse tumor model using anti-CD8 antibodies *in vivo* and *in vitro* to deplete these lymphocytes prior to adoptive immunotherapy in immunodeficient hosts to be treated with PDT (12, 6). Also if lymphocytes were depleted by anti lymphocyte antiserum prior to PDT in the rat rhabdomyosarcoma model, tumor growth retardation was not decreased, but was comparable to PDT treated control animals (de Vree, unpublished results). It is more likely that lymphocytes play a role in the generation of a specific tumor response after PDT. This may occur if direct damage to tumor cells leads to a massive accumulation of neutrophils. The occurrence of such a strong local inflammatory response may be regarded as characteristic for PDT enabling the immune system to mount an effective response to the tumor. In that respect PDT differs from radiation therapy for instance, which induces more apoptosis than necrosis of the tumor cells (12). Neutrophils attracted to the lesion site after PDT may degrade tumor cells that have escaped primary cell kill and attract lymphocytes by the release of chemotactic factors and cytokines. The cellular debris is then taken up by for example resident macrophages which are activated by photodynamically killed tumor cells (13). These macrophages can process this debris and function as antigen presenting cells (APC). Upon the presentation of these processed tumor cell antigens in the context of the proper MHC molecules, T-cells are activated and clonal expansion of T-cells recognizing tumor antigens leads to a specific memory immune response that will maintain

long-term resistance to rechallenge with the same tumor (12). Next to their role of non-specific effector cells evidence exists that granulocytes can act as APC themselves and thus may participate in the generation of a specific memory immune response as well (14). Whether this is the case in PDT induced specific immunity remains to be established.

PDT based on various types of photosensitizer often leads to vascular stasis and damage. This may seem an unwanted side-effect, because it limits the generation of toxic singlet oxygen. The general concept, however, is that this negative effect is fully compensated by the fact that the tumor cells will subsequently die as a consequence of the lack of oxgen. Experimental evidence for this is based upon the deterioration in clonogenicity of PDT treated tumor cells in time (15) and on the inhibitive effects on tumor cure of NSAID's that prevent vessel constriction (16-19). However these latter agents also affect granulocyte function (20). Therefore, the hypothesis that vascular damage due to blood flow stasis is essential to PII-based PDT at least needs shading. For preventing vascular stasis may also lead to increased tumor damage because it facilitates the accessibility of activated neutrophils to the tumor (chapter 6). We used a relative low light fluency for PDT. It could be reasoned that increasing the fluency would increase the direct tumor cell kill by PDT putting in perspective the necessity of the performance of the neutrophils. However, Veenhuizen & Stewart in attempt to classify the available literature on fluency rate effects in PDT came to the conclusion that the optimal fluence rate for PDT-induced tumor damage lies between 50 en 100 $mW.cm^{-2}$ (21). Furthermore, there are several recent studies in which low fluence rates or intermittence of illumination over several periods of time instead of one continuous period resulted in better tumor responses (22-27). This may be due to a renewed influx of oxygen so that more singlet oxygen can be generated. Although these approaches increase the direct effect of PDT and concomitantly the need for accessory mechanisms may decrease, the recent work of Korbelik et al. shows that even after a complete response of the tumor to PDT the presence of neutrophils cannot be missed to prevent remission (6).

To date Photofrin is the only sensitizer approved for clinical settings. In contrast to what is generally accepted we have shown here that vascular constriction may not be necessary per se for efficient PDT. Therefore, it remains to be established if second- and third-generation photosensitizers with less pronounced effects on the vasculature will indeed achieve better tumor responses than PII.

Taken together, the data presented in this thesis suggest the following sequence of events during and shortly after PDT (Figure 1).

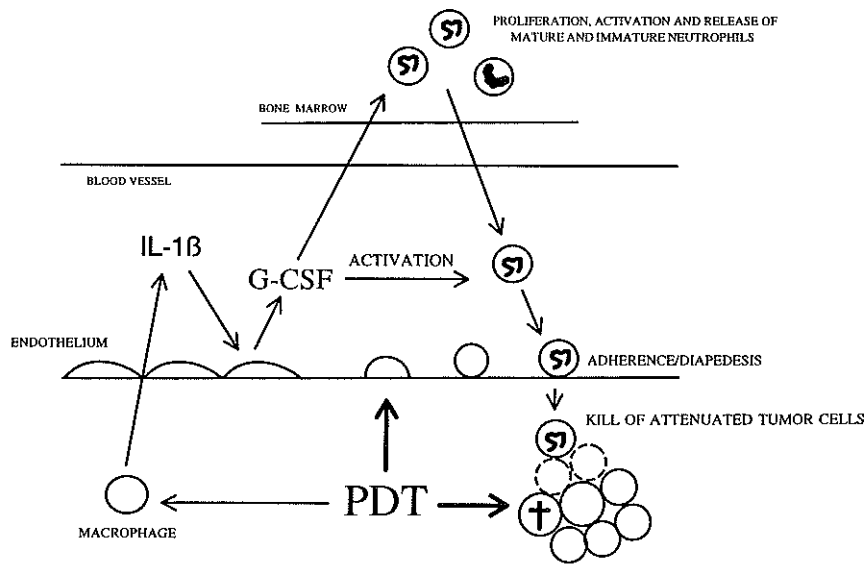

Figure 1. Proposed mechanism of tumor cell kill by PDT on the bases of the presented data in chapter 2 to 6.

PDT kills many tumor cells directly by the generation of singlet oxygen or other toxic molecules (directly killed tumor cells are represented by open circles with cross in Figure 1). At a certain distance from the capillaries the limited supply of oxygen diminishes the generation of singlet oxygen. As a result some tumor cells escape direct killing, and may become attenuated only (discontinuous circles) while other cells may even survive the treatment (open circles without cross). PDT not only kills the tumor cells but also affects the endothelial cells lining the wall of the tumor blood vessels. These latter cells contract upon PDT which results in the exposure of the subendothelial matrix. PDT did not increase the expression of cellular adhesion molecules on the endothelium and did not alter the production of

NO, prostacyclin or vWF of this tissue. Granulocytes (open circles with crinkle) adhere directly to the subendothelial matrix and then migrate to the lesion site attracted by the large amounts of debris released from killed tumor cells. Upon phagocytosis of this material neutrophils and resident macrophages produce pro-inflammatory cytokines like IL-1, IL-6 or TNF-α. IL-1β, which is demonstrable in the circulation after PDT, is capable of stimulating the subsequent production of G-CSF by endothelial cells and fibroblasts. G-CSF not only stimulates the production of neutrophils in the bone marrow, but activates neutrophils already present in the circulation as well. On their way to the lesion site, these activated neutrophils are hindered by thrombocytes adhering to the subendothelial matrix of the vessel wall and by stasis of the blood flow. However, once these neutrophils arrive into the tumor it is conceivable that large amounts of lysosomal proteinases and superoxide anions will be produced which kill the attenuated tumor cells. Furthermore, by their presentation of tumor antigens and the production of cytokines these phagocytes will enhance the specific immune response to the tumor preventing its regrowth.

Although the findings presented in this thesis are promising for increasing the efficacy of PDT, the use of G-CSF (and other growth factors) must be put into perspective. A recent report indicates that G-CSF can increase the invasive potential of a number of head-and-neck-carcinoma cell lines which express the G-CSF receptor (28). Moreover, Ferrario & Gomer (29) showed that PDT in the range of light doses required to obtain tumor cures induced a high level of acute lethality in mice consistent with a traumatic shock syndrome. Therefore further research is warranted before such an adjuvant therapy directed at increasing the number and activation status of neutrophils in the tumor can be safely applied in PDT-treated patients.

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Summary

The study described in this thesis was aimed at elucidating the mechanisms underlying the interaction between endothelial cells and granulocytes and at investigating the relevance of this interaction for the effect of PDT. First it was observed that granulocytes rapidly adhere to the vessel wall upon PDT. The mechanism underlying this adherednce was investigated in an *in vitro* model comprised of monocellular layers of human vascular endothelium and human granulocytes. In this model it was shown that granulocytes rapidly adhered after PDT dependent on sensitizer and light dose and on the time point of addition of granulocytes. Cellular adhesion molecules on the endothelial cells were not likely to play a role in the adhesion since their expression was not augmented by PDT. Also the adhesion of granulocytes could not be blocked by inhibitive antibodies to these molecules. In a related study it was shown that the increased neutrophil adherence was not dependent on a supposed decreased release of the anti-adhesive factors NO and prostacyclin by the PDT-treated endothelial cells. Antibodies directed to β_2-integrin adhesion receptors on granulocytes however blocked their adhesion substantially, showing the involvement of this ligand. Accurate macroscopic analysis of endothelial cells treated by PDT revealed a gradual contraction of the endothelium which coincided with the increased adhesion of granulocytes. Granulocytes only adhered to culture wells occupied by endothelial cells pointing at the involvement of the subendothelial matrix produced by these cells. At 4°C or by preincubation of the neutrophils with staurosporin their adherence to the subendothelial matrix exposed by PDT of endothelial cells could be prevented. Apparently, activation of the β_2-integrin receptor by interaction with the subendothelial matrix is necessary for the increased binding of neutrophils. These *in vitro* findings suggest that the PDT-induced contraction of the endothelial cells permits neutrophil adherence to the subendothelial matrix. It is conceivable that a similar mechanism contributes to the initial adherence of granulocytes to the vessel wall as observed after PDT *in vivo*.

Next we questioned whether the observation of adhering granulocytes was of relevance for the efficacy of PDT *in vivo*. This was studied in rhabdomyosarcoma-bearing rats in which the number of these phagocytes was decreased or increased before interstitial PDT by use of anti-rat neutrophil serum or G-CSF, respectively. The antiserum led to a complete depletion of circulating neutrophils. 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 growth rate occurred subsequent to an increase above the normal range of the number of circulating neutrophils. Administration of G-CSF on the other hand led to a specific increase in the numbers of circulating neutrophils. In these animals the tumor growth at day two after PDT was retarded as compared with PDT treated animals that received saline only. By statistical analyses of both experimental conditions it was shown that the efficacy of PDT was dependent on the number of circulating neutrophils present at the day of PDT. It is therefore concluded that neutrophils are indispensable for succesfull PDT *in vivo*.

Several investigators have observed a rapid accumulation of these neutrophils into the tumor lesion. In order to clarify the mechanism underlying this increased accumulation, we determined the effect of PDT on the course of mature and immature neutrophils in the circulation of rhabdomyosarcoma-bearing rats, and studied the changes in the level of IL1β as an important stimulator of the proliferation of precursor cells of granulocytes in the bone marrow. It was shown that the effect of PDT on tumor growth was preceded by a rapid and specific increase of the number of mature neutrophils in the peripheral blood reaching maximum values after 8 h. This increment was preceded by an increase of band neutrophil numbers and elevated serum levels of IL-1β being maximal at 2 h after the start of PDT. Further investigation showed that injection of anti G-CSF antibodies not only attenuated the increase in neutrophil numbers, but also decreased the efficacy of PDT. On this basis it is supposed that an IL-1-induced release of G-CSF by PDT underlies the nonspecific immune reaction to the tumor. Apparently, G-CSF not only stimulates the production rate of neutrophils in the bone marrow, but also increases their functional activity to become the indispensable tumor cell killers.

Next to neutrophils also thrombocytes can adhere to the exposed subcellular matrix causing blood flow obstruction after PDT. To investigate whether preventing this obstruction during PDT will improve the efficacy of the therapy, the number of thrombocytes in rhabdomyosarcoma-bearing rats was decreased before the start of PDT by the administration anti-rat thrombocyte antiserum. This treatment led to a depletion of thrombocytes and subsequent tumor growth retardation as compared to control animals. However treatment of animals with antiserum after PDT led to the same increased delay of tumor growth, thereby ruling out the possibility that enhanced production of singlet oxygen underlied the increased efficacy of PDT. Since we found that the myelopeoxidase content of the tumors upon PDT was increased under thrombocytopenia to a much higher level as

compared to normal rats, we then questioned whether the increased contribution of neutrophils to tumor growth retardation could be the underlying mechanism. To answer this the number of granulocytes was decreased by the administration of anti-granulocyte antiserum prior PDT followed by anti-thrombocyte antiserum post PDT. This treatment regimen led to a considerable loss of the thrombocytopenia-dependent gain in the efficacy of PDT. Apparently neutrophils underlie the observed enhanced efficacy of PDT under thrombocytopenia as well. It is hypothesized that in the absence of blood flow obstruction the attenuated tumor cells will be better accessible to these activated tumor cell killers. This would imply that under normal clinical conditions the full granulocyte-dependent killing potential of PDT is not utilised due to the presence of thrombocytes.

Samenvatting

Het onderzoek beschreven in dit proefschrift behelsde de interactie tussen endotheel cellen en granulocyten en het belang van deze interactie voor het effect van PDT . Er is waargenomen dat granulocyten snel aan de vaatwand kunnen hechten als gevolg van PDT. Het mechanisme dat ten grondslag ligt aan deze adhesie werd bestudeerd aan de hand van een *in vitro* model bestaande uit een monolaag van humaan vasculair endotheel cellen en humane granulocyten. Ook in dit *in vitro* model is aangetoond dat granulocyten snel hechten na PDT. De mate van hechting was afhankelijk van de gebruikte hoeveelheid fotosensitizer en licht en het tijdstip waarop granulocyten werden toegevoegd. Cellulaire adhesie moleculen op de endotheel cellen speelden waarschijnlijk geen rol in deze adhesie omdat hun expressie niet verhoogd was door PDT. Ook kon de hechting van granulocyten niet worden geremd met blokkerende antilichamen gericht tegen deze adhesie moleculen. In een gerelateerde studie werd aangetoond dat de verhoogde aanhechting van neutrofielen ook niet afhankelijk was van een veronderstelde verlaging van de anti-adhesie factoren NO en prostacycline door de PDT-behandelde endotheel cellen. Antilichamen gericht tegen de β_2-integrine adhesie receptoren op granulocyten blokkeerden hun adhesie echter grotendeels wel, wat de betrokkenheid van dit ligand bij de hechting aantoont. Nauwkeurige macroscopische analyse van PDT-behandelde endotheel cellen onthulde een graduele contractie van het endotheel welke samenviel met een toenemende adhesie van granulocyten. Granulocyten hechtten alleen aan kweek platen waarop endotheel cellen hebben gezeten. Dit wijst op de betrokkenheid bij de hechting van de subendotheliale matrix geproduceerd door deze cellen. Bij 4°C of door pre-incubatie van neutrofielen met staurosporine kon hun adhesie aan de subendotheliale matrix, die dus vrijkomt na PDT behandeling van het endotheel, worden voorkomen. Blijkbaar is activatie van de β_2-integrine receptor door interactie met de subendotheliale matrix nodig voor een verhoogde hechting van neutrofielen. Deze *in vitro* bevindingen suggereren dat de, door PDT-geïnduceerde, contractie van het endotheel neutrofiel adhesie aan de subendotheliale matrix mogelijk maakt. Het is zeer wel voorstelbaar dat een vergelijkbaar mechanisme bijdraagt aan de initiële adhesie van granulocyten aan de vaatwand zoals geobserveerd na *in vivo* PDT.

Vervolgens vroegen we ons af of de observatie van adhererende granulocyten wel van belang was voor de efficiëntie van PDT *in vivo*. Dit werd bestudeerd in ratten getransplanteerd met rhabdomyosarcoma tumoren. Het aantal van deze

fagocyten werd verlaagd of verhoogd vóór interstitiële PDT met behulp van respectievelijk anti-rat neutrofiel antiserum of G-CSF. De injectie van het antiserum had een complete depletie van circulerende neutrofielen tot gevolg. Onder deze omstandigheden werd de groei van de tumor na PDT behandeling helemaal niet geremd. Echter, toen de antiserum behandeling 5 dagen na PDT werd stopgezet trad een onverwachte verlaging van de groeisnelheid van de tumoren op, tegelijk met een toename van het aantal circulerende neutrofielen tot boven het normale niveau. Injectie van G-CSF vlak vóór interstitiële PDT veroorzaakte een snelle specifieke toename van het aantal circulerende neutrofielen. In deze dieren was de tumor groei 2 dagen na PDT vertraagd in vergelijking met ratten die alleen fysiologisch zout ontvingen. Na statistische analyse van beide experimenten bleek dat de efficiëntie van PDT afhankelijk was van het aantal circulerende neutrofielen dat aanwezig was op de dag van PDT behandeling. Daarom mag worden geconcludeerd dat neutrofielen onmisbaar zijn voor een succesvolle PDT *in vivo*.

Verscheidene onderzoekers hebben een snelle ophoping van deze neutrofielen waargenomen in de tumor laesie na PDT. Om het mechanisme dat ten grondslag ligt aan deze ophoping op te helderen bepaalden we het effect van PDT op het aantal volgroeide en onvolgroeide neutrofielen in de circulatie van ratten met rhabdomyosarcomas. Bovendien zijn de veranderingen in het serum niveau van IL1β -een belangrijke stimulator voor de proliferatie van voorloper cellen van granulocyten in het beenmerg- bestudeerd. Het bleek dat het effect van PDT op de tumor groei vooraf werd gegaan door een snelle en specifieke toename van het aantal volgroeide neutrofielen in het perifere bloed met maximale waarden 8 uur na PDT. Deze toename werd vooraf gegaan door een toename van het aantal staafkernige (onvolgroeide) neutrofielen en een verhoogd serum niveau van IL-1β met een maximum op 2 uur na de start van PDT. Nader onderzoek toonde aan dat na injectie van anti G-CSF antilichamen niet alleen de toename van het aantal neutrofielen verminderde maar dat ook de efficiëntie van PDT verslechterde. Om deze reden veronderstellen wij dat een IL-1 geïnduceerde productie van G-CSF door PDT de non-specifieke immuun reactie tegen de tumor tot gevolg heeft. Blijkbaar stimuleert G-CSF niet alleen de productie van neutrofielen in het beenmerg maar ook hun functionele activiteit met als gevolg dat ze tumor cel killers worden.

Naast neutrofielen kunnen ook thrombocyten hechten aan de geëxposeerde subcellulaire matrix. Dit kan na PDT gepaard gaan met een (tijdelijke) obstructie van de bloedstroom en daarmee mogelijk met een minder efficiente therapie. Om te bestuderen of het voorkomen van deze obstructie tijdens PDT de efficiëntie van de

therapie zal verbeteren, werd het aantal thrombocyten in ratten met rhabdomyosarcomas verlaagd voor de start van PDT door injectie van anti-rat thrombocyten antiserum. Deze behandeling leidde, zoals verwacht, tot een depletie van thrombocyten en had een vertraagde tumor groei in vergelijking met controle dieren tot gevolg. Echter, behandeling van dieren met antiserum na PDT leidde tot dezelfde mate van vertraging van tumor groei, hetgeen de mogelijkheid dat een verhoogde productie van singlet oxygen de verhoogde efficiëntie van PDT veroorzaakt uitsluit. Omdat we vonden dat het myeloperoxidase gehalte van tumoren na PDT onder thrombocytopenie was verhoogd tot een veel hoger niveau dan bij normale ratten, vroegen we ons af of een verhoogde bijdrage van neutrofielen aan de tumor groei vertraging ten grondslag lag. Om deze vraag te beantwoorden werd het aantal granulocyten verlaagd door toevoeging van anti-granulocyten antiserum vóór PDT gevolgd door het anti-thrombocyten antiserum na PDT. Deze behandeling leidde tot een aanzienlijke daling van de onder thrombocytopenie verkregen winst in de efficiëntie van PDT. Blijkbaar zijn neutrofielen dus ook verantwoordelijk voor de onder thrombocytopenie verkregen verhoogde efficiëntie van PDT. Onze hypothese luidt dan ook dat in de afwezigheid van obstructie van de bloedstroom de verzwakte tumor cellen beter bereikbaar zijn voor deze geactiveerde tumor cel killers. Dit zou impliceren dat onder normale klinische condities het volledige granulocyt-afhankelijke kill potentieel van PDT niet gebruikt wordt door de hinderlijke aanwezigheid van thrombocyten.

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Wil J.A. de Vree, Henrïette S. de Bruijn, Regina G. Kraak-Slee, and W. Sluiter. The effect of thrombocytopenia on the efficacy of photofrin-based photodynamic therapy *in vivo* (submitted for publication).

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Curriculum vitae

De schrijver van dit proefschrift werd geboren op 20 januari 1964 te Batenburg. Na het behalen van het VWO diploma aan het Pax Christi College te Druten in 1982 werd in september van datzelfde jaar begonnen met de studie biologie aan de Katholieke Universiteit te Nijmegen. Het doctoraal werd behaald in 1988 en omvatte o.a. de bijvakken immunologie (afdeling nefrologie, Academisch Ziekenhuis Nijmegen o.l.v. Prof. Dr. P.J.A. Capel) en moleculaire biologie (ITAL Wageningen o.l.v. Dr. B. Visser) en het hoofdvak microbiologie (afdeling microbiologie, Katholieke Universiteit Nijmegen o.l.v. Prof. Dr. Ir. G.D. Vogels). Van februari 1989 tot september 1991 was hij als wetenschappelijk medewerker verbonden aan de afdeling reumatologie van het Leids Universitair Medisch Centrum te Leiden o.l.v. Prof. Dr. F.C. Breedveld en Prof. Dr. M.R. Daha. Van oktober 1991 tot maart 1993 werd de vervangende dienstplicht verricht op de afdeling endotheel en lipiden van het TNO instituut voor Preventieve Gezondheidszorg te Leiden o.l.v. Prof. Dr. V.W.M. van Hinsbergh. Van maart 1993 tot april 1998 was hij als wetenschappelijk medewerker verbonden aan de afdeling biochemie van de Erasmus Universiteit te Rotterdam alwaar o.l.v. Prof. Dr. J.F. Koster en Dr. W. Sluiter het onderzoek werd verricht dat is beschreven in dit proefschrift.