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Acute phase response-associated systemic neutrophil mobilization in mice bearing tumors treated by photodynamic therapy

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Abstract

Photodynamic therapy (PDT) inflicts tumor tissue injury that is experienced by the host as a local trauma. This provokes a strong host response with pronounced neutrophilia as one of its manifestations. Mouse FsaR fibrosarcoma model was used for investigating photodynamic therapy (PDT)-induced neutrophilia and its link to the acute phase response. Compared to normal mice, the extent of neutrophilia induced following Photofrin-based tumor PDT in adrenalectomized host mice was less pronounced revealing the elicited engagement of the adrenal–pituitary axis, which is one of the principal characteristics of the acute phase response. Neutrophilia was demonstrated after tumor-localized PDT even in the host mice previously depleted of circulating neutrophils. The rise in serum levels of complement C3 protein, which is an acute phase reactant and a principal mediator of tumor PDT-induced neutrophilia, occurred at the post PDT time period when the neutrophilia was largely resolved. However, the activation of complement system (assessed by the standard erythrocyte hemolysis assay) peaked already at 6 h after PDT and correlated with the time kinetics of PDT-induced neutrophilia. The findings of this study uncover the link between tumor PDT-induced neutrophilia and key acute phase response manifestations, the activation of adrenal–pituitary axis and the expression of a complement C3 protein (major acute phase reactant).

Keywords: Photodynamic therapy; Neutrophilia; Acute phase response; Complement system

1. Introduction

Photodynamic therapy (PDT) has become an established treatment option for a variety of pre-malignant, malignant and non-cancerous lesions [1,2]. The destruction of targeted lesions is achieved by localized generation of photooxidative damage in biological molecules that is initiated by light-mediated excitation of drugs that can in this state interact with molecular oxygen to produce reactive oxygen species [3]. Treatment of solid tumors by PDT inflicts tissue injury at the targeted site that is experienced by the host as a local trauma provoking canonical responses evolutionally developed for dealing with this type of threat [4,5]. This host response associated with PDT treatment is of vital importance for the therapy outcome, because it includes activities of inflammatory and immune effectors that contribute to both early-phase nonspecific destruction of cancer cells and their long-term immune rejection [1,4,5].

It has recently become evident that the host reaction elicited by PDT-based tumor treatment is also manifested as

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an acute phase response [5,6]. Systemic changes associated with inflammation distant from inflammatory sites comprising the acute phase response include altered plasma concentrations of various proteins (acute phase reactants), enhanced hormone synthesis by adrenal and pituitary glands, and increased blood leukocyte levels [7,8]. The main purpose of the acute phase response is to create an overall protective environment required for coping with significant tissue injury and to contain the disrupted homeostasis. In relation to PDT, acute phase response was first mentioned in a study on normal peritoneum treatment describing the induction of leukocytosis and elevation of plasma levels of prototypic acute phase reactant in mice, serum amyloid P (SAP) [9]. Subsequently, the induction of neutrophilia was reported following tumor treatment by PDT [10–12]. Further evidence of instigation of acute phase response in hosts bearing PDT-treated tumors was provided in a recent report documenting the elevation of plasma levels of another important acute phase reactant, complement C3 protein [13].

In the present communication, we are further exploring the basic elements of PDT associated acute phase response and neutrophilia.

2. Materials and methods

2.1. Tumor model

Mouse FsaR fibrosarcoma [14] was maintained in syngeneic C3H/HeN mice by biweekly intramuscular passage. Cohorts of experimental tumors were implanted from cell suspensions prepared by enzymatic digestion of tumor tissue, with 1×10^6 cells per mouse injected subcutaneously at the sacral region on the dorsal side of 8–9 week old C3H/HeN females. In some experiments, FsaR tumors were implanted into mice of the same strain that were purchased as adrenalectomized from Charles River Canada (Saint-Constant, Quebec, Canada). The tumors were treated when reaching 6–8 mm in largest diameter (7–8 days after implant). All mouse protocols were approved and controlled by the Animal Ethics Committee of the University of British Columbia.

2.2. PDT treatment

Tumor-bearing mice were administered Photofrin (Axcan Pharma Inc., Mont-Saint-Hilaire, Quebec, Canada) by intravenous injection at a dose of 10 mg/kg. The tumors were treated 24 h later with 630±10 nm light to 150 J/cm^2 at average fluence rate of 100 mW/cm^2. A high throughput fiber illuminator (Sciencetech Inc., London, Ontario, Canada) equipped with a 150 W QTH lamp, integrated ellipsoidal reflector, and 630±10 nm interference filter was used for generating light that was delivered through an 8 mm core diameter liquid light guide (model 77638, Oriel Instruments, Stratford, CT) for superficial tumor illumination.

2.3. Neutrophil analysis

Blood samples collected from the tail vein were used for preparing air-dried smears on glass slides and for hemacytometer counts (after selective erythrocyte lysis). Only one blood collection was done from each mouse to minimize nonspecific stress induced by multiple collections [11]. Absolute numbers of blood neutrophils were derived by multiplying the total number of leukocytes from hemacytometer counts with the proportion rate of neutrophils obtained from differential counts from Wright's stained smears. In some experiments, lung, spleen, liver and bone marrow were taken from the same animals. Lung and liver tissues were digested in an enzyme cocktail containing collagenase, dispase and DNAse to obtain cell suspensions that were first subjected to erythrocyte lysis, and then used for hematocytometer counts and for preparing Wright's stained smears for differential neutrophil counts. Absolute neutrophil numbers in the bone marrow samples were expressed per femur, while those from lung, liver, and spleen were per tissue weight. Neutrophil depletion in mice was performed as described in detail earlier [15], by injecting 0.1 mg/mouse i.p. of rat anti-mouse GR1 (Ly-6G) antibody obtained from RB6-8C5 hybridoma. When combined with PDT treatment, anti-GR1 was administered 30 min before the onset of photodynamic light treatment (tumor illumination lasted around 15 min). Four identically treated mice were included in each experimental group.

2.4. Complement-mediated hemolysis assay

Serum obtained from the collected blood was diluted 10× in gelatin veronal buffer (Sigma Chemical Co., St. Louis, MO) and used for analyzing alternative complement pathway-dependent erythrocyte lysis employing standardized techniques [16] as described previously [11]. Briefly, hemolysis of rabbit erythrocytes was determined after 1 h incubation at 37 °C in the presence of ethylene glycol-bis(aminoethylether)-tetraacetic acid (EGTA) and magnesium salts based on absorbance reading at 412 nm. Each treatment group consisted of four mice.

2.5. Statistical analysis

Variance analysis with one-way ANOVA and post hoc comparison based on LSD test were used for determining difference between treatment groups, with $p<0.05$ considered a statistically significant difference.

3. Results

3.1. Role of adrenal hormones in PDT-induced neutrophilia

An important element of the acute phase response is the engagement of the adrenal–pituitary axis, accompanied with

enhanced release of adrenocortical hormones [8]. Since neutrophilia is the most prominent hematological alteration induced by these hormones [17], blood neutrophil numbers following Photofrin-based PDT of subcutaneous FsaR tumors were determined in adrenalectomized and normal host mice. As shown previously with various mouse and rat tumor models [10,11], the treatment of tumors by PDT (choosing a dose that cures about 50% of treated tumors) provokes a rapid and large increase in blood neutrophil counts in the host mice that decline by 24 h post therapy (Fig. 1). Neutrophilia (defined as the occurrence of any blood neutrophil levels significantly higher than normal) induced by tumor PDT in normal mice was found to vary in magnitude from experiment to experiment (as exemplified by differences seen between Figs. 1 and 2), but significant rises in neutrophil counts were consistently demonstrated in all our experiments. This neutrophilia is characterized by two phases, an early phase seen in Fig. 1 to culminate around 3 h after PDT and advanced phase that peaks around 8–10 h after PDT [11,12]. The two phases are promoted by different chemotactic mediators and reflect different roles of neutrophils in the early and advanced phases of the induced host response [5]. The rise in neutrophil levels was less pronounced in adrenalectomized mice bearing PDT-treated tumors than in corresponding normal mice, with particularly large differences evident at the 3 and 8 peak hours after PDT treatment (Fig. 1). Neutrophil level changes in the light only treatment groups (the best controls for all relevant nonspecific shock associated with handling of mice [11]) indicate that PDT-unrelated effects were an important contributing factor to neutrophilia at 1 h time-point, but were much less significant at later time-points (Fig. 1 insert).

3.2. PDT-induced neutrophilia in "neutrophil-depleted" mice

The neutrophilia observed after tumor-localized PDT treatment was shown to be instigated by powerful chemotactic signals emanating from PDT-treated tumors that sequester neutrophils from their non-circulating pools [11,12]. To further

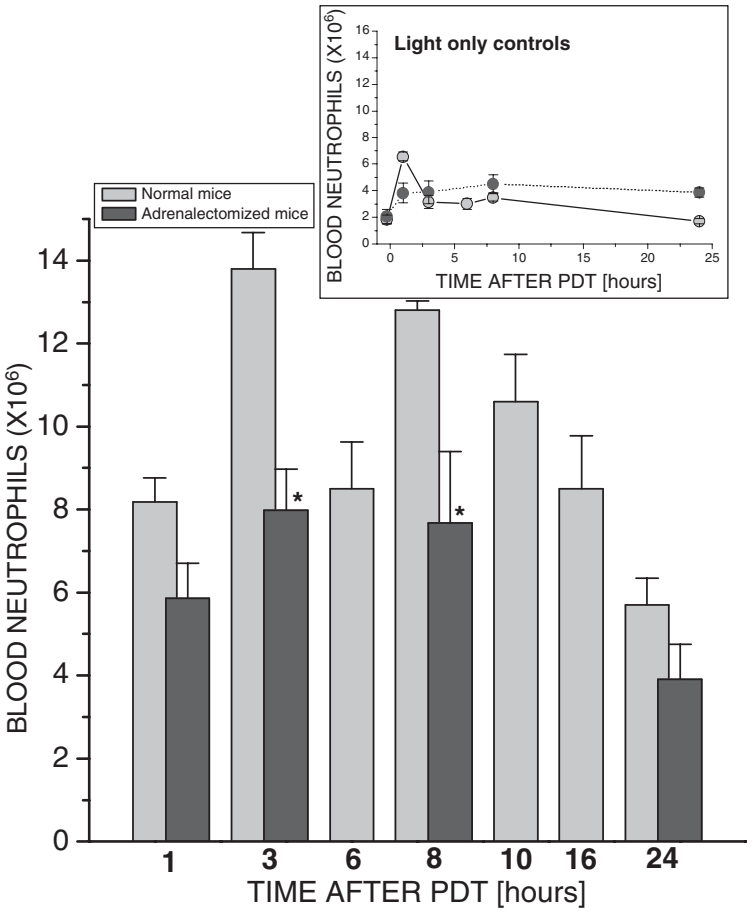

Fig. 1. PDT-induced neutrophilia in normal and adrenalectomized mice. Blood samples for total and differential cell counts were collected at indicated times from mice bearing FsaR tumors treated by PDT (Photofrin 10 mg/kg; 150 J/cm^2). Values in the ordinate are the absolute numbers of neutrophils per ml of blood. Blood neutrophil level in untreated normal mice was 1.85±0.31 (SE), and in untreated adrenalectomized mice was 2.07±0.55 (SE)×10^6 per ml. The effects on neutrophil counts in control groups exposed to light only (no Photofrin) are depicted in the insert. N=4, bars are SE; *value statistically different than the corresponding value obtained with normal mice (p<0.05).

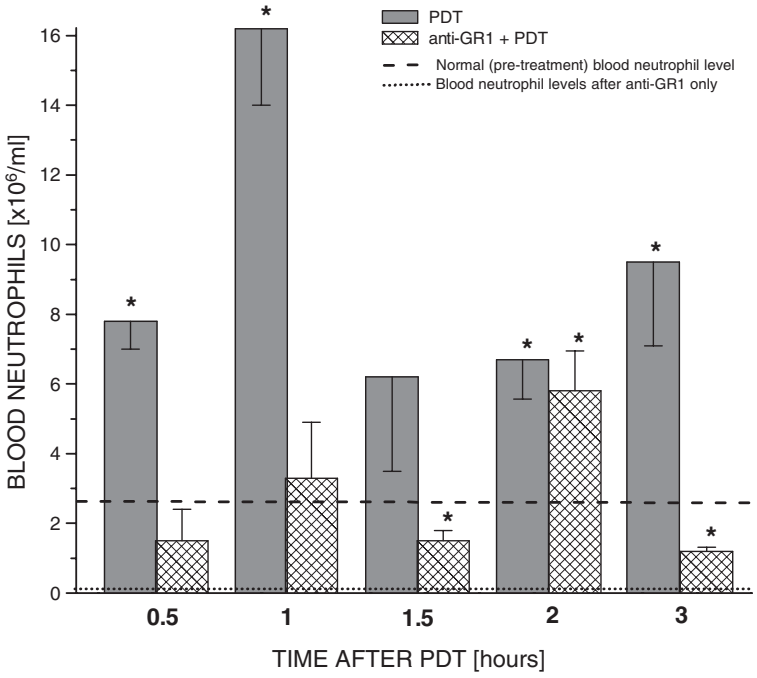

Fig. 2. PDT-induced neutrophilia in mice treated for neutrophil depletion. FsaR tumors growing in normal C3H/HeN mice were PDT treated as described for Fig. 1. Anti-mouse GR1 antibody (0.1 mg/mouse) was injected i.p. at 30 min before the onset of photodynamic light treatment. Blood samples were collected at different times after therapy and used for determining the levels of circulating neutrophils. Blood neutrophil level in control untreated mice was 2.59 ± 0.80 (SE) $\times 10^6$ per ml. $N=4$, bars are SE; *value statistically different than the normal (pre-treatment) neutrophil level ($p<0.05$).

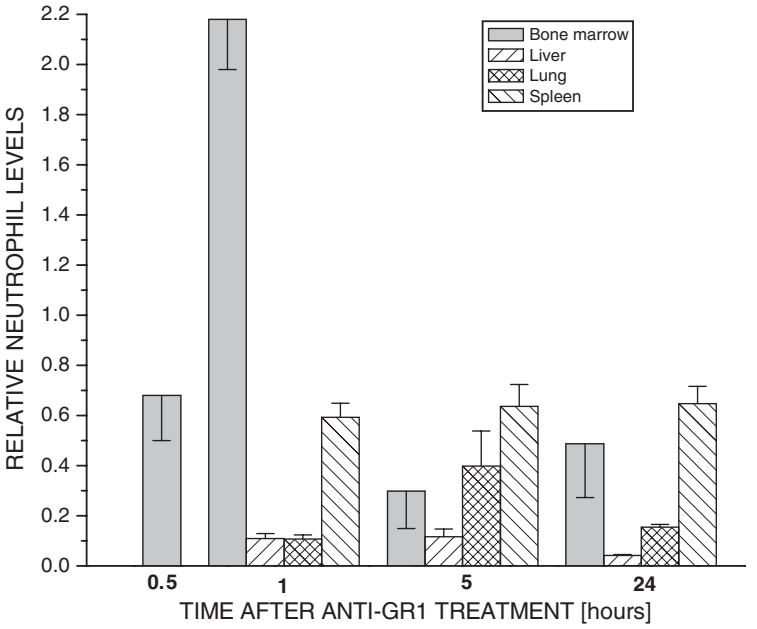

Fig. 3. Neutrophil levels in the bone marrow, liver, spleen, and lungs of control mice (not treated by PDT) injected with neutrophil-depleting antibody. Indicated tissues were collected from mice sacrificed at different times after they were administered anti-mouse GR1 antibody as described for Fig. 2. Neutrophil levels determined in these samples are shown as values relative to the pre-treatment level. $N=4$, bars are SE; all the depicted values are statistically different than the pre-treatment levels ($p<0.05$).

investigate the potency of these neutrophil-mobilizing signals generated by PDT, mice were subjected before PDT to a standard neutrophil depletion treatment using i.v. administration of antibodies raised against mouse myeloid membrane antigen GR1. This immunodepletion protocol reduces the numbers of circulating neutrophils (in control mice) to ≤5% of their pretreatment levels (dotted line in Fig. 2). However, when these "neutrophil-depleted" mice were treated by tumor-localized PDT there was a remarkable increase in their circulating neutrophil levels. A marked recovery in neutrophil numbers was observed in these mice already at 30 min after the termination of photodynamic light delivery, and levels comparable to normal were detected at 1 h post PDT (Fig. 2). After a temporary decline, the neutrophil levels in these "neutrophil-depleted" mice rose even further reaching about twice the normal level at 2 h post PDT, and were similar to those found in PDT-treated mice that were not subjected to the neutrophil depletion protocol. After this time-point neutrophilia increased again in PDT only treated mice but it could not be matched in "neutrophil-depleted" mice.

3.3. Neutrophil levels after depletion treatment

In order to better understand this PDT-induced neutrophilia in "neutrophil-depleted" mice, neutrophil levels in various organs of control mice (not treated by PDT) were examined at various times following neutrophil depletion treatment (Fig. 3). The results reveal that while neutrophil numbers in the liver and lungs were reduced to the extents comparable with blood, this was not the case in the spleens and especially not in the bone marrow. Relatively high neutrophil levels in the spleens could be explained by the role of this organ as the body's largest filter of the blood and the site of elimination of neutrophils (opsonized with complement proteins bound to GR1 antibodies). However, even more striking are high neutrophil levels in the bone marrow. At 30 min after anti-GR1 injection the bone marrow neutrophil numbers were around 70% of the pre-treatment levels, and this was followed 30 min later by a dramatic surge in the number of these cells exceeding the normal levels more than two-fold (Fig. 3). Such surge is presumably achieved by rapidly upregulated maturation of neutrophil precursors in the bone marrow; this is supported by our finding of a several-fold increase in the prevalence of band neutrophils at this site at 45 min after anti-GR1 injection (data not shown). Hence, regenerated pools in the bone marrow seem to be a major source for the neutrophils re-appearing in the circulation of "neutrophil-depleted" mice following PDT.

3.4. PDT-induced complement activation

Activation of the complement system following tumor-localized PDT was identified as a major event responsible for the elaboration of potent neutrophil chemoattractants [11,12], which are capable of sequestering into circulation neutrophils that would normally remain in non-circulating pools. A previous study with mouse Lewis lung carcinoma model showed that serum levels of C3 (key protein in the complement cascade and acute phase reactant) after an initial decline significantly increased in the host mice at 24 h after PDT [13]. Similar results were obtained with the FsaR tumor model; for instance, the levels of C3 (means±SD) determined in serum obtained from mice treated as described for Figs. 1 and 2 were 1.00±0.19, 0.61±0.05, 1.81±0.16, and 1.80±0.35 mg/ml for non-treated controls, and 6, 24, and 72 h post PDT groups, respectively. Hence, these changes in serum C3 levels show no correlation with the development of PDT-induced neutrophilia. However, neutrophil migration is not influenced by the C3 protein itself but by anaphylatoxins C3a and C5a [12,18] which are fragments liberated by the cleavage of C3 and C5 upon complement cascade activation. Hence, the assessment of the activation status of the complement system for deciphering the impact on neutrophil trafficking is more relevant than the C3 level determination. This was obtained by measuring the extent of the lysis of erythrocytes incubated with serum samples collected from PDT-treated mice, which is a standard hemolytic assay for documenting complement engagement via the alternative pathway. The results reveal that complement activity became elevated immediately after PDT, increased about two-fold at the peak interval at 6 h post PDT, remained elevated during the next 6 h, and declined to the pre-treatment levels at 24 h post PDT (Fig. 4). Thus, the time kinetics of post PDT complement activation correlates with the time scale of PDT-induced neutrophilia.

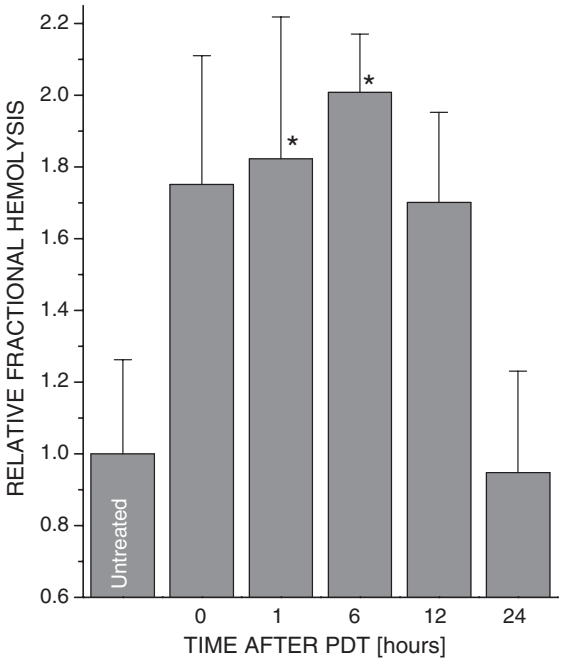

Fig. 4. Complement-mediated hemolytic activity in serum of mice bearing PDT-treated tumors. FsaR tumors were treated by PDT and blood collected from mice as described for Fig. 1. The serum samples obtained from the blood were used in the rabbit erythrocyte lysis assay that reveals the extent of complement activity depending on its alternative pathway. $N=4$, bars are SE; *value statistically different than that obtained with samples from PDT-untreated mice ($p<0.05$).

4. Discussion

The evidence presented in this study reveals the scope of systemic impact of tumor PDT by characterizing the avidity of systemic neutrophil mobilization in the development of the induced neutrophilia, and uncovering its link with the activation of adrenal–pituitary axis and the expression of complement C3 as acute phase reactant. The engagement of the endocrine system, a major hallmark of the acute phase response, was demonstrated using adrenalectomized mice and shown that adrenal hormones have a significant role in the expression of PDT-induced neutrophilia. The release of adrenal hormones is linked with the intensity of induced inflammatory reaction, and, therefore, will be more pronounced with PDT doses that induce stronger inflammatory response. Reinforced in this study are also recent findings with a different tumor model and mouse strain on post PDT rise in systemic levels of complement C3 protein [13], which is a major acute phase reactant [19]. Neutrophilia, manifested as a component of PDT-induced acute phase response, was already described in our previous reports [11,12,18] and further insight allowing its better understanding is provided in this study.

The potency of chemotactic signals for neutrophils emanating after PDT treatment is illustrated by the exhibition of neutrophilia in mice previously subjected to a standard neutrophil depletion treatment. Neutrophil depletion in mice using the same antibody as in this work (rat anti-mouse GR1 (Ly-6G), clone RB6-8C5) is routinely performed in mouse studies, as it reduces the levels of blood neutrophils in the treated mice by >95% [15,20,21]. However, the results depicted in Fig. 2 demonstrate that it is erroneous to consider the mice subjected to this anti-GR1 treatment as truly neutrophil-depleted since high numbers of neutrophils can rapidly re-appear in the blood of these mice in the case of a strong pro-inflammatory insult. This prompted us to examine in more detail the levels of neutrophils in various organs of PDT-untreated mice after they were injected with anti-GR1. The results reveal that in the bone marrow neutrophils depleted by anti-GR1 are rapidly replenished by what seems to be highly accelerated differentiation of neutrophil precursors and their maturation (Fig. 3). Such acceleration in the maturation rate of less differentiated precursors in the bone marrow is promoted by cytokines IL-1 and G-CSF [22] that are known to be induced by PDT treatment [10]. Hence, this compensatory mechanism may be additionally boosted in hosts bearing PDT-treated tumors. In addition, relatively high neutrophil levels were found persisting in the spleen (Fig. 3), the organ which normally holds a large part of the marginating pool of these cells and the site where anti-GR1 induced neutrophil clumps and neutrophils with bound immune complexes are filtered. An insight into this may be derived from a similar situation in humans occurring in the Felty's syndrome, as in this condition (caused by neutrophil autoantibodies and characterized by neutropenia due to the retention of these cells in the spleen) the residual marginating pools of neutrophils are shifted towards the spleen [23].

A particularly extensive rebound in the level of bone marrow neutrophils can be detected at 1 h after anti-GR1 injection. In absolute numbers, over 8 million of these cells per femur were detected at this time-point, which is more than sufficient for providing the source of circulating neutrophils shown in Fig. 2. These restocked neutrophils would normally regenerate non-circulating pools in the bone marrow and other sites if they were not opsonized by anti-GR1 antibody and eliminated by complement. During the first several hours after the administration of anti-GR1, the bone marrow is attempting to compensate for the loss of depleted neutrophils (by maximizing its capacity for the production of new neutrophils) until its reserves of immature neutrophil precursors are exhausted. Our results suggest that PDT-induced neutrophil chemotaxis is so powerful that it can mobilize from the bone marrow into the circulation these newly produced neutrophils at a faster rate than the rate of their anti-GR1-mediated immunodepletion.

As shown in earlier reports [10–12,18], multiple mediators including complement, cytokines (IL-1, IL-6, G-CSF, TNF-α, IL-10), chemokines, histamine, arachidonic acid metabolites, and coagulation factors are responsible for the development of PDT-induced neutrophilia. The findings of the present study add adrenal hormones to this list. Low to intermediate levels of adrenocortical hormones are known to exhibit stimulatory effects on neutrophil migration, while high levels are inhibitory [24]. The stimulatory effects have been attributed to both demarginalization of neutrophils increasing their influx from non-circulating pools and to decreasing the egress of neutrophils to inflammatory exudates [25]. This appears to result from the enhancement by adrenocortical hormones of the expression of cytokine receptors on various cells and of pro-inflammatory cytokine release (the impact of the latter including altered expression of leukocyte adhesion molecules) [24].

Interestingly, C3 protein appears to get engaged (as a source of anaphylatoxins) in PDT-induced neutrophilia

before the rise of its levels as an acute phase reactant. Indicative in this respect is the drop in serum level of this protein observed during the first several hours after PDT that can be explained by its consumption (generating anaphylatoxins and other active fragments) associated with the activation of the complement system. During this period preceding the rise in systemic C3 levels triggered by its production as acute phase reactant, a critical source of C3 and other complement proteins could be tumor-associated leukocytes because they exhibit upregulated expression of genes encoding these proteins [26].

It can be concluded that, during the first day post PDT, acute phase response manifests principally as a strong neutrophilia whose purpose is to maximize the mobilization of neutrophils for their deployment as inflammatory and innate immune effectors in treated tumors [4,5,27]. While adrenal hormones and probably certain acute phase proteins become involved in supporting/regulating PDT-induced neutrophilia, other acute phase reactants mediate important functions of acute phase response (such as removal of dead cells and local healing) during the later phase peaking between one and three days post PDT. C3 protein belongs to this latter group of acute phase reactants that include several other complement proteins, as well as serum amyloid P (SAP) and other pentraxins [5,6]. Although acute phase response is elicited after PDT treatment of both normal and tumor tissue, and is orchestrated within the tumor antigen-nonspecific host-protecting action mediated by the innate immune system, owing to its relevance for the process of dead cell disposal it has an important indirect impact on the development of antigen-specific immune response against PDT-treated tumors [5].

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