

Mediators of peripheral blood neutrophilia induced by photodynamic therapy of solid tumors

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Abstract

Photodynamic therapy (PDT) of tumors elicits a strong host immune response and one of its manifestations is a pronounced neutrophilia. By blocking their function prior to Photofrin-based PDT of mouse EMT6 tumors, we have identified multiple mediators whose regulated action is responsible for this neutrophilia. In addition to complement fragments (direct mediators) released as a consequence of PDT-induced complement activation, there are at least a dozen secondary mediators that all arise as a result of complement activity. The latter include cytokines IL-1 β , TNF- α , IL-6, IL-10, G-CSF and KC, thromboxane, prostaglandins, leukotrienes, histamine, and coagulation factors. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Although photodynamic therapy (PDT) is now an established clinical treatment for a variety of malignant and non-malignant diseases, its mechanism of action is still under intense investigation and there is excellent potential for further development of this modality [1]. Recent advances in the understanding of PDT-mediated destruction of solid tumors make it increasingly clear that the elicited engagement of host inflammatory and immune responses has a crucial role in the therapeutic outcome [1–3]. It has become evident that the strong acute inflammatory response prompted rapidly after PDT treatment mobilizes potent tissue-destructive effector systems including complement as well as activated neutrophils and

other inflammatory cells that converge in large numbers at the treated site [3–5].

We have recently demonstrated that the treatment of mouse tumors with Photofrin-based PDT induces a strong acute phase response [4]. Its manifestations include a profound rise in the level of peripheral blood neutrophils (neutrophilia) due to the recruitment of these cells from their storage/marginated pools and accelerated maturation of their progenitors in the bone marrow. In the present study we show that these systemic effects are instigated by signals emanating from the PDT-treated lesion and we identify multiple mediators responsible for the induced neutrophilia.

2. Materials and methods

One million EMT6 mammary sarcoma cells were inoculated subcutaneously in the lower dorsal region of syngeneic 6–8-week-old female BALB/c mice. The

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only exception was the blood flow measurement, which was done with tumors implanted in the dorsal side of the hind left footpad. In some experiments, EMT6 tumors were also implanted in the lower dorsal region of NOD-*scid* mice. The tumor size used in experiments was 6–8 mm in largest diameter.

For PDT treatment, mice were given an i.v. injection of Photofrin (porfimer sodium; Axan Pharma Inc.) at a dose of 10 mg/kg. Twenty-four hours later, the mice were restrained unanesthetized in special holders and the tumors treated with the light dose of 60 J/cm² (power density 110–120 mW/cm²). The same photosensitizer and light dose were used throughout this study. The light produced by a High throughput fiber illuminator (Sciencetech Inc., London, Ontario, Canada) equipped with a 150 W QTH lamp with integrated ellipsoidal reflector and 630 ± 10 nm interference filter was delivered through an 8 mm diameter liquid light guide, model 77638 (Oriol Instruments, Stratford, CT).

The procedure for tumor blood flow measurement was described in detail elsewhere [6]. Briefly, blood flow in superficial regions of EMT6 tumors growing in the footpad was recorded using a laser Doppler flowmeter (Laserflo Perfusion Monitor, Model BPM 403; TSI, Inc., St. Paul, MN). Relative blood flow values were derived by normalizing the readings to the mean pre-treatment value. The flowmetry was stopped during the PDT treatment and then resumed immediately after the termination of PDT illumination, by carefully repositioning the needle probe on the tumor precisely at the pre-treatment site. A winged needle infusion set connected to a syringe containing the solution of rat anti-mouse GR-1 (Ly-6G) antibody produced by RB6-8C5 hybridoma was installed into the tail vein of mice before the onset of blood flow measurement. The antibodies (5 mg/kg) were injected in a bolus volume of 0.1 ml per 20 g body weight instantly after the PDT light was switched off. Control experiments verified that this injection has no significant effect on the tumor blood flow of non-treated mice.

Absolute blood levels of neutrophils and other leukocytes were determined by multiplying the total number of white blood cells obtained from hemacytometer counts with the proportion rate of each leukocyte type derived from the differential count obtained from Wright's stained blood smears [4].

Blood from the tail vein was collected only once from each mouse to minimize non-specific stress interference [4]. In clamping experiments, metal tumor clamps were kept for indicated time intervals and were removed after blood collection.

Blocking agents used for the investigation of neutrophilia mediators were administered intraperitoneally at 30 min before the onset of PDT light treatment. For blocking complement component C5, we used anti-mouse C5 antibodies produced by hybridoma BB5.1 (kindly provided by Dr B. Stockinger). The dose used was 750 µg/mouse. Antibodies of the same immunoglobulin type (mouse IgG1) obtained from hybridoma 1B7.11 (ATCC TIB-191) that recognize trinitrophenyl were used as isotype controls. Functional blocking of mouse TNF-α, G-CSF, IL-10, and KC was done with rabbit polyclonal antibodies raised against these cytokines (50 µg/mouse) that were purchased from PeproTech Inc. (Rocky Hill, NJ). Monoclonal antibodies blocking mouse IL-1β (clone 1400.24.17, Endogen Inc., Woburn, MA), IL-6 (clone 20F3, Endogen) and ICAM-1 adhesion molecule (clone 3E2, BD PharMingen, San Diego, CA) were also used at 50 µg/mouse. Receptor antagonists were employed for blocking the action of thromboxane (SQ 29,548), prostaglandin E2 (SC-19220), leukotrienes (REV 5901), and platelet-activating factor (PAF) (trans-BTP Dioxolane), all obtained from Cayman Chemical Co. (Ann Arbor, MI) and used at 10 mg/kg. The same dose was used for warfarin, the antagonist of vitamin-K dependent synthesis of coagulation system factors II, VII, IX and X. For blocking the activity of xanthine oxidase and poly(ADP-ribose) polymerase (PARP), we used their specific inhibitors oxypurinol and 3-aminobenzamide, respectively, both at 20 mg/kg. Pyrilamine (H₁ histamine receptor antagonist) was used at 0.5 mg/kg to block the effect of histamine. Warfarin, oxypurinol, 3-aminobenzamide and pyrilamine were all from Sigma (St. Louis, MO).

For immunohistochemical detection of complement activity, sections (5 µm) from paraffin-embedded untreated and PDT-treated dorsal EMT6 tumors were re-hydrated and treated with antigen retrieval citrate buffer (Biogenex, San Ramon, CA). Monoclonal mouse anti-human C5b-9 (cross-reactive with murine C5b-9) obtained from Quidel (Mountain View, CA) or isotype-matched control antibody (mouse IgG2ak

from Sigma) were used as primary antibody. Immunohistochemistry was performed using a standard ABC procedure in a mouse-on-mouse Vectastain kit (Vector Laboratories, Burlington, Ontario, Canada) containing the biotinylated anti-mouse IgG (H + L) secondary antibody. Color was developed using a diaminobenzidine substrate (DAB, Sigma). Images were collected using the NIKON coolpix 995 digital camera attached to a NIKON Eclipse E400 microscope and Nikonview software.

Statistical analysis was based on an unpaired Student's *t*-test.

3. Results and discussion

The rapid and massive accumulation of neutrophils in PDT-treated tumors initiated already during photodynamic light treatment has been well documented [5,7]. The importance of these cells for the response of tumors to PDT is exemplified by their impact on tumor blood flow shown in Fig. 1. The treatment of EMT6 tumors growing in the left hind footpad of BALB/c mice by Photofrin-based PDT promptly reduced blood flow in these lesions to approximately 50% of normal levels, which gradually recovered to pre-treatment levels within 30 min. We have observed a similar response in other tumor models [6]. However, tumor blood flow in mice depleted of circulating neutrophils by *in vivo* administration of anti-GR-1 monoclonal antibodies [8] immediately after PDT remained reduced and showed signs of recovery only at 60 min post-treatment (Fig. 1). This finding suggests that after PDT neutrophils localized in tumor vasculature release vasoactive mediators facilitating the normalization of blood flow in these tumors. One such vasodilator known to be produced by activated neutrophils could be nitric oxide [9] whose importance in the PDT response has already been documented [6,10]. Since accelerated re-oxygenation post-PDT can exacerbate the insult from ischemia-reperfusion injury [11], the improved normalization of tumor blood flow will not necessarily protect from PDT-mediated tumor damage. We have previously reported that the depletion of neutrophils dramatically reduces the cure rate of PDT-treated EMT6 tumors [8], and a similar effect was also observed with PDT-treated rat tumors [12].

In a recently published work [4], we have shown that neutrophils not only accumulate in large numbers in PDT-treated tumors but that they concomitantly also appear in highly elevated levels in the blood (neutrophilia) of the hosts. In order to determine how important the factors released from the PDT-treated site are for this neutrophilia, we have examined the impact of tumor clamping. To completely occlude blood vessels leading to and from the tumor growing in the lower dorsal region, clamps were mounted immediately after the termination of photodynamic light treatment. Notably, no visible signs of damage nor edema were detected in these lesions even if they were clamped for up to 24 h after PDT. In contrast, the non-clamped tumors showed signs of a strong edema within 2 h post-PDT and became completely ablated 10–12 h later. The examination of blood samples collected from mice bearing clamped PDT-treated EMT6 tumors shows that up to 6 h post-PDT there

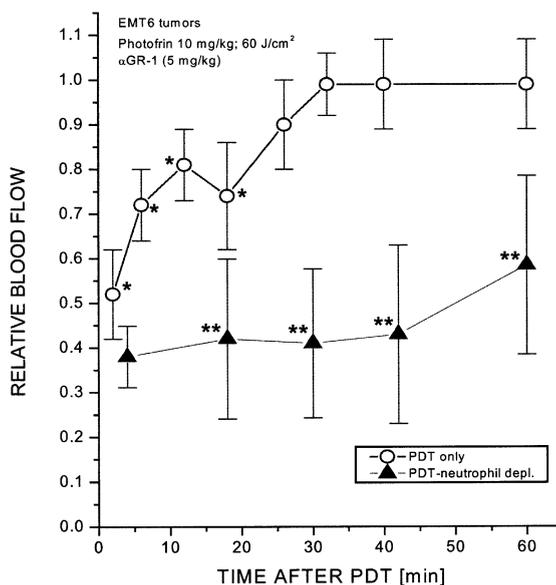


Fig. 1. The effect of neutrophil depletion on the blood flow in PDT-treated tumors. Mice bearing EMT6 foot tumors were given Photofrin (10 mg/kg *i.v.*) followed 24 h later by tumor-localized light treatment (60 J/cm²). Circulating neutrophils were depleted in some mice by injecting anti-GR-1 antibodies (5 mg/kg *i.v.*) immediately after photodynamic light treatment. Tumor blood flow was recorded before and after light treatment using laser Doppler flowmetry. Bars are SD, *n* = 4. **P* < 0.05 for statistical difference from normal blood flow; ***P* < 0.05 for statistical difference from PDT only.

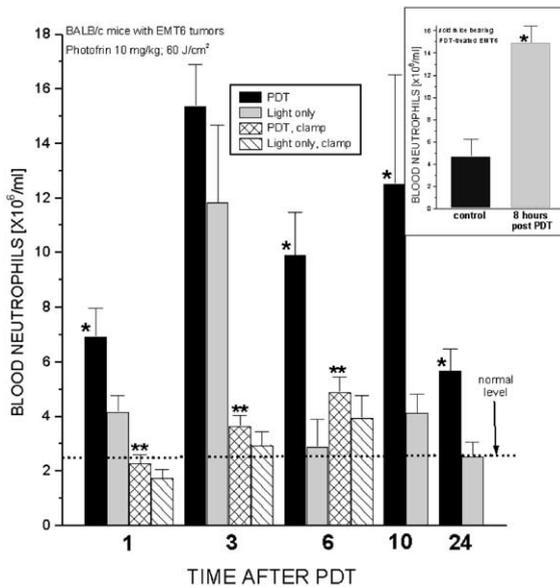


Fig. 2. PDT-induced neutrophilia in BALB/c and scid mice, and the effect of tumor clamping. Dorsal EMT6 tumors growing in either BALB/c or scid mice were treated by PDT as described in Fig. 1, and blood samples were collected (once from each mouse) at different times thereafter and used for differential leukocyte analysis. Tumors in some of the mice were clamped immediately after photodynamic light treatment and the clamps were not removed until after blood collection. Light-only control groups were treated in the identical way except that photosensitizer administration was omitted. The data obtained with the scid mice are shown separately (inset). Bars are SEM, $n = 4$. * $P < 0.05$ for statistical difference from non-clamp light-only levels; ** $P < 0.05$ for statistical difference from the level in the respective non-clamp group.

was no significant increase in the levels of neutrophils (compared to respective light-only controls) (Fig. 2). This is in contrast to a pronounced rise that occurs in mice bearing non-clamped PDT-treated tumors (depicted in the same figure), which is already known from our previous report [4]. For control groups in these experiments we used mice with either clamped or non-clamped tumors treated with light only (no photosensitizer), as their data reveal the extent of the rise in blood neutrophil levels caused by non-specific stress effects [4]. The results of the clamping experiments infer that agents released from PDT-treated tumors are responsible for the induction of peripheral blood neutrophilia, as reaction is prompted in the host engendering an influx of factors into the treated site that are indispensable for the

subsequent destruction of tumor tissue. We have already identified complement as a major mediator responsible for PDT-induced neutrophilia [4]. Such neutrophilia is also induced in scid mice bearing PDT-treated EMT6 tumors (Fig. 2, inset). Since the antibody-mediated activation of the classical complement pathway is not functioning in scid mice due to impaired activity of lymphoid populations, this result supports our earlier conclusion that PDT induces complement activation via the alternative pathway [4].

Formation of the terminal complement membrane attack complex (MAC) from C5b-9 proteins in PDT-treated EMT6 tumors was demonstrated using immunohistochemistry and is depicted in Fig. 3. It can be seen that the PDT treatment induced a widespread MAC assembly and deposition throughout the tumor parenchyma and also on endothelial cells. Staining demonstrates that deposition of MAC is not nuclear but concentrated to the cytoplasm and outer membrane of affected cells. Faint positive staining in the section from a non-treated tumor suggests weak complement activation likely related to inflammatory pressures associated with tumor progression.

In further experiments we examined whether other mediators in addition to complement participate in the induction of neutrophilia following PDT. The example with one such candidate, IL-6, which is a known regulator of neutrophil activity [13], is shown in Fig. 4. It can be seen that blocking IL-6 function with anti-mouse IL-6 antibodies significantly reduced the rise in the levels of circulating neutrophils at 8 h post-treatment without affecting the levels of other major blood leukocyte populations (lymphocytes, monocytes and immature band neutrophils) in mice bearing PDT-treated EMT6 tumors. This confirms our previous finding [4] that PDT selectively induces changes in blood neutrophils and not in other circulating leukocyte populations.

The same investigative approach of *in vivo* blocking the action of putative candidates enabled us to identify other mediators involved in the development of PDT-induced neutrophilia. Two and 8 h after PDT were chosen in this investigation as the time intervals representative of the 'early' and 'advanced' phases of neutrophilia, respectively. The results are summarized in Fig. 5. The data are presented as the relative extent of neutrophilia obtained with and without the block-

ing agent in mice bearing PDT-treated tumors. This clearly depicts the magnitude of the impact of individual agents and enables the presentation of data from a series of independent experiments.

The top twin columns in Fig. 5 show the results

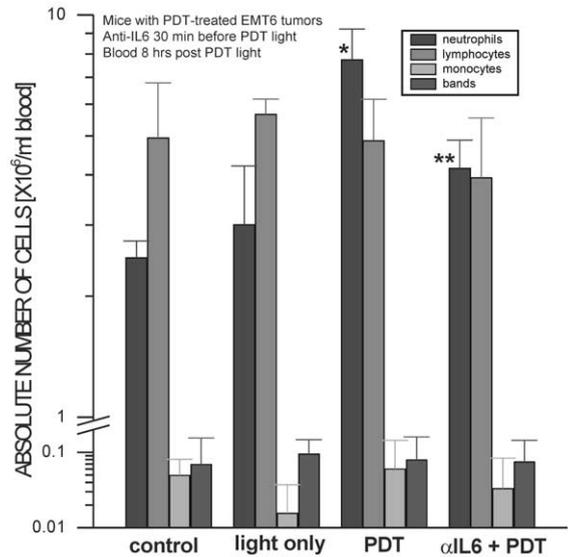
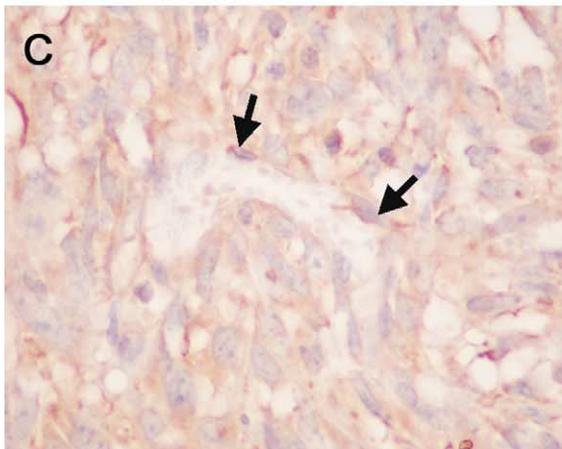
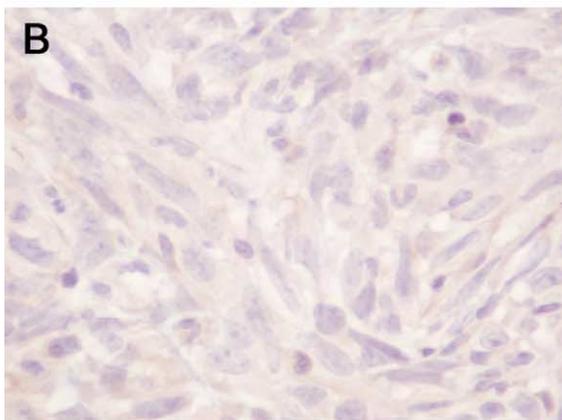
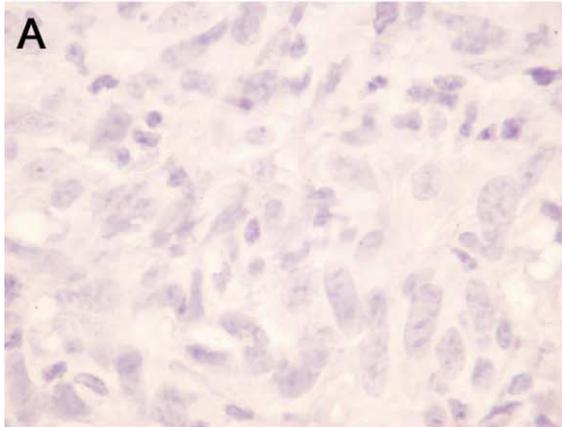


Fig. 4. Levels of blood leukocytes in mice bearing PDT-treated tumors and the effect of IL-6 blocking antibodies. Dorsal EMT6 tumors growing in BALB/c mice were treated by PDT and blood samples were taken for leukocyte analysis as described in Figs. 1 and 2. One group of mice also received anti-mouse IL-6 antibodies (50 μ g/mouse i.p.) 30 min before PDT light treatment. Also used was the light-only control group described in Fig. 2. Bars are SEM, $n = 4$. * $P < 0.05$ for statistical difference from control neutrophils; ** $P < 0.05$ for statistical difference from PDT-only level.

obtained with antibodies blocking the activity of the fifth component of murine complement (C5), which prevents the generation of C5a anaphylatoxin and the C5b-9 attack complex [14]. While C5 blocking had no significant effect on the development of early-phase, it reduced by close to 30% the extent of late-phase post-PDT neutrophilia. Whereas our earlier findings with the C3 convertase inhibitor *N*-acetyl-L-aspartyl gluta-

Fig. 3. Immunohistochemical detection of the complement membrane attack complex (MAC) formation in PDT-treated tumors. Sections from dorsal EMT6 tumors were stained for MAC (C5b-9) complexes as described in Section 2 and counterstained with hematoxylin. The PDT treatment was as described in Fig. 1. (A) Negative control (secondary antibody only) of a tumor excised 30 min post-PDT demonstrating no detectable unspecific (background) staining with the staining protocol used. (B) A section from an untreated tumor showing faint positive staining. (C) A section of a tumor excised 30 min post-PDT (as in A). (C) demonstrates extensive outer membrane and cytoplasmic MAC staining on cells throughout the tumor parenchyma as well as on the endothelial cells of a small capillary (arrows). Magnification 400 \times .

mic acid (NAAGA) demonstrate that complement is a potent mediator of PDT-induced neutrophilia [4], the results with C5 blocking provide additional elements to the understanding of its action. The anaphylatoxin C5a is well characterized as a neutrophil chemoattractant [15], whereas the MAC is also known to possess leukocyte chemotactic properties at sublytic concentrations [16]. While either one or both of these components participate in the advanced phase of PDT-induced neutrophilia, it is obvious that other mediators are of importance in the early phase and that there are additional participants mediating the advanced phase. This could include components generated in the earlier stage of the complement activation cascade

(for instance anaphylatoxins C3a and C4a, and this requires further investigation) and also a variety of other secondary mediators released as a consequence of complement activation. Since NAAGA treatment completely inhibited the induction of neutrophilia at both 2 and 8 h post-PDT [4], it appears unlikely that mediators independent of complement can have a major role.

To rule out non-specific effects of blocking agents utilized in this investigation on blood neutrophil levels, control EMT6 tumor bearing mice were not PDT-treated but only administered the blocking agent and their blood was collected at the same time interval post-injection as with PDT-treated mice. For example, we verified that blood neutrophil levels in mice injected with anti-C5 antibodies were not significantly different than normal neutrophil levels in mice of the same age. In addition, the treatment with isotype control antibodies non-specific for C5 was found to produce no obvious effect on either blood neutrophil levels in PDT-non-treated mice or on the extent of neutrophilia induced in PDT-treated mice.

Biological activity of complement involves the release of various secondary mediators including agents known to possess the capacity to affect neutrophil migration and activity [17,18]. This prompted us to examine whether the contribution to post-PDT neutrophilia can be linked to the activity of cytokines, arachidonic acid metabolites, histamine and the coagulation system associated with complement activation. The results obtained with blocking IL-1 β and TNF- α show that both act as potent promoters of the early phase of PDT-induced neutrophilia but appear to not have a significant role in the advanced phase (Fig. 5). Cytokine IL-6 is not only contributing to the advanced phase (as shown in Fig. 4) but is an even more prominent contributor in the early phase. The data attained with blocking two other cytokines, G-CSF and IL-10, demonstrate that they are important contributors to the advanced-phase neutrophilia with no apparent influence in the early phase (Fig. 5). G-CSF may be indirectly linked to complement activation by IL-1-mediated induction [19]. It is worthy to note that in the reports of various investigators the activity of all these cytokines has been implicated in the PDT response [7,19,20]. Since complement activation has also been associated with chemokine release [17], we tested for KC (mouse analogue of

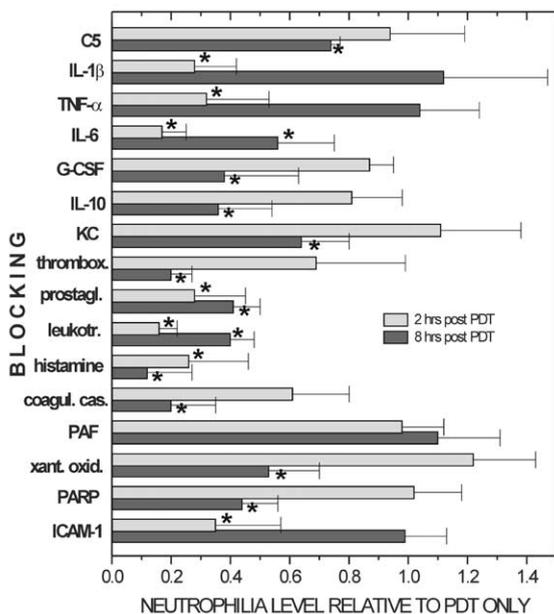


Fig. 5. Mediators of PDT-induced neutrophilia. Dorsal EMT6 tumors growing in BALB/c mice were treated by PDT as described in Fig. 1 and blood was collected for leukocyte analysis at either 2 or 8 h post-PDT. Neutrophilia mediators were identified by specific blocking of their activity as exemplified for IL-6 in Fig. 3. The results are presented as the ratio of the PDT-specific increase in absolute blood neutrophil counts obtained with and without the blocking agent. The agents used were the antibodies against C5, IL-1 β , TNF- α , IL-6, IL-10, G-CSF, KC, and ICAM-1, receptor antagonists of thromboxane (SQ 29,548), prostaglandin E₂ (SC-19220), leukotrienes (REV 5901), histamine (pyrilamine) and PAF (trans-BTP Dioxolane), anticoagulant warfarin, and inhibitors of xanthine oxidase (oxypurinol) and PARP (3-aminobenzamide). The doses used for all these agents are specified in Section 2. Bars are SD, $n = 4$. * $P < 0.05$ for statistical difference from PDT only.

human GRO- α) as a representative. Blocking the activity of this chemokine reduced the extent of advanced-phase neutrophilia, while showing no obvious impact in the early phase (Fig. 5). Given that immune blocking of KC, TNF- α , G-CSF and IL-10 was done with rabbit polyclonal antibodies, the fact that in some cases there was no detectable effect on the early-phase while in other cases no obvious influence was found on the advanced-phase neutrophilia excludes the possibility of non-specific effects of these antibodies on the endpoint under examination.

Arachidonic acid metabolites are well known participants in tumor response to PDT [21,22]. Pre-treatment of EMT6 tumor bearing mice with either aspirin or indomethacin (non-specific cyclooxygenase inhibitors) markedly reduced the development of PDT-induced neutrophilia (data not shown). The results obtained with specific blocking of the main metabolites, thromboxane, prostaglandin E₂ and leukotrienes are depicted in Fig. 5. The treatment with thromboxane receptor antagonist SQ 29,548 markedly diminished the extent of the advanced phase whereas it exhibited no significant influence on the early phase of PDT-induced neutrophilia. Pronounced reductions of both early- and advanced-phase neutrophilia occurred with the receptor antagonists for prostaglandin E₂ (SC-19220) and leukotrienes (REV 5901).

Further examination identified histamine (blocked by pyrilamine) as another potent mediator similarly effective during early- and advanced-phase neutrophilia (Fig. 5). Histamine is released from activated mast cells that accumulate in PDT-treated tumors [5]. The next pair of columns in Fig. 5 shows the results obtained with anticoagulant warfarin, which indicate that components of the coagulation and/or other plasma cascade systems also participate in the induction of advanced-phase neutrophilia with no apparent involvement in the early phase. Factors blocked by warfarin are linked to the regulation of potent mediators of neutrophil activity including thrombin, factor XII, kallikrein and bradykinin [23–26].

While complement activation also induces the release of PAF [17], which is a potent mediator of neutrophil chemotaxis and activation [15], we have not detected any significant role of this mediator in PDT-induced neutrophilia when using its receptor antagonist trans-BTP Dioxolane (Fig. 5).

We next examined several other inflammatory events associated with complement activation, which may indirectly influence neutrophil chemotaxis. Xanthine oxidase, a key enzyme in ischemia-reperfusion injury [11], is effectively inhibited by oxypurinol whose administration attenuated the induction of advanced- but not early-phase neutrophilia (Fig. 5). Ischemia-reperfusion injury appears to contribute to PDT-mediated tumor destruction [3,6]. Endothelial damage caused by this insult elicits complement activation and triggers other events that impact upon neutrophil sequestration [11]. A similar effect, i.e. a reduction in the extent of the advanced- but not early-phase neutrophilia, was obtained by inhibiting PARP with 3-aminobenzamide. The inhibition of this enzyme was reported to attenuate neutrophil recruitment [27], which is probably reflecting the role of PARP in controlling the activation of nuclear transcription factor NF- κ B that induces the expression of various inflammation-related genes [28]. Using immunohistochemistry, we have detected activated PARP in PDT-treated mouse tumors (Korbelik, unpublished data). The bottom twin columns in Fig. 5 depict the effect of ICAM-1 blocking antibodies. The expression of this endothelial cell adhesion molecule is upregulated following complement activation [17] and this promotes neutrophil invasion [29]. Our results show that blocking ICAM-1 diminishes the intensity of the early-phase (but not advanced-phase) neutrophilia.

Neutrophilia investigated in this study is a manifestation of the systemic host response to PDT of solid tumors and it reflects the mobilization of neutrophils for their engagement as activated inflammatory effector cells in treated tumors [3,4]. Although various neutrophilia mediators identified in the present work have already been characterized as important participants in PDT response, this study reveals their integrated role in PDT-elicited neutrophil activity. In agreement with the present study, elevated post-PDT serum levels of complement, histamine, eicosanoids, IL-1 β , KC and other chemokines have been determined by various investigators [4,19,21,31], while decreased PDT-mediated cure rates have been reported after blocking complement, IL-1 β , IL-6, KC and other chemokines, histamine, thromboxane and other eicosanoids, xanthine oxidase and PARP [3,21,30–32]. The extent of the influence of these

various neutrophilia mediators on the neutrophil accumulation and activity in PDT-treated tumors is under investigation in our ongoing study. The contribution to PDT response by many of these agents is not limited to neutrophilia mediation, but their particular role in the mediator network integrated in the tumor-host cross-talk is essential for the therapeutic outcome.

3.1. Conclusions

Neutrophilia elicited in mice bearing PDT-treated tumors results from the integrated action of multiple mediators that are massively released from the targeted lesion. A key event responsible for this phenomenon appears to be PDT-induced complement activation. While some components of the complement cascade act directly as neutrophilia mediators, a number of secondary mediators generated as a consequence of complement activity are also involved; some are produced rapidly during the PDT treatment and others at later time periods. Some of these mediators participate in both the early- and advanced-phase neutrophilia (IL-6, prostaglandins, leukotrienes, and histamine), while others contribute selectively to either early-phase (IL-1 β and TNF- α), or advanced-phase (G-CSF, IL-10, thromboxane and coagulation cascade components) neutrophilia. Moreover, there are indirect mediators (exemplified by the activity of xanthine oxidase, PARP and ICAM-1) that appear to exert influence in early- or late-phase neutrophilia. Also participating are probably other complement-associated mediators not examined in the present study, for instance, chemokines such as IL-8/MIP-2. The effect on neutrophil chemotaxis can be augmented by a synergistic interaction of various mediators [33]. Rather than acting independently, these mediators appear integrated in a network that renders the neutrophil trafficking regulated within the inflammatory framework aimed at resolving the lost homeostasis of the inflamed (tumor) site and thereby contributes to the destruction of the PDT-treated tumor.

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