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Adjuvant treatment for complement activation increases the effectiveness of photodynamic therapy of solid tumors

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Phototoxic lesions generated in tumor tissue by photodynamic therapy (PDT) are recognized by the host as a threat to the integrity and homeostasis at the affected site. Among the canonical pathways invoked by the host for dealing with this type of challenge is the activation of the complement system, integrating proteins that serve as molecular sensors of danger signals produced by PDT and those initiating signalling cascades coupled into the network of inflammatory and immune responses. Since the activated complement system is a salient participant of the antitumor response produced by PDT, it is worth exploring whether its manipulation can be exploited for the therapeutic benefit. Using mouse tumor models, the present study examined the potential of representative complement-activating agents to act as effective adjuvants to PDT. Tumor-localized treatment with zymosan, an alternative complement pathway activator, reduced the recurrence-rate of PDT-treated tumors, markedly increasing the percentage of permanent cures. In contrast, a similar treatment with heat aggregated gamma globulin (complement activator *via* the classical pathway) was of no significant benefit as a PDT adjuvant. Systemic complement activation with streptokinase treatment had no detectable effect on complement deposition at the tumor site without PDT, but it augmented the extent of complement activity in PDT-treated tumors. This finding based on immunohistochemistry analysis explains the results of tumor therapy experiments, which showed that systemic treatment with streptokinase or a similar agent, urokinase, enhances the PDT-mediated tumor response. Zymosan and streptokinase administrations produced no beneficial results with PDT of tumors growing in complement-deficient mice. This study, therefore, establishes the potential of complement-activating agents to serve as effective adjuvants to PDT for cancer treatment.

Introduction

Photodynamic therapy (PDT) is applied for eradicating cancerous or other diseased tissue by local activation of photoreactive drugs capable of transferring the absorbed light energy to molecular oxygen to generate highly reactive oxygen species that damage surrounding biological molecules.[1] Photooxidative lesions produced by PDT are recognized by the host as 'altered self' presenting danger that requires intervention of the host-protecting mechanisms.[2] This host reaction, mediated through a network of inflammatory and immune responses, is a key contributor to the efficacy of PDT-mediated destruction of cancerous lesions.[1,3]

Activation of the complement cascade, the major non-cellular effector system of innate immunity,[4] has an important role in the initiation and orchestration of the PDT-induced host response.[3] Complement engagement is triggered after binding of the recognition component of one of the three independent activation routes (classical, alternative and lectin pathway) to invading pathogens, foreign cells or autologous cells altered by injury.[4] Complement proteins that act as opsonins bind to PDT-treated cells flagging them for detection by sensors of the innate immune recognition system.[2,4,5] Complement-opsonized PDT-treated cells attract neutrophils, macrophages, dendritic and other immune cells displaying complement receptors. Among these receptors are CR3 and CR4 whose ligands also include fibronectin, laminin and collagen,[5] which may get exposed following PDT-mediated degradation of extracellular matrix.[2] The ligation of complement receptors triggers specific intracellular signalling pathways that lead to downstream activation of nuclear transcription factors enabling the expression of genes encoding inflammatory cytokines and chemokines.[2] The deposition of lytic membrane attack complex (MAC) of complement, detected in the vascular endothelium of PDT-treated tumors,[6] is likely to contribute to the collapse of blood supply in these lesions. Association of complement molecules with tumor proteins facilitates the recognition of tumor antigens and harnesses the activity of lymphoid populations engaged in the adaptive immune response against the tumor;[7] this type of engagement may also be invoked in the PDT response.[2,3]

Extensive involvement of the complement system in tumor response to PDT may offer the opportunity to develop effective adjuvant treatments for PDT based on the exploitation of the antitumor activity of complement. Various substances that specifically activate complement are known to possess antitumor activity.[8,9] Zymosan, particles consisting of protein–carbohydrate complexes prepared from the cell wall of *Saccharomyces cerevisiae*, is a standard complement activator long known to stimulate host response against cancer.[10] Since its systemic administration is associated with toxic side-effects,[11] we used zymosan as a model agent applied by tumor-localized injection. Complement-activating agents that are safe for systemic application include clinically used anticoagulants streptokinase and urokinase.[12,13] The present study investigates the potential use of these agents for tumor-localized or systemic complement activation to enhance the therapeutic effect of PDT.

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Materials and methods

Lewis lung carcinoma (LLC) and squamous cell carcinoma SCCVII (both poorly immunogenic tumors) were implanted in syngeneic C57BL/6 and C3H/HeN mice, respectively, by injecting subcutaneously 1×10^6 tumor cells into lower dorsal region of 7–9 week old mice, as described earlier.[14] LLC were also inoculated in the same way into immunodeficient mice, B6.129S4-C3^{tm1Crr} (complement C3 knockout, C3KO) and NOD-*scid* (B and T cell deficient, complement C5 deficient), where their growth rate was similar to that in immunocompetent mice. About one week after implantation, the tumors reached 7–8 mm in the largest diameter and were ready for treatment. All animal procedures were approved by the Animal Care Committee of the University of British Columbia.

Complement-activating agents zymosan (Z42500) and streptokinase (S8026) were purchased from Sigma Chemical Co. (St Louis, MO, USA), urokinase (from human urine) from Calbiochem (Merck KGoA affiliate, Darmstadt, Germany) and heat aggregated gamma globulin from Quidel Corp. (San Diego, CA, USA). Streptokinase and urokinase were dissolved in phosphate buffered saline (PBS) and administered i.v. at doses of 800 and 300 units per mouse, respectively. A fine suspension of zymosan dispersed in PBS, was injected intratumorally (volume 50 μl) for achieving the dose of 0.5 mg mouse^{-1}.

Treatment of mouse tumors with Photofrin- or mTHPC-based PDT was performed as described in detail elsewhere.[15] Photofrin (porfimer sodium, supplied by Axcan Pharma Inc., Mont-Saint-Hilaire, Quebec, Canada) was administered i.v. at 7.5 or 10 mg kg^{-1} 24 h before exposing the tumors to 630 ± 10 nm light from a 150 W QTH lamp equipped high throughoutput fiber illuminator (Sciencetech Inc., London, Ontario, Canada) delivered through an 8 mm core diameter liquid light guide model 77638 (Oriel Instruments, Stratford, CT, USA). The photosensitizer 5,10,15,20-tetra-(m-hydroxyphenyl)chlorine (mTHPC), also known as foscan (provided by Biolitec Pharma Ltd., Edinburgh, UK), was i.v. injected at 0.1 mg kg^{-1} 24 h before exposing the tumors with 652 ± 1 nm light from a 0.25 W laser diode model SDL-7422-HI (Spectra Diode Labs, San Jose, CA, USA) also delivered through the liquid light guide model 77638. The fluence rate in all PDT treatments was around 110 mW cm^{-2}. In the experiments where tumor cure or regrowth was the endpoint, the mice (8 in each treatment group) were inspected every two days for signs of tumor growth for up to 90 days after PDT, and tumors showing no signs of recurrence at that time were considered cured.

Immunohistochemical detection of the deposition of the MAC complement component in PDT-treated mouse tumors was described in detail earlier.[6] Briefly, 5 μm sections cut from paraffin-embedded tumors were first re-hydrated and treated with antigen retrieval citrate buffer, and then stained with monoclonal mouse anti-human C5b-9 antibody (cross-reactive with mouse C5b-9) purchased from Quidel Corp., or the isotype-matched control mouse IgG2aκ (Sigma). A standard ABC procedure in mouse-on-mouse Vectastain kit (Vector Laboratories, Burlington, Ontario, Canada) was used and color developed using a diaminobenzidine substrate (DAB, Sigma). Sections from at least 3 identically treated tumors were included in scoring the intensity of C5b-9 staining.

For mouse C3 ELISA, the tumors were homogenized immediately after excision using a Polytron PT 3100 homogenizer (Kinematica AG, Switzerland). Based on the method described by Taktak and Stenning,[16] wells were first coated with goat anti-mouse C3 $F(ab')_2$ fragments (Cappel, ICN Pharmaceuticals, Inc., Aurora, OH, USA), then samples (tumor homogenates or C3 standard) were added, and following 1 h incubation at 37 °C the wells were washed and finally stained with horseradish peroxidase-conjugated goat anti-mouse C3 (Cappel). Mouse C3 protein used for the ELISA standards was prepared from plasma of DAB/2J mice (The Jackson Laboratory, Bar Harbor, ME, USA) following the isolation and purification protocol based on SP/QAE-Sephadex chromatography described by Gyongyossy and Assimeth.[17]

Statistical analysis for tumor response evaluation was based on Log-rank test, while the unpaired Student's *t*-test was applied to test the difference between means for the data from ELISA measurement.

Results

The LLC represents a mouse tumor model of limited sensitivity to PDT. Although LLC tumors initially respond well to PDT treatment and appear flat and necrotic at one day after the therapy, they all re-grow within the next two weeks (Fig. 1). Single tumor-localized treatments with two different complement-activating agents, zymosan and heat aggregated gamma globulin (HAGG), were examined when applied immediately after photodynamic light treatment. The protocol with zymosan, activator of complement *via* the alternative pathway, markedly reduced the recurrence of PDT-treated LLC tumors resulting in over 60% cures of these lesions (Fig. 1). The same zymosan protocol performed without PDT produced no significant effect on the growth of LLC tumors (not shown). In comparison to zymosan, HAGG (complement activator *via* the classical pathway)[18] was a much less effective adjuvant to PDT, since it attained only a moderate delay in the recurrence of PDT-treated tumors but no statistically significant cures (Fig. 1).

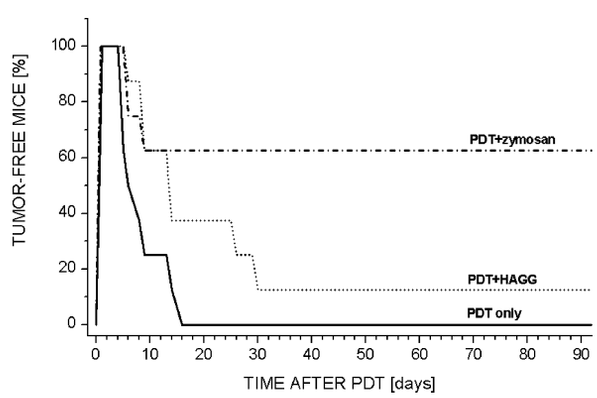

Fig. 1 The effects of zymosan and HAGG on the response of LLC tumors to Photofrin-based PDT. C57BL/6 mice bearing subcutaneous LLC tumors were administered Photofrin (10 mg kg^{-1}, i.v.) and 24 h later the tumors were exposed to the 630 nm light dose of 150 J cm^{-2}. Immediately after light treatment some of the mice were given an intratumoral injection of zymosan (0.5 mg mouse^{-1}) or HAGG (1.25 mg mouse^{-1}). The mice were thereafter monitored for 90 days for signs of tumor growth; those showing no visible/palpable tumor mass were defined as 'tumor-free'. The difference in response between PDT only and PDT plus zymosan groups was statistically significant ($p < 0.05$), while the response of PDT plus HAGG group was not significantly different than PDT only group. Each treatment group consisted of 8 mice.

ELISA-based analysis of the levels of the key complement protein C3 in LLC tumors treated by PDT, zymosan or their combination is shown in Fig. 2. C3 was measured in tumor homogenates excised at 3 h post treatment, the time-point of its peak with PDT only.[22] The C3 content increased significantly after both PDT and zymosan injection compared to the content in non-treated tumors. The combined treatment, PDT plus zymosan, produced average C3 levels higher than those found after each of the individual treatments but due to experimental variations the difference was not statistically significant (Fig. 2).

When zymosan was applied as PDT adjuvant for treatment of LLC tumors growing in immunodeficient NOD-*scid* mice it produced no therapeutic benefit (Fig. 3). Both PDT only and

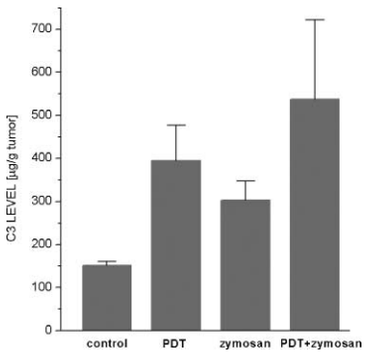

Fig. 2 The effects of PDT and zymosan treatments on the levels of C3 protein in LLC tumors. C57BL/6 mice bearing LLC tumors were treated with PDT, zymosan or their combination as described in Fig. 1. The mice were sacrificed and tumors excised at 3 h post treatment; nontreated tumors were included as controls. The level of C3 protein was determined in tumor homogenates by mouse C3 ELISA. The C3 tumor content was in all treatment groups significantly higher than in control tumors ($p < 0.05$). While the average C3 content in PDT plus zymosan group was higher than in PDT only and zymosan only groups the difference was not statistically significant. Bars are SD, $N = 4$.

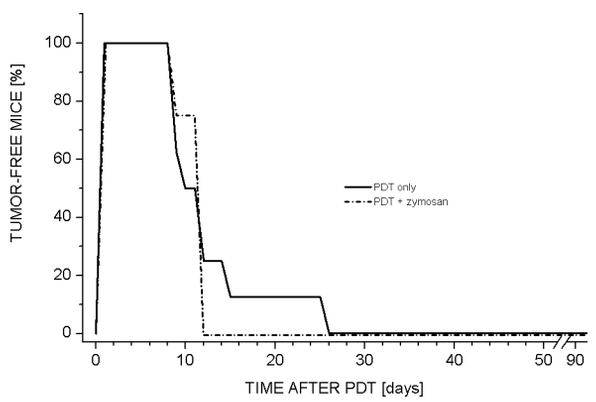

Fig. 3 The effect of zymosan on the response of LLC tumors growing in NOD-*scid* mice to Photofrin-based PDT. NOD-*scid* mice bearing LLC tumors were treated with PDT and zymosan as described in Fig. 1, except that Photofrin dose was increased to 10 mg kg^{-1}. The tumor response was then evaluated as in Fig. 1. There was no significant difference between the tumor response in PDT only and PDT plus zymosan groups. $N = 8$.

PDT plus zymosan treatments were ineffective in producing tumor cures in these mice characterized by both complement C5-deficiency and impaired lymphoid cell function.[19]

Intravenous injection of streptokinase results in its binding to plasminogen, which leads to plasmin-mediated activation of serum complement cascade.[20] The effect of streptokinase treatment on PDT response of LLC tumors is shown in Fig. 4. The photosensitizer mTHPC is more potent than Photofrin and was used in this experiment to reach a curable range in the PDT only group. The treatment with streptokinase performed immediately after photodynamic light treatment raised the cure rate of LLC tumors growing in syngeneic immunocompetent C57BL/6 mice to almost 80% and thus significantly improved the therapy outcome (Fig. 4a). Similarly to zymosan, the streptokinase treatment in the absence of PDT showed no obvious effect on the growth of LLC tumors (not shown).

The effect of streptokinase used in conjunction with PDT was also examined for the treatment of LLC tumors implanted in complement deficient C3KO mice of C57BL/6 genetic background. The tumors growing in these mice exhibit a greater sensitivity to PDT. The lack of complement-mediated vascular effects during photodynamic light delivery appears to ameliorate the restraint in supply of oxygen in tumors that is needed for the generation of phototoxic species.[22] Hence, a lower PDT light dose was needed than for tumors in wild-type mice to

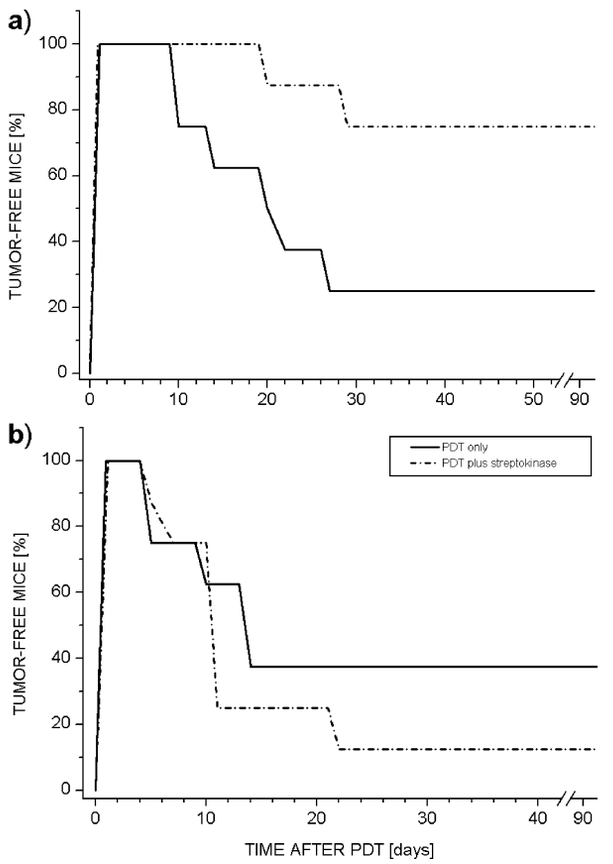

Fig. 4 The effect of streptokinase on the response to mTHPC-based PDT of LLC tumors growing in either complement-deficient mice or their wild-type counterparts. Mice bearing LLC tumors were administered mTHPC (0.1 mg kg^{-1} i.v.) and 24 h later the tumors were exposed to the 652 nm light dose of either (a) 100 J cm^{-2} (C57BL/6 hosts), or (b) 20 J cm^{-2} (C3KO hosts). Streptokinase (800 units $mouse^{-1}$) or control saline was injected i.v. immediately after light treatment. The tumor response was then evaluated as in Fig. 1. The difference in tumor response between PDT only and PDT plus streptokinase groups was in both cases statistically significant ($p < 0.05$). $N = 8$.

produce a similar tumor cure rate (20 compared to 100 J cm^{-2}, respectively) (Fig. 4b). The administration of streptokinase immediately after photodynamic light delivery was not beneficial for the therapy outcome with these complement-deficient mice, but instead had the opposite effect, reducing the rate of tumor cures.

The effect on PDT response of a systemic complement activation mediated by streptokinase and a similar agent, urokinase, was also examined with the squamous cell carcinoma model SCCVII (Fig. 5). The treatment with both of these agents (showing no detectable effect on tumor growth when used alone) improved the cure rate of SCCVII tumors treated by Photofrin-based PDT.

We used immunohistochemistry analysis to demonstrate that systemically administered streptokinase produces an increased complement deposition in PDT-treated tumors (Fig. 6). The SCCVII tumors excised at 2 h after the treatment with streptokinase, PDT or their combination, together with control nontreated tumors, were fixed and embedded. Staining of tumor sections with the antibody raised against the lytic MAC component of complement revealed only a very faint positivity in slides from non-treated tumors and from tumors excised from mice treated systemically with streptokinase. The samples from PDT-treated tumors showed a strong MAC staining; however, even higher intensity was observed in the samples from the PDT plus streptokinase group (1 and 1.5–2, respectively) based on the standard blind scoring system with the scale from 0 to 3.

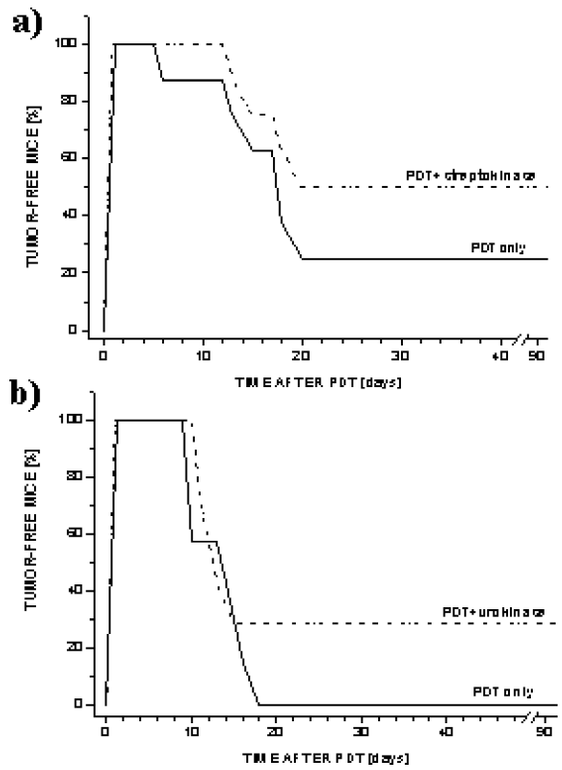

Fig. 5 The effect of streptokinase and urokinase on the response of SCCVII tumors to Photofrin-based PDT. C3H/HeN mice bearing subcutaneous SCCVII tumors were administered Photofrin (7.5 mg kg^{-1} i.v.) and 24 h later the tumors were exposed to the 630 nm light dose of either (a) 150 J cm^{-2} or (b) 120 J cm^{-2}. Streptokinase, 800 units $mouse^{-1}$ (a) or urokinase, 300 units $mouse^{-1}$ (b) were injected i.v. immediately after light treatment. The tumor response was then evaluated as in Fig. 1. Both PDT plus streptokinase and PDT plus urokinase groups showed response that was significantly different than their corresponding PDT only groups ($p < 0.05$). $N = 8$.

Discussion

This work demonstrates that a class of agents activating the complement system of innate immune response are effective adjuvants to PDT for the treatment of solid cancerous lesions. As established in our previous studies, PDT treatment induces the activation of the complement cascade and the deposition of complement proteins within the targeted lesion, which are the events that significantly influence the therapy outcome.[3,6,21] Adjuvant treatment with complement activating agents amplifies the engagement of the complement system in mediating the eradication of cancer cells in PDT-treated lesions, and in this way improves the efficacy of this modality for cancer therapy.

The results presented in this report show that both tumor-localized treatment with complement-activating agents and systemic induction of complement activation are effective in reducing the recurrence of PDT-treated tumors. Our findings suggest that, for tumor-localized treatment, more effective PDT adjuvants are complement activators engaging the alternative pathway of this cascade than those acting through the classical pathway (Fig. 1). Systemic complement activation with agents such as streptokinase does not result in the enhanced complement deposition in non-treated tumors (Fig. 6). However, streptokinase is known to enhance complement activation induced by other complement activators.[20] Localized complement engagement and other inflammatory changes evidently converge the impact of systemically activated complement cascade to the PDT-treated tumor, or render the targeted lesion less protection from further deposition of complement proteins emerging from the peripheral circulation.

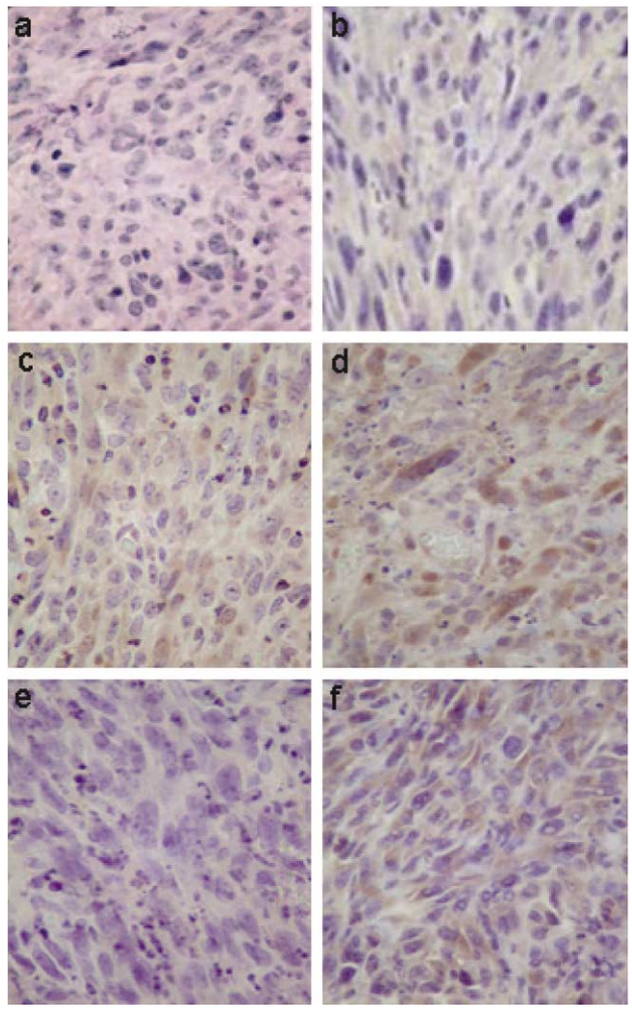

Fig. 6 Immunohistochemical detection of complement MAC component deposition in SCCVII tumors treated by streptokinase, Photofrin-based PDT and their combination. C3H/HeN mice bearing SCCVII tumors were treated with streptokinase, PDT or their combination as described in Fig. 5a. An additional group of SCCVII tumors was clamped for 30 min (allowing 2 h for reperfusion) for obtaining a positive control group based on the complement activation following the induction of ischemia–reperfusion injury. The tumors were excised at 2 h after treatment and tissue sections (including those prepared from nontreated control tumors) were stained for mouse C5b-9 as described in Materials and methods. (a) Nontreated control. (b) Streptokinase only treatment. (c) PDT only treatment. (d) PDT plus streptokinase treatment. (e) Staining control (no primary antibody). (f) Treatment for ischemia-reperfusion induction (positive control). Magnification 400×.

The observation that zymosan and streptokinase are not effective PDT adjuvants with C3KO and NOD-*scid* mice is in agreement with the assumption that complement activity is the basis of the therapeutic advantage demonstrated with these agents in immunocompetent hosts. The effect of streptokinase in complement-deficient mice (increased tumor resistance to PDT depicted in Fig. 4b) exposes a possible negative impact of co-activated coagulation cascade[13] on the antitumor effect of PDT that is offset by the engagement of complement system. Use of other types of immunocompromised mice may prove helpful in further elucidating the participation of various immune effectors in the mechanism underlying the observed enhancement of PDT-mediated tumor control by complement-activating agents.

The present report establishes the potential of complement-activating agents to be employed as PDT adjuvants. This prompts the continued investigation aimed at identifying optimized and clinically relevant protocols for effective potentiation of PDT-mediated cancer treatment based on exploiting the potency of the complement system.

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