Complement activation cascade and its regulation: Relevance for the response of solid tumors to photodynamic therapy

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The complement system has emerged as a prominent participant in host response elicited following treatment of solid tumors by photodynamic therapy (PDT). Activity of the complement system is tightly controlled by a series of endogenous regulatory proteins. The expression of decay-accelerating factor (DAF), complement-receptor-1-related protein y (Cry), and protectin, which are the three major mouse membrane-bound complement regulatory proteins (mCRPs), was examined following treatment of murine squamous cell carcinomas SCCVII by PDT mediated by the photosensitizer Photofrin. A marked decrease was detected in the expression of all three mCRPs on cancer cells from tumors following PDT, indicating that these cells were made more vulnerable to complement attack. In order to amplify this effect, following PDT mice were injected with antibodies neutralizing either Cry, protectin, or DAF. With anti-Cry and anti-protectin this resulted in increased tumor cure rate compared to PDT alone, while the contrary was observed with PDT plus anti-DAF combination (presumably owing to additional role of DAF in T cell signaling). Further examination including other complement regulatory proteins showed that combining antitumor PDT with antibody-mediated neutralization of factor H (soluble negative complement regulator) or injection of properdin (positive complement regulator) increased the cure rates of PDT-treated tumors. The use of various agents promoting complement activity appears promising for employment as adjuvants to PDT.

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1. Introduction

Photodynamic therapy (PDT), a clinical modality used for the destruction of an increasing variety of malignant and other lesions [1,2], produces a localized phototoxic insult triggered by the generation of reactive oxygen species [3,4]. However, direct cancer cell kill by lethal photooxidative damage is only one component contributing to tumor therapy outcome with PDT since the effects on tumor vasculature interlinked with elicited host response also play a critical role [3,5,6]. The strong and rapid host-protective reaction is provoked because tumor-localized injury inflicted by PDT is perceived as a trauma not dissimilar to physical insults that threatens the integrity and homeostasis at the affected site [6]. The presence of such trauma is revealed to the sensors of innate immune system as the appearance of “altered self”, and this provokes a regulated and integrated engagement of a broad spectrum of response elements including two major effector processes, inflammation and acute phase response, and the two principal arms of immunity, innate and adaptive immune response [6–8]. The execution of this tumor PDT-associated host response is orchestrated by innate immunity with a key role played by one of its major components, the complement system [6].

The complement system is a biochemical cascade integrating over 30 serum and cell surface proteins [9,10]. Its major functions relevant for tumor PDT response include: immune danger recognition, instigation and propagation of inflammatory response, disposal of waste (clearance of dead cells, products of inflammatory injury, and immune complexes), and bridging innate and adaptive immunity [6,11–13]. PDT-induced activation of complement system has been demonstrated both locally and systemically as an upregulation of genes encoding complement components and the elevation of protein products of these genes in the treated tumors, as well as in the host serum, liver and spleen [8,13–15].

Due to its considerable toxic potential, complement activation is very tightly controlled by a complex set of plasma and cell surface regulatory proteins (Fig. 1). The species-specific membrane-bound complement regulatory proteins (mCRPs) protect host cells from inadvertent or misdirected complement attack [16]. Complement activation in humans is controlled primarily by three mCRPs: membrane cofactor protein (MCP, CD46), decay-accelerating factor (DAF, CD55), and protectin (CD59) [16–18]. An additional mCRP in mice is complement-receptor-1-related protein y (Cry), functional and structural analogue of MCP and DAF, which effectively blocks
complement deposition on self-membranes [18,19]. Factor H is the principal soluble inhibitor of the alternative pathway (AP) of complement activation; this plasma glycoprotein blocks the formation and activity of C3 convertase, and in combination with factor I degrades the activated fragment C3b [20,21]. The only known physiological positive regulator of the complement activation cascade is the plasma protein properdin, which is generally regarded to function by stabilizing the nascent C3bBb convertase but was recently demonstrated to operate also by serving as a platform for new C3bBb assembly and ostensibly act as a pattern recognition molecule for the initiation of AP [22,23]. Almost all of these inherent regulators of complement activity are of significant therapeutic interest for various applications including cancer treatment [24–26]. We have proposed that the engagement of complement system in the response to tumor PDT can be exploited by adjuvant amplification with complement-activating agents for the enhancement of therapeutic benefit [27]. In support of this idea, we have shown that the cures of PDT-treated mouse tumors can be significantly increased by the combined administration of various complement activators including zymosan, streptokinase, urokinase and γ-inulin [27,28]. In the present study, we examined the mCRP expression changes following PDT treatment and the impact of modulating complement regulatory elements in mice bearing PDT-treated tumors on the therapy outcome.

2. Experimental

2.1. Tumor models

Poorly immunogenic tumor models, squamous cell carcinoma SCCVII [29] and B16BL6 melanoma [30] were grown in syngeneic immunocompetent C3H/HeN and B57BL/6 mice, respectively. Cohorts of experimental tumors were inoculated by injecting 1 million cells (obtained by enzymatic dissociation of maintenance tumors) into the lower dorsal region of 7–9 week old female mice. The tumors were treated around 8 days post implant when their largest diameter reached 6–8 mm. The protocols were approved by the Animal Care Committee of the University of British Columbia.

2.2. PDT treatment

The photosensitizers Photofrin (provided by Axcan Pharma, Inc., Mont-Saint-Hilaire, Quebec, Canada) used with SCCVII tumors and m-tetrahydroxyphenylchlorine (mTHPC, received from Scotia Pharmaceuticals Ltd, Stirling, UK) used with B16 tumors were injected intravenously at 10 and 0.1 mg/kg, respectively. This was followed 24 h later by the exposure of tumors to superficial light generated by a 150 W QTH lamp-based high throughput source.
with integrated ellipsoid reflector (model FB-QTH-3, Scientech Inc., London, Ontario, Canada) with included 630 ± 10 nm (Photofrin) or 655 ± 10 nm (mTHPC) interference filters. The light dose with Photofrin-PDT ranged from 120 to 180 J/cm², the choice depending on the impact (positive or negative) on tumor cure rates of various treatments combined with PDT. The light fluence rate, delivered through a liquid light guide (model 77638, Oriel Instruments, Stratford, CT) was 80–90 mW/cm². Mice were restrained during light treatment in special holders designed to expose the surgical region of their back. After therapy, they were monitored for tumor growth and those showing no sign of palpable tumors at 90 days post-treatment qualified as cured.

2.3. Adjutant complement activation immunotherapy

 Supernatants of hybridomas clone 1F2 (kindly provided by Dr. Michael Holers, University of Colorado) and clone RIKO-2 (a generous gift of Dr. Noriko Okada, Nagoya University) were used for isolating monoclonal antibodies against mouse Crry (rat IgG₂a) and against mouse DAF (Armenian hamster IgG), respectively. Rabbit polyclonal antibody (R-79) against mouse protectin (CD59) and goat polyclonal antibody (I-20) against mouse factor H were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Isotype immunoglobulin controls were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA) except for Armenian hamster IgG that was from ebioscience, Inc. (San Diego, CA). The antibody treatments were done as a single intraperitoneal injection (50 μg/mouse unless otherwise specified) performed immediately after PDT light treatment. Perseptron, known also as factor P, isolated from human serum (Calbiochem-Novabiochem Corp., San Diego, CA) was injected intratumorally (15 μg/mouse) also immediately after PDT. The same timing and administration route was used for the treatment with zymosan (Sigma Chemical Co., St. Louis, MO) injected at 0.5 mg/mouse, as described in more detail elsewhere [27].

2.4. C3 ELISA

 Determination of complement C3 protein content in SCCVII tumors was based on the ELISA assay described previously in full detail [15]. Briefly, goat F(ab’)2 fragment to mouse C3 and HRP-conjugated goat anti-mouse C3 (both from Cappel Laboratories, Durham, NC) were used as capture and detection antibodies, respectively. Mouse C3, isolated and purified from DAB/2J mice plasma, was used as the standard.

2.5. Flow cytometry

 Tumors excised from mice sacrificed at defined time intervals after PDT treatment and control untreated tumors were minced and disaggregated into single cell suspension by enzymatic digestion [31]. Following Fc block treatment with supernatants of HB-197 hybridoma (producing antibody raised against mouse Fc gamma receptor), the cells were stained with fluorophore-combined antibodies against mouse antigens CD45 (Pharmingen BD Biosciences, San Jose, CA), DAF (from RIKO-2 hybridoma), CD59 (R-79, Santa Cruz Biotechnology), Crry (Pharmingen), or appropriate isotype immunoglobulin controls. The results of antibody staining were determined by flow cytometry using the Coulter Epics Elite ESP (Coulter Electronics, Hialeah, FL) with 20,000 cells included for each test. An additional flow cytometry analysis was done with SCCVII cells collected 1 h after treatment in vitro with PDT (Photofrin 5 μg/ml for 1 h in serum-free medium followed by 1 J/cm² of 630 ±10 nm light) and stained with FITC-conjugated annexin V plus biotin-conjugated rat-anti-mouse Crry (followed by PE-streptavidin) all from Pharmingen.

2.6. Statistical analysis

 Log-rank test was used for statistical analysis of tumor response, while the differences between the means of the data from other experiments were evaluated by Mann–Whitney test. The threshold for statistical significance was p < 0.05.

3. Results

 Elevation in the levels of C3 component, the pivotal protein in the complement cascade, is one of the best indications of the activation of complement system. Measurement using C3 ELISA in samples from untreated SCCVII tumors growing subcutaneously in C3H/HeN mice detected 37 ± 16 μg C3 per gram of tumor tissue. In SCCVII tumors excised 24 h after PDT (Photofrin i.v. at 10 mg/kg followed 24 h later by 150 J/cm² light dose) the C3 levels were about 9-fold higher (334 ± 60 μg per gram of tumor tissue, p < 0.05). This result confirms that PDT treatment of SCCVII tumor induces the activation of complement system at the treated site, and similar findings were obtained following PDT with other mouse models [15,28].

 Since the activation of complement is sometimes prompted or promoted by a downregulation of protective mCRPs in the affected tissue [32], we wanted to test the possibility that PDT treatment precipitates changes in the expression of these regulatory molecules on SCCVII tumor cells. To examine this, SCCVII tumors were excised after PDT and disaggregated into single cell suspension that were stained with antibodies against the three mCRPs of major relevance in mice: DAF, Crry and protectin. Flow cytometry analysis of these cells revealed a marked decrease in the expression of all these three molecules on the surface of cancer cells (identified by negative co-staining with pan-leukocyte marker CD45) (Fig. 2). Most profoundly affected was DAF, whose expression decreased significantly already at 30 min after PDT and dropped further to around one fifth of its normal level at 3 h after PDT. The expression of both Crry and protectin have not significantly decreased at the 30 min time point but declined to around a half of their normal level at 3 h after PDT. Therefore, the downregulation of mCRPs including DAF, Crry and protectin can be at least partially responsible for the activation of complement system seen in PDT-treated tumors. Cells dying by apoptosis are known to have a decreased expression of mCRPs [33] and this may contribute in the above observed effects. In support of this, SCCVII cells found to be dying (annexin V positive) when examined one hour after treatment in vitro by Photofrin-based PDT exhibited more than 2-fold lower Crry-associated fluorescence than those remaining viable (annexin V negative) (p < 0.05).

 Bearing in mind that functional suppression of mCRPs using blocking antibodies is known to exacerbate complement-mediated injury [23,34], we tested next whether such treatment aimed at augmenting the PDT-induced regression in the availability of mCRPs can improve the therapeutic outcome of tumor PDT. Hence, SCCVII tumors growing in C3H/HeN mice were treated by Photofrin-PDT and immediately after photodynamic light exposure the host mice received a peritoneal injection of either a mCRP-blocking antibody or control saline. The treated tumors in all groups became impalpable (the lesion site flat and growth mass indiscernible) within 24 h after PDT, but this was followed by re-growth of the lesions in some mice mostly within the next three weeks. The mice were monitored for signs of tumor recurrence up to 90 days post-PDT and those found tumor-free at that time were considered cured. The results in the first series with PDT used alone achieving moderate levels of tumor cures showed that PDT plus single treatment with either anti-CDprotectin or anti-Crry (50 μg/mouse) increased the response to high tumor cure rates (Fig. 3a). Similar
results were obtained when the dose of the antibodies was changed to either 25 or 100 μg/mouse, while additional testing revealed that the treatments with these antibodies in the absence of PDT have no detectable effect on tumor growth, and that the controls in which anti-protectin and anti-Crry were substituted by isotype control IgG showed no significant influence on PDT response (not shown). Interestingly, in the experiment where anti-protectin and anti-Crry were combined in post-PDT administration the benefit seen with their individual applications was lost (Fig. 3b). A completely different effect was obtained with immuno-inactivation of DAF after PDT, because the tumor cure rates in the PDT plus anti-DAF group were significantly reduced compared to the PDT only group (Fig. 3c). Further testing with an antibody cocktail combining anti-DAF, anti-protectin and anti-Crry that was injected after PDT also revealed a negative effect, i.e. diminished tumor cure rates in relation to PDT only group (not shown).

Since complement activation can be promoted also by blocking the soluble negative regulator factor H [35], this approach was examined next following the protocol of injecting the blocking antibody immediately after PDT. The PDT dose applied in this experiment resulted in no tumor cures, but in combination with anti-factor H therapy resulted in cures of ~50% of the treated tumors (Fig. 4). Included controls were the same as with the anti-mCRPs series and attested that there was no significant impact from non-specific effects associated with antibody treatment with/without PDT.

The objective of the above examined approaches based on blocking the negative complement regulators can in some circumstances be reached by the contrasting approach of boosting a positive regulator. This was evaluated by injecting human properdin intratumorally immediately after PDT. Indeed, this combination proved also capable of significantly improving therapy outcome as evidenced by around 25% cure rate of SCCVII tumors compared to no cures obtained with PDT only (Fig. 5). In the absence of PDT, the injection of properdin produced no detectable effect on tumor growth.
Positive therapeutic results with the examined agents prompted us to examine in final experiments whether such agent can further boost the effect of a classical complement activator zymosan when applied for potentiating the curative effect of PDT with a poorly responsive tumor model. Hence, this treatment was tested with B16 melanomas whose growth can be only temporarily arrested by PDT when mediated by photosensitizer the mTHPC which is more potent than Photofrin [36]. Although B16 tumors were initially ablated by this PDT treatment, local recurrence was evident in most of them less than a week after therapy (Fig. 6). While the same PDT dose followed by adjuvant treatment with either zymosan or anti-Crry produced no significant improvement in tumor response, around 40% of tumor cures were attained when both zymosan and anti-Crry treatments were performed immediately after PDT. No detectable impact on tumor growth was seen with either zymosan or anti-Crry alone or with their combined treatment in the absence of PDT (not shown).

4. Discussion

For the complement system, a key element in discriminating between self and the target is the reliance on a series of control proteins that prevent attack on host tissue and allow attack on everything else [37]. To evade complement attack and immune surveillance, tumor cells often overexpress mCRPs on their surface [17,38]. However, the expression of these mCRPs on tumor cells is influenced by various agents including cytokines, ischemia, apoptotic signals, and certain pharmacological agents [17]. The present study reveals that PDT induces a marked decrease in the expression of mCRPs on tumor cells in treated lesions. Possible causes for this effect include the induction of ischemia, local activity of cytokines such as IL-1β, IL-6, TNF-α and TGF-β, and apoptosis all known to occur in PDT-treated tumors [7,39]. These PDT-induced changes render tumor cells in the treated lesions more vulnerable to complement attack and are likely to be at least partly responsible for the observed complement activation in PDT-treated tumors [13–15,27,28].

An established approach for overcoming the protection by mCRPs, which also hamper the efficacy of therapeutic antibodies whose effects largely depend on complement-dependent toxicity [26], is blocking the activity of mCRPs employing various agents including specific neutralizing antibodies [40–43]. This study demonstrates that promoting complement activity in PDT-treated tumor with the use of antibodies blocking either Crry or protectin (CD59) serves as an effective adjuvant that potentiates the therapeutic impact of PDT on mouse tumors. The treatment with these antibodies was done as a single intraperitoneal injection and it was scheduled immediately after PDT. The rationale for this timing stems from our earlier studies showing that complement activation during light treatment of tumors for Photofrin-PDT has a negative impact on the therapeutic outcome with this modality [44]. The administration of complement-activating antibodies before PDT could result in escalated activity of complement anaphylatoxins during PDT light delivery, which would increase the vasoconstriction in the blood vessels of treated tumor enhancing the decline in tumor oxygenation and consequently impair the PDT-induced generation of phototoxic oxidative species. Owing to this effect, the timing for adjuvant treatment promoting complement activation has to be restricted to post-PDT intervals.
Blocking Crry in mice would be analogous to doing the same to MCP and DAF in humans with the result of removing restrictions for the deposition of C3 opsonins on target tumor cells and generation of soluble inflammatory activation fragments (anaphylotoxins C3a and C5a) [45]. On the other hand, blocking protec tin in mice or humans would prevent this regulatory protein to bind to the terminal proteins of the complement cascade (C8 and C9) and obstruct their assembly into a lytic membrane attack complex [46]. In view of the concern that owing to their ubiquitous expression the inhibition of some mCRPs by systemic injection of blocking antibodies may result in serious side-effects due to tissue cytotoxicity from unhampered complement activation, which was exemplified by findings with rats injected anti-Crry [47], this problem appears not to exist in mice and may be possible to circumvent in humans. Injections of anti-DAF, anti-Crry and anti-protectin antibodies to mice in our studies produced no signs of either short-term or long term toxicity detectable either by gross observation or necropsy.

Interestingly, for the reasons that are not entirely clear, integrating anti-Crry and anti-protectin in the same treatment resulted in the abrogation of their individual positive effects on tumor cures when combined with PDT (Fig. 3b). A complicating factor in dissecting the effects of mCRPs is their capacity of complement-independent modulation of T cell activity that was recently discovered with at least some of these proteins [18]. This appears particularly pronounced with DAF whose role in complement-independent activation of T cells is becoming increasingly clear [48]. Hence, blocking DAF in the present study may have hampered the development of an immune response mediated by T cells recognizing the treated tumor as their target; such an immune response is known to occur after PDT and contribute to the therapy outcome [4–6]. Consequently, DAF inactivation after PDT had not increased but decreased the cures of treated tumors.

Results of this study also demonstrate that complement-activating interventions based on soluble complement regulatory proteins, either negative or positive, are also feasible as adjuvant treatments with tumor PDT. Blocking of the negative soluble regulator factor H by neutralizing antibodies was shown to sensitize lung cancer cells to complement attack and reduce in vivo tumor growth [35]. The same type of treatment done as a single anti-factor H antibody administration performed immediately after PDT was shown in this work as effective in elevating the cure rates of PDT-treated tumors.

Recent discovery that properdin has binding affinity toward molecular patterns associated with infection or injury [23,49] suggests that this protein may become localized on the surface of tumor cells altered by treatment such as PDT. Properdin localized on the cell surface triggers autologous complement system activation through the AP, which could be exploited for targeting destruction of tumor cells [50]. This supports the approach tested in this work in which tumor-localized injection of properdin isolated from human serum was used for directing complement activation. The results suggest that such treatment (or other protocols based on properdin) can serve as another option for securing effective enhancement of PDT-mediated tumor cures based on adjuvant complement activation.

From this work and related studies [27,28] it is becoming clear that a variety of complement-activating agents can be successfully employed for promoting complement activation in joint treatment with tumor PDT. These agents include inert polysaccharides providing optimized complement-activating surface (zymosan, γ-inulin), anticoagulants that induce plasmin-mediated activation of complement cascade (streptokinase, urokinase), positive regulator of complement cascade (properdin), inactivators of mCRPs, and blockers of soluble negative complement regulators. Some of these agents give better results when administered systemically while localized injection works better with others. It does not seem that one of these agents emerges as superior to the others as the complement activator for use in conjunction with PDT, but is more likely that the choice should vary depending on the tumor type treated or could be even governed by characteristics of cancerous disease in individual patients.

With this wide choice of interventions in complement cascade, we were prompted to explore the possibility of gainfully combining two agents promoting complement activation in different way. Such combinations should be chosen with care to prevent a counterproductive response as exemplified by the results with anti-Crry plus anti-protectin. Nonetheless, a very encouraging outcome was observed with the combination of a solid phase activator zymosan (providing a matrix with complement-activating surface) [51] and blockage of Crry (prolonging the activity of complement C3 and C5 convertases) [19,45]. Although zymosan proved an affective PDT adjuvant with mouse Lewis lung carcinoma model [27] it produced no observable benefit with PDT for treating B16BL6 melanomas (Fig. 5). The strong resistance of these PDT-incurable melanomas was, however, overcome by the additional inclusion of anti-Crry as seen by attaining substantial rates of tumor cures [around 40%]. Importantly, this therapeutic gain would not have been achieved without the induction of a strong systemic anti-tumor immunity, because the B16BL6 melanoma model is highly metastatic and cannot be controlled solely by local control [28,30].

In summary, this work demonstrates that PDT dampens the expression of mCRPs on the surface of treated tumor cells that leaves them more vulnerable to complement attack. Further amplification of this effect by using mCRP-neutralizing antibodies as PDT adjuvant can be exploited for therapeutic gain. Modulating the action of other regulators of complement activity also appears to be a promising approach within this type of combined treatment. From the clinical standpoint, effective PDT and immunotherapy combination modalities offer encouraging prospects, particularly for controlling both local and systemic recurrence of treated cancer.

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