Cancer Immunol Immunother (2007) 56:649–658
DOI 10.1007/s00262-006-0221-z

ORIGINAL ARTICLE

Activation of complement C3, C5, and C9 genes in tumors treated by photodynamic therapy

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Received: 30 May 2006 / Accepted: 2 August 2006 / Published online: 1 September 2006

Abstract Cancer therapies, which deliver a rapidly induced massive tumor tissue injury, such as photodynamic therapy (PDT), provoke a strong host response raised for dealing with the inflicted local trauma. Activated complement system was identified as an important element of host response elicited by tumor PDT. The expression of genes encoding complement proteins C3, C5, and C9 was studied following tumor PDT mediated by photosensitizer Photofrin using mouse Lewis lung carcinoma (LLC) model. Treated tumors and the livers of host mice were collected at different times after PDT and the expression of the investigated genes was analyzed by RT-PCR. The results show a significant up-regulation of C3, C5, and C9 genes in PDT-treated tumors at 24 h after therapy, while no significant increase in the expression of these genes was found in the liver tissues. The expression of C3, C5, and C9 genes also became up-regulated in untreated tumor-associated macrophages (TAMs) co-incubated in vitro with PDT-treated LLC cells. This effect was abolished or drastically reduced in the presence of antibodies blocking heat shock protein 70 (HSP70), Toll-like receptor (TLR) 2 and TLR4, and specific peptide inhibitors of TIRAP adapter protein and transcription factor NF-κB. The presented study reveals that complement genes C3, C5, and C9 become up-regulated in tumors treated by PDT, but not in the host's liver. Tumor-localized up-regulation of these genes can be largely attributed to monocytes/macrophages invading the treated lesion after PDT. This effect appears to be induced by the recognition of danger signals from PDT-treated tumor cells such as HSP70 by TAMs that involve the TLR2- and TLR4-triggered signal transduction pathways leading to the activation of NF-κB.

Keywords Complement gene expression · Photodynamic therapy of cancer · Mouse tumor model · Toll-like receptors 2 and 4 · Heat shock protein 70 · Tumor-associated macrophages

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Introduction

Some clinically established and experimental cancer therapies deliver rapidly induced massive tumor tissue injury, an insult that the host perceives not much differently than a local trauma sustained in any other part of the body threatening the integrity and homeostasis at the affected site. Such cancer therapies include photodynamic therapy (PDT) [11], laser thermotherapy and phototherapy [10, 32], high intensity focused ultrasound ablation [16, 34] and cryotherapy [23, 30]. Upon experiencing tumor-localized insult produced by these therapies, the host is provoked to launch canonical responses that have evolutionarily evolved for coping with this type of trauma. The purpose of the engaged host-protecting mechanisms is to prevent the spreading of tissue damage by containing the disrupted homeostasis, eliminate the incapacitated injured tissue, remove dying cells and debris found at the afflicted location, and to promote healing with the restoration of tissue function at that site [21]. The recognition of tumor-localized insult and

orchestration of host response is mediated by the innate immune system, which mobilizes two major effector processes, inflammation and acute phase response. Of major relevance from the therapeutic endpoint, during the phase of containing and eliminating damaged tissue the elicited host response amplifies the therapy-instigated eradication of tumor cells [21]. Moreover, in the course of dealing with this type of tumor-localized injury the innate immune system becomes capable of priming and instructing the adaptive immune system to develop a response recognizing the treated tumor as its specific target, which can produce further therapeutic benefit [11, 17, 25, 33].

In PDT, which is a clinically relevant modality for the treatment of cancer and other indications [11, 15], the destruction of targeted lesions is initiated by reactive oxygen radicals formed by the transfer to molecular oxygen of energy captured by the light excitation of photosensitizing drugs administered to patients before their tumor illumination [14]. Investigation of the host response associated with tumor treatment by PDT has identified the complement system as one of its important elements. Following tumor PDT, elevated levels of C3 protein and mannose-binding protein-A (MBL-A) were detected in the plasma of the host mice (expressed as acute phase reactants) and in the treated tumors [6, 20]. Binding of C3 and its cleaved fragments as well as the assembly of C5b9 terminal complex was demonstrated in vitro on PDT-treated tumor and endothelial cells in the presence of homologous serum [7], which reveals that the complement system recognizes cellular damage inflicted by this therapy and reacts by a full activation of its cascade. Complement anaphylatoxins (predominantly C3a) act as major mediators of neutrophilia that develops in mice bearing PDT-treated tumors [8] and their blocking diminishes PDT-mediated tumor cures [6].

The major source of complement C3 component are liver hepatocytes [2]. However, the extrahepatic production of this and other complement proteins is also well established and considered critically important for local inflammatory and immune responses [24, 26]. The objective of this study was to determine the expression of C3 gene as well as C5 and C9 genes, encoding key proteins of the complement cascade [27], in mouse tumors treated by PDT and in the liver of host animals. The results demonstrate that in the treated tumors PDT induces an increased expression of C3, C5, and C9 genes whose origin can be attributed to macrophages invading the targeted lesions after the therapy.

Materials and methods

Tumor model and cells

Subcutaneous Lewis lung carcinomas (LLC) [31] were implanted in the lower dorsal region of syngeneic immunocompetent C57BL/6 mice and were used for experiments when reaching 6–8 mm in largest diameter. In some experiments, LLC tumors were implanted in Toll-like receptor (TLR) four mutant mice, C57BL/6.KB2-cln8mnd/MsrJ (purchased from The Jackson Laboratory, Bar Harbor, ME, USA). These mice carry a spontaneous mutation in TLR4 gene responsible for producing the inactive protein [3]. The animal protocols used were approved by the Animal Ethics Committee of the University of British Columbia. Alpha minimal essential medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT, USA) was used for culturing LLC cells in vitro. Non-treated LLC tumors were also used for generating the cultures of tumor-associated macrophages (TAMs) following a differential attachment procedure employed in our earlier studies [19]. Briefly, single cell suspensions pooled from 8 to 10 disaggregated LLC tumors were resuspended in serum-free medium, placed in Petri dishes and left 15 min in a 37°C incubator. The nonattached cells were then washed away leaving attached >95% pure TAM cultures. Splenic macrophage cultures of a similar purity were obtained by releasing cells from mouse spleens (of C57BL/6 or TLR4 mutant mice), removing erythrocytes by lysis, incubating the cells (pooled from six spleens) overnight resuspended in complete growth medium in Petri dishes kept at 37°C, and then washing away nonattached cells.

PDT treatment

The protocol for PDT started with the administration of photosensitizer Photofrin® (provided by Axcan Pharma Inc., Mont-Saint-Hilaire, Que., Canada) injected intravenously in tumor-bearing mice at 10 mg/kg. The tumors were exposed to light treatment 24 h later with mouse restrained unanesthetized in holders exposing their backs. The light source was a 150 W QTH lamp-based high throughput fiber illuminator (Sciencetech Inc., London, Ont., Canada) equipped with integrated ellipsoidal reflector and a 630 ± 10 nm interference filter, and was coupled with an 8 mm diameter liquid light guide, model 77638 (Oriel Instruments, Stratford, CT, USA). The total dose of light delivered superficially and encompassing the tumor plus ~1 mm of surrounding skin was 150 J/cm^2 at a fluence rate of 100 mW/cm^2.

For in vitro PDT, LLC cells or TAMs attached in 3-cm diameter Petri dishes or 25-mm tissue culture inserts with a porous 0.02-μm anapore membrane base (Nalge Nunc International, Naperville, IL, USA) were exposed to Photofrin (20 μg/ml) for 24 h in complete growth medium. The cells were then washed twice with PBS and exposed to the light dose of 1 J/cm^2 (fluence rate 20 mW/cm^2). The LLC cell survival after this treatment was 10–20%.

Gene expression analysis

Total RNA was obtained from tumor and liver samples, immersed in ice-cold TRI reagent (Sigma Chemical Co.) and homogenized, by phenol-chloroform extraction. Using acid phenol (pH 5) and chloroform, RNA was "cleaned" and excess protein and TRI reagent/Trizol removed. The samples were then precipitated with 10% 3 M NaOaC (pH 5.2) and 100% ethanol (2.5× volume) and incubated for 1 h at −20°C. The RNA pellet was washed with 1 ml of 75% ethanol, resuspended and dissolved in dietheylpyrocarbonate (DEPC) treated ddH_2O (DEPC water) and kept at −80°C until use.

For semi-quantitative RT-PCR, complementary strand DNA (cDNA) was synthesized from 1 μg of total RNA using products from Invitrogen Canada Inc. (Burlington, Ont., Canada). One microgram of RNA was added to a PCR tube containing 1 μl dNTP mix (10 mM each of dATP, dTTP, dGTP, and dCTP) and 1 μl of oligo $(dT)_{12–18}$ (500 μg/ml) primers, and topped to a total of 12 μl with DEPC water. This was incubated at 65°C for 5 min in the thermocycler (MJ Research, Waltham, MA, USA) and immediately chilled on ice. Eight microliter of master mix containing 4 μl Superscript II buffer (5×), 2 μl DTT (0.1 M), 0.1 μl RNAse inhibitor-cloned (10 U/μl), 0.9 μl DEPC water, and 1 μl superscript II reverse transcriptase (200 U/μl per 20 μl reaction) was added to each tube. This was spun down using a PCR mini centrifuge and left at room temperature for 10 min followed by 50 min at 42 and 70°C for 10 min in the thermocycler.

Primers designed (by ourselves) to be specific to the 3′ end of the mRNA sequence of the genes of interest were constructed to be 18–22 nucleotides in length and were ordered from Qiagen (Valencia, CA, USA). The respective primer pair sequences, their melting temperatures (T_m), and their product size are listed in Table 1.

All PCR reactions were performed in a PTC-100 Thermal Cycler (MJ Research). PCR was done using reagents from Invitrogen and according to their outlined procedure. For a 20 μl reaction, 2 μl of magnesium-free 10× PCR buffer [200 mM Tris–HCl, 500 mM KCl], 0.6 μl of Mg2+ (50 mM), 0.4 μl dNTP mix (10 mM each), 1 μl of each oligo-DNA primer (10 mM) (Qiagen), 1 μl of cDNA, 0.2 μl Taq DNA polymerase (units) and 13.8 μl of sterile DEPC water were used. A master mix was made for all primer sets and 19 μl added to 1 μl of cDNA in 0.15 ml PCR tubes. Thirty cycles with the following parameters were done: step 1–95°C for 2 min, step 2–95°C for 30 s, step 3–54°C (*C3*, *C9*), 55°C (*C5*), 58°C (*GAPDH*), step 4–72°C 1 min, step 5–72°C for 7 min, step 6–4°C for 10 min.

For poly-acrylamide gel electrophoresis, PCR products or negative control samples were run on 12% acrylamide gels by loading 10 μl of the PCR product and stained with SYBR-green nucleic acid stain (Invitrogen). One hundred base pair markers (Invitrogen) were used for verifying the PCR product size. Gels were scanned on the STORM Imager (Amersham Biosciences, Piscataway, NJ, USA) under phosphor-blue selection and the generated images were used for gene expression quantification employing ImageQuant software (Amersham). Individual band intensity was measured in terms of pixels per unit area compared to the background intensity. For each sample the intensity of the gene of interest (*C3*, *C5*, and *C9*) was normalized against the intensity of glyceraldehide-3-phosphate dehydrogenase (GAPDH) for that same sample thus giving a new "normalized" value for gene intensity, which can be used for the analysis of gene expression. This normalizing

Table 1 Description of oligonucleotide primer pairs used in PCR reactions

Gene	Primer sequence (5′–3′)	Alignment	T_m(°)	PCR product size (bp)
GAPDH	TGGCCTCCAAGAGTAAGAA	Left	63.4	147
	GGCCCTCCTGTTATTATGG	Right	62.7	
C3	GAAAAGCCCAACACCAGC	Left	56.3	151
	GGACAACCATAAACCACCATAG	Right	57.2	
C5	CCTCTGGCTTGGAAACCTA	Left	59.2	157
	ACCAACACCCCTGACTGCTA	Right	60.1	
C9	TTGGAAAAGGCTGTTGAAGAC	Left	57.2	109
	CACTGCCCATCCAGAAGAAT	Right	57.2	

acted as an endogenous control correcting for differences in RNA content, RT deficiencies, RNA degradation and sample handling.

Complement gene expression in vitro

Following PDT treatment (or sham treatment with controls) of exponentially growing LLC cells or freshly prepared TAMs attached to 3-cm Petri dishes, PBS was replaced with serum and protein-free medium (S8284, Sigma), and the cultures prepared in triplicates (each with 1×10^6 cells) were left 8 h in a CO_2-incubator at 37°C. The cells were then harvested using a cell scraper, pelleted by centrifugation, collected in 1 ml of cold TRI reagent and processed for RNA isolation followed by the RT-PCR protocol.

For testing the impact of the presence of PDT-treated tumor cells on the complement gene expression in macrophages, LLC cells growing in tissue culture inserts (1×10^6/sample) were PDT-treated or sham-treated and the inserts transferred immediately to 3-cm Petri dishes containing 1×10^6 TAMs or spleen macrophages. The two cultures, kept in serum and protein-free medium, were co-incubated in CO_2-incubator at 37°C for different time intervals. The inserts were then discarded and macrophages harvested for the RT-PCR protocol. In some experiments, blocking antibodies or selective inhibitors were added (using 100× concentrated stock solutions) to be present during the co-incubation period. The antibodies tested were chicken anti-heat shock protein-70 (HSP70) polyclonal antibody (K-20, Santa Cruz Biotechnology Inc., San Diego, CA, USA), mouse monoclonal antibody against mouse TLR2 (clone T2.5, Hycult Biotechnology, Uden, The Netherlands), and rat monoclonal antibody against mouse TLR4 (clone MTS 510, Serotec Ltd., Oxford, UK). The concentration used of these antibodies and their isotype controls was 20 μg/ml. Also tested were cell permeable peptides inhibiting Toll receptor-IL1R domain-containing adaptor protein (TIRAP) (613570, Calbiochem, Merk KGaA, Darmstadt, Germany) at 150 μg/ml (with separate DMSO solvent only controls) and NF-κB (SN50, Calbiochem 613570) at 50 μg/ml.

Statistical analysis

The data are presented as mean ± standard deviation. A non-paired Student's *t*-test was applied for testing the difference between means. Differences with $p < 0.05$ were considered significant.

Results

Expression of complement genes in tumor and liver tissues

Mice bearing subcutaneous LLC tumors were administered photosensitizer Photofrin and 24 h later their tumors were exposed to light treatment producing a PDT dose that typically renders around 25% cures of these lesions. Groups of mice were sacrificed 3, 5, 8, and 24 h later, and RNA isolated from the collected tumors and livers was used analysis of the expression of complement C3, C5, and C9 genes. Also included were samples from control non-treated tumor-bearing mice. The relative "semi-quantitative" values of gene expression obtained by the employed two-step semi-quantitative RT-PCR is a reliable means for effectively comparing the levels of mRNA transcripts in cells or tissues before and after various treatments. An example of the generated electrophoresis gel with the bands of investigated genes is shown in Fig. 1a. It can be seen that C3, C5, and C9 genes were expressed in both liver and tumor tissues of non-treated mice. Based on band intensity measurement, the liver : tumor ratios for C3, C5, and C9 expression were 1.46, 1.48, and 1.45, respectively. From the gel it is also obvious that the expression of these genes in tumor tissues were increased at 24 h after PDT. The PDT treatment had no detectable effect on GAPDH expression. The details of band intensity measurements, with the values first normalized for GAPDH expression in the same tissue and then calculated as ratios relative to the level of expression in non-treated control mice, are presented in Fig. 1b–d. While no significant changes can be seen at 3 and 8 h post PDT, all the three complement genes were significantly up-regulated in tumors at 24 h after PDT. This increase was over threefold with C3 and C5 genes and somewhat lower with the C9 gene. In comparison, the expression of these genes in the liver has not significantly changed after PDT, except for a 43% decrease in the expression of C9 at 8 h post PDT. The measurement of gene expression in tumors beyond 24 h post PDT was not reliable because these lesions became largely ablated. The hepatic expression of C3, C5, and C9 assessed at 5 days post PDT revealed no significant changes at that time point (not shown). Similar analysis with TLR4 mutant mice as tumor hosts is described later in this section.

Expression of complement genes in tumor cells and macrophages

The PDT-induced up-regulation of complement genes was further analyzed in vitro using cultured LLC cells

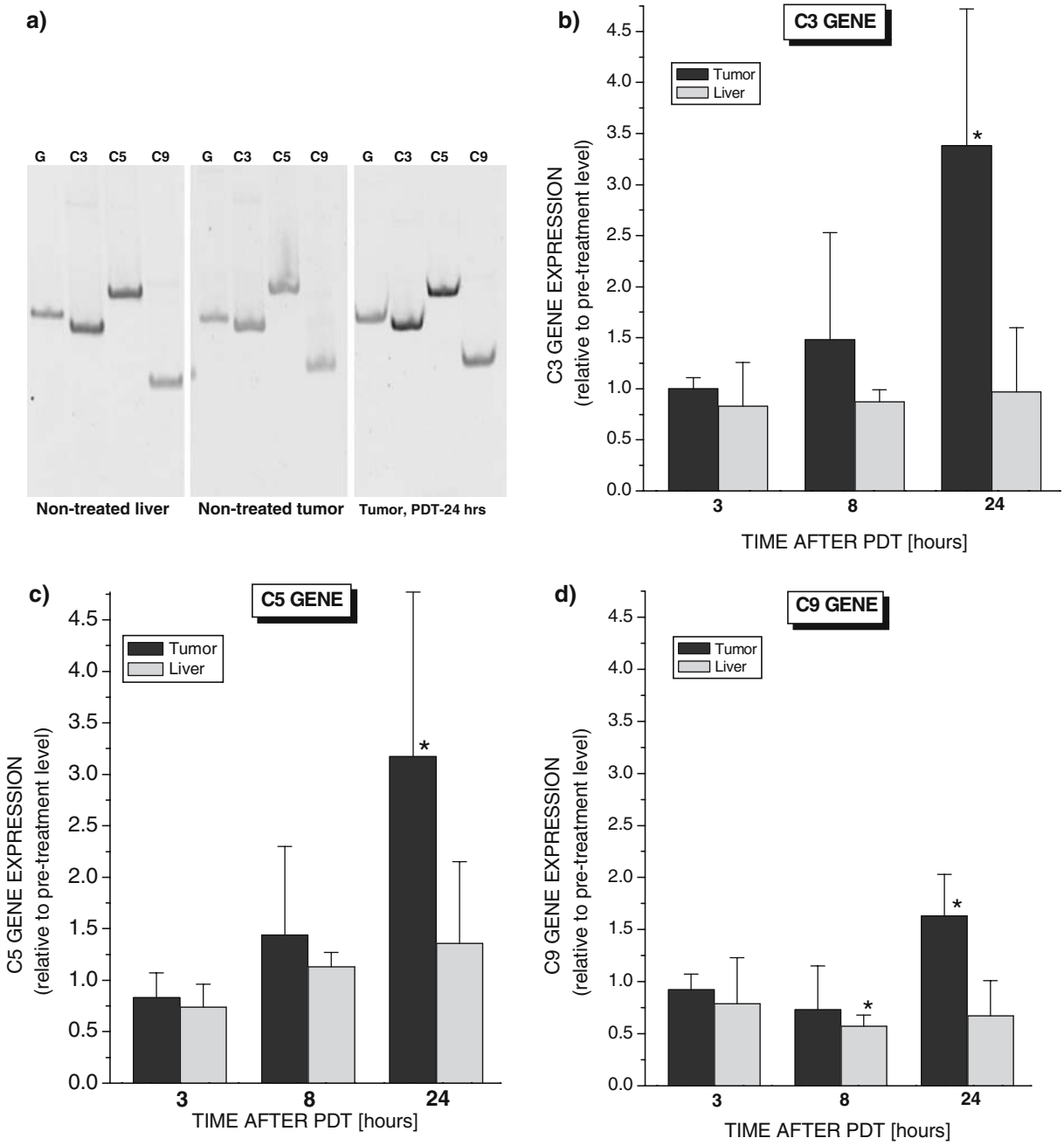

Fig. 1 The expression of complement genes C3, C5, and C9 in tumors treated by PDT and the livers of host mice. Mice bearing subcutaneous LLC tumors were treated by PDT (Photofrin 10 mg/kg i.v. followed 24 h later by tumor-localized light dose of 150 J/cm^2). Tumor and liver samples were collected from mice sacrificed at different times after PDT light treatment and used for the determination of the expression of investigated genes by RT-PCR. **a** Representative example of generated electrophoresis gels with bands of G (reference gene GAPDH), C3, C5, and C9 genes (composed using three individually run gels from the same experiment). The values obtained from band intensity measurement, that were first normalized for GAPDH expression in the same tissue and then calculated as ratios relative to the level of expression in non-treated control mice, are shown separately for C3 (**b**), C5 (**c**) and C9 gene (**d**). *Bars* represent SD ($n = 4$), *asterisk* denotes statistically significant difference ($p < 0.05$) compared to the pre-treatment levels in the respective control group

and macrophages isolated either from LLC tumors (TAMs) or spleens from mice bearing non-treated LLC tumors. The expression of C3, C5, and C9 genes in LLC cells had not significantly changed after their treatment with PDT in vitro, as shown for the 8-hour post PDT time point in Fig. 2. As seen in the same Figure, a similar finding was obtained with PDT-treated TAMs or non-treated TAMs co-incubated with non-treated LLC cells except for a small increase in C5 expression. However, a marked up-regulation of these

genes was seen in non-treated TAMs that were co-incubated for 8 h with PDT-treated LLC cells (Fig. 2). It should be noted that, due probably to biological fluctuation, the extent of this up-regulation showed some variations from experiment to experiment (as exemplified in the following figures), but was always significant.

The time kinetics of changes in C3 gene expression in non-treated TAMs and spleen macrophages co-incubated for different intervals with PDT-treated LLC cells are depicted in Fig. 3. It can be seen that the C3 expression becomes significantly increased in TAMs after 8 h of co-incubation and persists for at least another 8 h. In comparison, a significant increase in C3 expression was also evident in spleen macrophages co-incubated for 8 h with PDT-treated LLC cells but was much less pronounced. Furthermore, the C3 expression in these cells markedly declined at the 16-hour time point.

Role of TLR2/4 signaling

Our earlier studies have shown that the induction of cytokine TNFα production in TAMs co-incubated with PDT-treated tumor cells is at least in part mediated by the activation of cellular signaling pathways triggered by the engagement on TAMs of TLR2 and TLR4 upon binding HSP70 released from PDT-treated cells [19]. In order to examine whether a similar intracellular signaling pathway is involved in the PDT-induced up-regulation

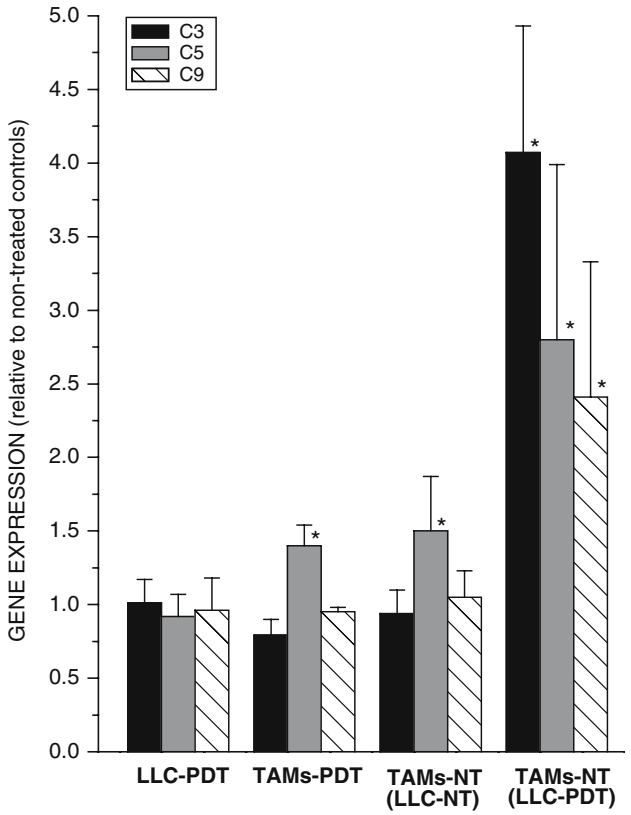

Fig. 2 The effect of PDT treatment in vitro on the expression of C3, C5, and C9 genes in LLC tumor cells and TAMs. For PDT treatment, LLC cells or TAMs obtained from non-treated LLC tumors were incubated with Photofrin (20 μg/ml for 24 h) and then exposed to the light dose of 1 J/cm^2 (LLC-PDT and TAMs-PDT, respectively). In addition, non-treated TAMs (TAMs-NT) were co-incubated with non-treated LLC (LLC-NT) or PDT-treated LLC cells contained in tissue culture inserts with a porous base. Following 8 h of incubation, the cells were harvested for the RT-PCR based analysis of the expression of genes of interest. The results are shown as GAPDH-normalized values relative to those obtained with respective PDT-non-treated control cells incubated alone. *Bars* represent SD ($n = 4$), *asterisk* denotes statistically significant difference ($p < 0.05$) compared to the values for the respective gene obtained with PDT-non-treated TAMs incubated alone

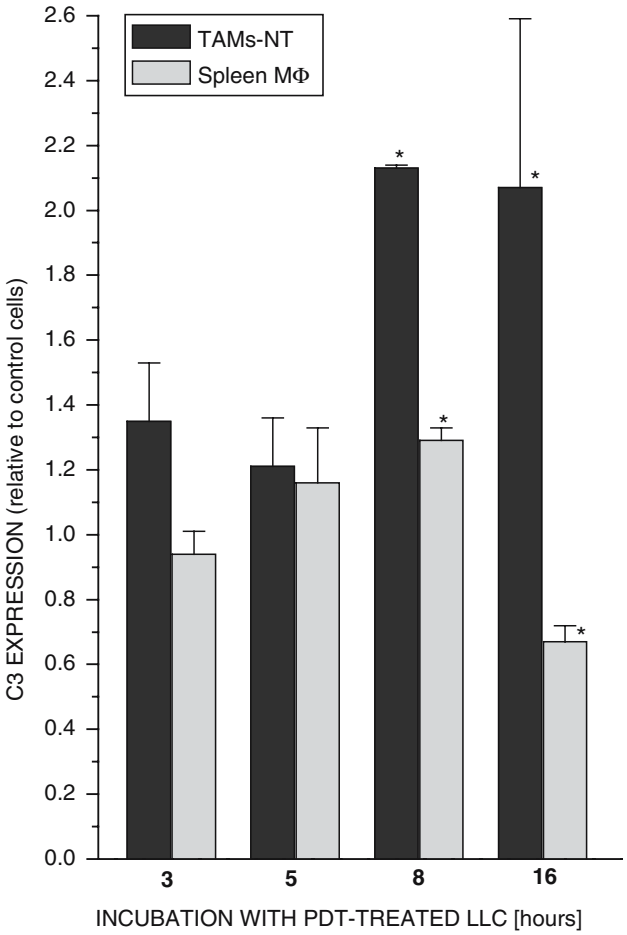

Fig. 3 Time kinetics of C3 gene expression in TAMs and spleen macrophages co-incubated with PDT-treated LLC tumor cells. Tissue culture inserts with PDT-treated LLC cells (prepared as described for Fig. 2) were added to Petri dishes with non-treated TAMs or spleen macrophages and the samples were incubated for different time intervals before the macrophages were collected for gene expression analysis. The results are shown as GAPDH-normalized values relative to those obtained with PDT-non-treated TAMs or spleen macrophages incubated alone. *Bars* represent SD ($n = 4$), *asterisk* denotes statistically significant difference ($p < 0.05$) compared to the values obtained with respective PDT-non-treated control cells incubated alone

of C3, C5, and C9 genes, we tested the effects of a series of blocking antibodies and specific inhibitors on the expression of these genes in TAMs co-incubated 8 h with PDT-treated LLC cells. The results, shown as C3, C5, and C9 expression values relative to the levels in control TAMs incubated alone, are presented in Fig. 4. The data with HSP70-blocking antibodies confirm the role of this HSP as a major molecular signal released from PDT-treated tumor cells. The presence of these antibodies completely inhibited PDT-induced up-regulation of C9 gene and greatly inhibited the increase in expression of C3 and C5 genes. Blocking of TLR2 and TLR4 had in both cases a pronounced impact but with important differences. Anti-TLR2 produced a complete and near complete inhibition of C3 and C9 up-regulation, respectively, while it was about a half that effective with C5. In contrast, with anti-TLR4 there was close to 50% inhibition of C3 up-regulation and around 80% inhibition of C5 and C9 up-regulation. The effects of TIRAP inhibitor peptide were similar to anti-TLR4, with a halfway inhibition of C3 and completely inhibited C5 and C9 up-regulation. Finally, selective inhibition of NF-κB completely prevented the induction of increase in C9 expression, greatly inhibited C3 up-regulation and halved the extent of C5 gene up-regulation. Control treatments (isotype control antibodies, solvent controls) produced no significant impact, verifying the absence of non-specific effects with the tested agents.

For an alternate experimental strategy, spleen macrophages freshly harvested from TLR4 mutant mice were used for testing based on the same protocol as for Fig. 4. The co-incubation for 8 h with PDT-treated LLC cells produced no evidence of an increase in C5 gene expression in these cells, while the up-regulation of C3 and C9 genes was reduced to 53 (±1) and 62 (±7)%, respectively, compared to that found in spleen macrophages from wild-type mice under the same experimental conditions. Consistent with this in vitro evidence were the results obtained in vivo with LLC tumors growing in TLR4 mutant mice. The analysis of tumor tissues excised at 24 h following tumor PDT as described for Fig. 1 and untreated controls revealed no detectable up-regulation of C5 gene, the increase in C9 expression was not significant, while the induced C3 up-regulation was lower than in tumors growing in wild-type mice.

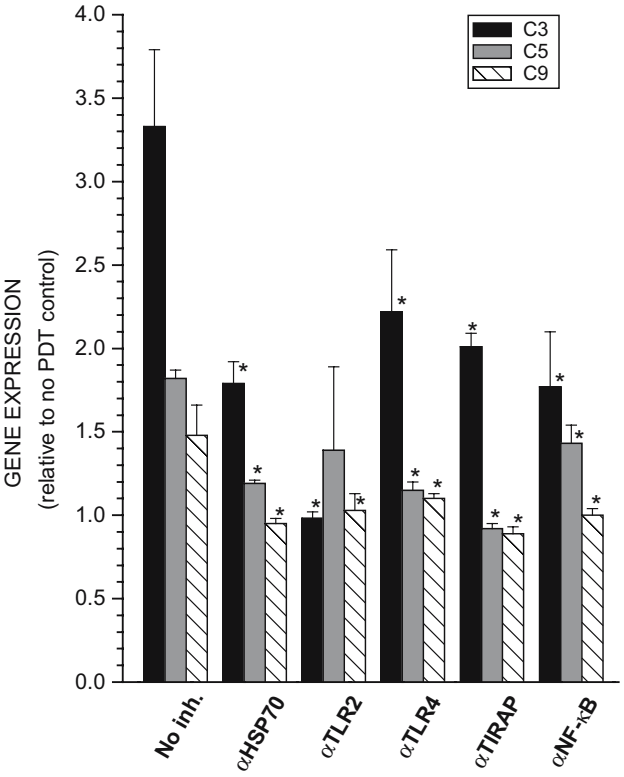

Fig. 4 The effects of various blocking antibodies and selective inhibitors on the up-regulation of C3, C5, and C9 genes induced in presence of PDT-treated LLC cells. Samples with non-treated TAMs were prepared for co-incubation with PDT-treated LLC cells as described for Figs. 2 and 3. During the co-incubation, the following agents were present in the incubation medium of various samples: anti-HSP70, anti-TLR2, anti-TLR4 (all 20 μg/ml), TIRAP inhibitor peptide (150 μg/ml) and NF-κB inhibitor SN50 (50 μg/ml). After the co-incubation period of 8 h, the TAMs were collected for gene expression analysis. The results are shown as GAPDH-normalized values relative to those obtained with PDT-non-treated TAMs incubated alone. *Bars* represent SD ($n = 4$), *asterisk* denotes statistically significant difference ($p < 0.05$) compared to the values for the respective gene obtained with TAMs incubated with PDT-treated LLC cells with no inhibitor present

Discussion

This study reveals that complement genes C3, C5, and C9 become up-regulated in tumors treated by PDT. The accumulation of protein products of these genes and localized complement cascade activation in PDT-treated tumors has already been well documented with LLC and other tumor models employing ELISA and immunohistochemistry [5, 6, 9]. The functional significance of this complement system engagement and its relevance for the therapy outcome is demonstrated by the decrease in cure rates of PDT-targeted tumors upon treatment with agents specifically inhibiting/blocking complement activity [6, 21]. One of the main characteristics of tumor PDT is that it provokes a strong host response which is an integral component of the therapeutic impact of this modality [4, 21]. Activated complement system has emerged as an important element of this host response with critical roles in: (a) initial recognition of danger from tumor-localized

insult inflicted by PDT, (b) instigation and propagation of the elicited inflammatory response, (c) efferocytosis (dead cell removal), and (d) promoting the development of a vigorous adaptive immune response recognizing the PDT-treated tumor as its target [21].

Our recent findings have shown that tumor PDT is also associated with the induction of acute phase response [8, 9, 20]. In addition to the rise of the prototypic murine acute phase reactant, serum amyloid P component (SAP), MBL-A, C3, and other complement and pentraxin proteins become expressed as acute phase reactants in mice bearing PDT-treated tumors [6, 20]. About a fourfold rise in C3 plasma levels was measured in mice bearing LLC tumors at 24 h after PDT, which was followed by a gradual decline toward the pretreatment levels over several days [6]. However, no significant increase in hepatic expression of C3, C5, and C 9 genes was detected in the mice following the same treatment (Fig. 1). The average hepatic expression levels of C3 and C9 in this experiment showed even a decline compared to the pre-treatment level that has not reached statistical significance except for a significant drop with the C9 gene at 8 h post PDT. This negative trend makes less likely the possibility that the hepatic expression of C3, C5, and C9 in mice bearing PDT-treated tumors was up-regulated post-transcriptionally, as it is known to occur with some acute phase reactants [12]. The more likely explanation is that the source of elevated plasma levels of these proteins was the PDT-treated tumor.

During strong inflammatory reactions, a significant proportion of circulating C3 levels in humans was shown to originate from extrahepatic sources and was suggested to be mainly secreted from activated monocytes/macrophages accumulating in large numbers at inflammatory sites [28]. Indeed, massive numbers of monocytes/macrophages were demonstrated to accumulate rapidly after PDT in treated tumors [13, 22]. In vitro experiments performed in this study demonstrate that TAMs receive signals from PDT-treated cancer cells that induce them to up-regulate the expression of their C3, C5, and C9 genes. When localized in tumor microenvironment, macrophages appear to become primed for up-regulating these genes more strongly than macrophages residing at other sites, as illustrated by the less pronounced response seen with spleen macrophages (Fig. 3). Even in untreated tumors, the levels of C3, C5, and C9 gene expression reach close to 70% of the extent found in the host's liver tissue (Fig. 1a).

The up-regulated production of complement proteins by macrophages localized at inflammatory sites was attributed to the influence of inflammatory cytokines [28]. However, our results suggest that, at least with the tumor PDT model, a major role is played by the engaged TLR2 and TLR4 signaling with downstream activation of nuclear transcription factor NF-κB. The data also indicate that HSP70, one of agonistic ligands for these receptors, is as a significant participant. This HSP was suggested to represent a major endogenous danger signal released from PDT-treated tumors whose recognition by pattern-recognition receptors (PRRs) such as TLRs largely contribute to the induction of host response associated with this therapy [21]. The involvement of TLR2- and TLR4-triggered signaling in the PDT-instigated up-regulation of C3, C5, and C9 genes is further supported by the strong inhibitory effects obtained with the antibodies blocking these receptors and by the peptide specifically blocking TIRAP (Fig. 4). The latter is an adaptor protein exclusively involved in signaling through these receptors, with a particularly prominent role with TLR4 [1]. We have also found that the TLR2 and TLR4 expression on TAMs is increased after co-incubation of these cells with PDT-treated tumor cells (M. Korbelik and J. Sun, unpublished results).

Interestingly, our results also suggest (based on the diverse inhibitory effects of the tested inhibitory/blocking agents) that there are specific differences in the roles of TLR2 and TLR4 signaling pathways in the activation of the C3, C5, and C9 genes. The co-operative interaction between TLR2 and TLR4 signaling seems to be involved in the activation of all these three genes. However, C3 and C9 were manifestly more dependent on TLR2 than TLR4, while the reverse seems to be the case with C5. In addition, the NF-κB activity appeared absolutely critical for C9 but not so exclusive for C3 and C5. Consistent with such conclusions are the findings on PDT effects obtained in vitro with spleen macrophages from TLR4 mutant mice and in vivo with PDT-treated LLC tumors growing in these hosts, showing a lack of C5 gene up-regulation and diminished increase in the expression of C3 and C9 genes.

The expression of complement genes at disparate sites such as liver and inflamed tumor is differently regulated by local environmental factors and optimized for the requirements at particular sites [24]. Local production of complement proteins has been recognized as important in tissue homeostasis and immune defense [26]. Complement proteins secreted locally in PDT-treated tumors secure their immediate supply at the specific stages of the elicited host response, which could be critical for the execution of host-protecting mechanisms and the eventual outcome of this therapy. The functional significance of this event is revealed by the reduction in cures of PDT-treated tumors following

blockage of the complement activation cascade by inhibitor FUT-175 [21], or by neutralization of complement anaphylatoxins C3a and C5a [6].

Macrophages residing in tumors accumulate elevated levels of the administered photosensitizer [18] and are therefore preferentially killed by PDT treatment. Hence, the likely source of tumor-localized complement proteins after PDT are not these cells but monocytes/macrophages invading promptly the lesion in massive numbers after therapy. This is in agreement with the finding that the up-regulation of C3, C5, and C9 genes in PDT-treated tumors has not reached significant levels at 8 h post PDT (as seen in vitro) but at 24 h after therapy.

Of note, different patterns of PDT-induced gene expression changes than with C3 appear to be exhibited by some other complement components. Initial results of our investigation indicate that after PDT a down-regulation of expression occurs for C1q and MBL-A genes while the genes encoding ficolins A and B become highly up-regulated in the treated LLC tumors; concomitantly, the MBL-A gene becomes up-regulated in the host's livers (S. Merchant and M. Korbelik, unpublished results). These differences, presumably reflecting specific roles of individual complement and pentraxin proteins in the disposal of killed tumor cells [29] and other aspects of host response to tumor-localized injury, warrant further detailed examination.

Acknowledgments The authors wish to thank Dr. Wan Lam for helpful advice in gene expression analysis. This study was supported by the National Cancer Institute of Canada, with funds from the Canadian Cancer Society.

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