Dual role of complement in tumour growth and metastasis (Review)

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Abstract. Complement-dependent cytotoxicity is one of the mechanisms by which therapeutic monoclonal antibodies are successful against cancer. Complement is one of the innate immune defence systems, whose activation products and membrane-bound regulators interact with cells of the adaptive immune response. The complement system is currently undergoing a re-appreciation in its role within the immune surveillance of tumour. The majority of human tumours are low immunogenic. Complement may be involved through direct, 'danger signal'-elicited activation or via infiltration of inflammatory cells, which express complement components. Inflammatory cells may be associated with malignant transformation and tumour regression. The evidence for the effects of complement activation and regulation on tumour progression and expansion will be reviewed using *in vivo*, *in vitro*, and patient studies, and conclusions drawn for the implications in therapy and management of tumour patients.

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1. Background

Complement is a local and systemic mediator of the first line response to inflammatory or infectious perturbations of the steady state. It is a system of zymogens, which are activated

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in a hierarchical cascade on microbes or altered cells via the classical (antibody mediated), lectin (carbohydrate moiety mediated) or alternative pathway. From the concept of complement being 'a guardian of the extracellular space' (1), it follows that its functional limitation in this compartment relies on fluid phase regulators. Importantly, complement components and regulators are also expressed in lymph nodes and spleen. Complement has emerged as a player in the adaptive humoral and cellular immune responses and has been described as 'nature's premier adjuvant' (1). It exerts this newly appreciated function by tagging antigen and increasing B-cell signalling via CD19/21 or retaining antigen on dendritic cells via CD21 (2), by priming and activating T cells (3,4), by supporting differentation and maturation of dendritic cells (5), and by aiding immunologic memory (6).

Complement has become an entity to consider in a variety of (non-infectious) conditions such as: autoimmune disease, ischemia reperfusion, and tumour immunology. Evidence in support of a role of complement in the latter is contradictory; while some studies show a role for complement in immune surveillance, others demonstrate a role for complement in promoting tumour growth. These studies will be summarised hereafter.

2. Two opposing roles for complement in tumour control

Binding of natural, anti-carbohydrate antibodies (7) or tumour-specific antibodies initiates activation of complement via the classical pathway and effects tumour cell lysis via the membrane attack complex (complement-dependent cytotoxicity). Leukemic cells have been described to efficiently activate both, lectin and alternative pathways of complement, leading to opsonisation and uptake via NK cells and leukocytes (8). Antibody-dependent cellular cytotoxicity is enhanced via C5a-mediated upregulation of FcγR (8). In a mammary sarcoma model, C5a has been shown to exert an inhibitory effect on tumour growth (9). Further, binding of complement components C1q, MBL and/or properdin contributes to apoptotic clearance (10) (Table I). Homoiostatic removal of apoptotic cells avoids tumour development (11).

Tumour cells are protected from complement attack via membrane bound and soluble/membrane-associated regulators (CD55, termed decay accelerating factor or DAF; CD59, termed protectin; CD46, termed membrane cofactor protein; and factor H). CD55 is particularly well researched; tumour-expressed metalloproteinase MMP-7 releases CD55 from the extracellular matrix (12). In addition to its downregulatory

Table I. Complement as adjuvant mediator of anti-tumour immune surveillance.

Enhancement of antibody-dependent cellular cytotoxicity
Complement-dependent cytotoxicity
Inhibition of tumour growth
Contribution to apoptotic clearance

Table II. Complement as adjuvant promotor of tumour growth.

Protection of tumour cells from complement attack and NK cell mediated cell lysis via membrane bound and soluble regulators
Cell activation via sublytic concentrations of terminal complement complex
Activation and migration of myeloid derived suppressor cells
Suppression of anti-tumour T cell immunity

Table III. Complement in tumour immuno-editing.

Elimination:
Antitumor activity of IL-12 (IL-12 production - IFNγ induced, complement mediated; ref. 24)
Danger signals: Calreticulin exposure and ensuing complement activation (25)
Carbohydrate-directed cytotoxicity of natural IgM antibodies (26)
Equilibrium:
The phenotype of the tumour is the result of an individual interplay of host immunity and tumour.
Escape:
Suppression of T-cells via mast cells (complement-mediated uptake of serotonin; ref. 27)
Procachectic effects via complement-mediated cytokine induction (IL-6)

role of complement lysis, CD55 acts to downregulate NK-cell mediated cell lysis (13) (mechanism unknown, ref. 14) and suppress anti-tumour T-cell immunity (15). The inhibitory relationship between CD55 expression and T-cell activity was derived from the use of specific complement-deficient mice (16). Similarly, Crry, the mouse homologue of human CR1/2, suppresses the anti-tumour CD8$^+$ T-cell response and contributes significantly to the tumour burden (17). Sublytic amounts of terminal complement complex, as they are likely to arise during downregulation of complement activation, have as a consequence; proinflammatory cell activation, as described for endothelial cells (18), and protective effect for tumour cells, so-called complement-induced protection (19). A fundamental recent realisation is that C5a significantly affects activation and migration of myeloid derived suppressor cells (20). Together with the observation of a pro-metastatic action of C5a (21), it is possible that C5a may be a so-called tissue derived stromal factor, which mobilises myeloid cells from bone marrow (22). In fact, a bone marrow mobilising effect has been described for C5a (23). Thereby, C5a could attract myeloid derived suppressor cells and lead to immune escape (Table II).

Complement is a factor in the interrelationship between tumour and the host immune system. It has a role in tumour immuno-editing (Table III).

In the early 1970s, a co-incidence of complement activation in tumour patients with the presence of antibodies was noted. It is now understood that the two observations are causally linked; the changes in complement activity are secondary to the presence of circulating immune complexes (28). Immune complexes are prognostically unfavourable. Patients (50-80%) with lung cancer have circulating immune complexes (29). These can be initiated by natural antibodies against carbohydrate modifications on tumour cells, which exceed the ability of complement-mediated solubilisation.

On the other hand, complement activation is a significant effector mechanism for the success of, e.g., rituximab treatment (30) and is likely to step to the forefront with the development of tetravalent monoclonal antibodies, which exhibit enhanced ability to activate the complement (31). Confounders in the interpretation of the dual nature of the role of complement in tumour growth and metastasis, is the fact that tumours and their stromal tissue harbour a continuum in activated cells in an altered micro-environment; tumour-infiltrating leukocytes may be growth promoting or reducing, opposing effects likely to be a reflection of acute and chronic events, which manifest over time (32). Therefore, knowledge of presence or absence of necrosis, types of tissue, tumour-specific mechanisms (stroma/ malignant cells), integration of time and co-morbidities are needed to achieve a clearer understanding of the specific impact of complement on development and control of tumour. Polymorphisms in innate immune players may be under-appreciated determinants in these processes (33).

3. Tumour-associated inflammation, determinants and effects

Tumour begets inflammation and chronic inflammation begets tumour. Lymphocytic infiltration and complement activation in tumour parenchyma appear to control malignancy in papillary thyroid carcinoma because of a significant preponderance of cellular deposits of IgG, C3d, C4d and C5 in cancerous tissue compared to non-cancerous tissue (34). Chronic bacterial *(H. pylori)* or viral (hepatitis B/C virus) infection may lead to malignant transformation of host cells. Acute inflammation is a risk factor for cancer progression, while chronic inflammation, involving, apart from complement, a range of other factors such as reactive oxygen species, vascular endothelial cell growth factor (VEGF), matrix

metalloproteinases, is involved in cancerogenesis (35). Necrosis acts in a proinflammatory way and matures dendritic cells in tumour tissue. In the absence of necrosis, immature dendritic cells predominate (36) and increase T regulatory cells (37).

Complement activation leads to an increase in VEGF (38). VEGF in turn increases synthesis of CD55, which promotes neoangiogenesis via binding to seven-span transmembrane receptor CD97 (12). Neoangiogenesis is co-determined by the interactions of CD36 and TSP1 (39). Thrombospondin has 'properdin-like' type I repeats (40), and properdin appears to have a conserved stretch of amino acids encompassing the CD36 binding motifs of TSP1 (41), but its relation to angiogenesis has not yet been explored. Of note, terminal complement components also harbour thrombospondin modules (C6, C7, C8, C9).

Inflammatory conditions have an impact on the glycosaminoglycan decoration of eukaryotic cells. Binding of negative and positive complement regulators to the surface glycosaminoglycan heparansulfate is a significant determinant of growth and metastasis in mouse melanoma model (42). This binding may occur via histidine-rich glycoprotein, which binds to heparansulfate. Properdin, key protein in amplification of complement activation, has been found to bind to glycosaminoglycan moieties on the cell surface (43 and own unpublished data), but the experimental approach chosen (digestion of heparan sulfate) does not exclude involvement of histidine-rich glycoprotein.

Through its interaction with heparin and its derivatives, complement becomes a player in anticoagulation approaches in the management of cancer patients. A recent meta-analysis has reported a favourable outcome for patients survival in studies using low molecular weight heparin, but more systematic studies are needed to refine patient and cancer groups (44,45). A greater understanding of the contribution made by the interaction of complement with low molecular weight heparin would be useful in this endeavour, as knowledge in this area is fragmented; it is known that low molecular weight heparin binds C1q and interferes with its assembly to form hemolytically active C1, the complex of C1q, C1r2, C1s2 (46). Binding of low molecular weight heparin to immobilised C1 increases the activity of C1 inhibitor (47). The interference in complement activity could be an additional factor, alongside the anticoagulant activity of low molecular weight heparin, which mediates its significant anti-metastatic action. The complement effectors are unknown, but may receive attention as non-anticoagulant heparins are put to use, which successfully inhibit tumour progression (48,49).

Hypoxia downregulates expression of complement inhibitors by tumour cells (not endothelial cells; ref. 50) *in vitro* (51); downregulation of factor H and I secretion and of CD46 expression in non-small cell lung cancer cells exposed to hypoxia, is coincident with increased deposition of C3b and C9. These results call into question the conclusion of recent studies in which CD59 (CD59 restricts formation of membrane attack complex) or CD55 (CD55 accelerates decay of convertases) and CD46 (acts as cofactor in factor I-mediated cleavage of C3b and C4b) were targeted by retrovirus mediated RNAi or antisense-ODNs, respectively and proposed as gene therapy targets, as these cells were grown in normoxia (52,53).

Chronic inflammation induces glycosylation changes in certain serum glycoproteins, which are present also in cancer, and may have functional consequences, favoring anti-apoptotic mechanisms (54). C3 and histidine-rich glycoprotein are affected by this phenomenon and have been characterised as markers of colorectal cancer (55). It is intriguing to speculate that the increased sialylation and fucosylation observed for C3 and histidine-rich glycoprotein convey for these proteins a greater reactivity, affinity or stability.

4. Mouse models of tumour growth and metastasis involving complement

Cathepsin, isolated from highly metastatic melanoma, inactivates complement-mediated lysis. After transfection of cells from a non-metastasising melanoma clone, the rate of metastasis is significantly increased and relates to the ability of cathepsin to cleave C3 (56). Similarly, matrix metalloproteinase-1 cleaves complement C3 and protects tumour cells from injury. Its prometastatic activity is directly related to the ability of matrix metalloproteinase-1 to cleave C3, as seen from *in vivo* models using matrix metalloproteinase-1 transfected tumour cells, C3-deficient mice, and their controls (57).

Tumour-associated complement activation may significantly enhance tumour growth by suppressing the antitumour cellular immune response (20). This study, using a subcutaneous cervical carcinoma model and several specific knock out mouse strains, revealed that tumour growth depended on the presence of complement C3, C4, and C5aR. C3 cleavage products and C1q were found associated with tumour vasculature. C5aR blockade led to a greater infiltration of CD8+ T-cells and smaller tumours. Enhanced CD8+ antitumour activity is also observed when Crry, the mouse homologue of CR1 and 2, is downregulated in a mouse model of metastatic bladder cancer. Survival in this model depends on CD8+ T-cells and C3 (17). Human tumours, by contrast, generally escape CD8+ T-cell mediated elimination due to a loss of MHC I expression.

Silencing of the genes for complement downregulators CD59 and CD55 diminishes the tumour burden in mouse models of ovary and prostate cancer, respectively (52,58). However, instillation of tumour cells transfected with a C5a expressing plasmid leads to tumour regression. The implications are not clear as unmanipulated tumour cells, not control plasmid transfected cells, were used as controls (9). Studies exploring the relevance of complement in tumour immunity are limited (Table IV). Therefore, what is the clinical relevance of complement activation in tumour expansion?

5. Complement as targetable modulator in cancer therapy.

TLR-dependent signalling may exert tumour growth-restricting or -promoting effects, which are mediated by a polarisation to either Th1 or Th2, respectively (37). Complement and TLRs are surface pattern recognition systems, and their integration has only recently been explored (16,59). The modulation of their interactions through targeting of complement components has not yet been investigated. So far, the clinical studies focus on evaluating the beneficial effect of complement activation in controlling tumour growth and metastasis:

Table IV. Complement in mouse models of tumour immunity.

Gene interference	Models	Effect	Refs.
C3 KO, C4 KO, C5aR KO[a]	Syngeneic, *in vivo*	Impaired activation of MDSC, less tumour growth	20
Crry siRNA	Syngeneic, *in vivo*	Enhanced CD8^{+} anti-tumour activity	17
CD59 siRNA	Xenografts, *in vivo*	Less tumour burden (increased lysis)	52
CD55 siRNA	Syngeneic, *in vivo*	Less tumour burden (increased lysis)	58
C5a	Syngeneic, *in vivo*	Blocks cell cycle, increases apoptosis, tumour regression	9

[a]KO, gene targeted deficiency; MDSC, myeloid-derived suppressor cells.

Complement receptor CR3 mediates the beneficial effect of the adjuvant therapy using ß-glucans (60). Concomitant complement activation is beneficial in the success of photo-dynamic therapy of murine squamous cell carcinoma, where a decrease in the expression of Crry, CD55 and CD59 was noted. This is further enhanced by the adjuvant, intraperitoneal administration of anti-factor H, anti-Crry, anti-CD59 or, by intratumoural instillation, properdin (61). An antibody-mediated blockade of CD59 *in vitro* (using blood from patients with colorectal carcinoma) was shown to enhance the antigen specific CD4^{+} T-cell response (62). In fact, the combination of specific, anti-tumour monoclonal antibodies with blockade of membrane bound complement regulators is heralded as a therapeutic concept with promise, especially when bispecific antibodies can be used, which have significantly decreased ability to bind to cells not expressing the tumour antigen (63).

It is possible that the success of the approach to increase complement activation is due to interference with a property conveyed by a complement protein that has not yet been characterised. The following example serves to illustrate this: colorectal carcinoma patients overexpress CD55 and have significantly reduced 7-year survival (64). A human anti-idiotypic antibody, which mimics CD55 (DAF), has shown success in vaccination trials involving patients with colorectal cancer and osteosarcoma (65). Tumour-expressed complement downregulators such as CD55 are thought to provide an advantage to survival and expansion of tumour cells by protecting them from complement-mediated attack. However, more recently, it has been shown that CD55 binds to CD97, thereby effecting mediation of intercellular adhesion. This binding appears to be related to the depth of invasion of gastric carcinoma (66). Further, CD55 synthesis is increased by prostaglandin E2 (67), a cyclooxygenase derived mediator, which favors tumour progression (68). Therefore, CD55 blockade is likely to target two functions of the membrane bound regulator; a function exerted with the host interface (complement downregulation) and another, defining malignancy of the tumour (invasiveness).

6. Conclusions and outlook

Complement modifies tumour growth, metastasis and responses to therapeutics. Complement is a player in the host interactions with neoplastic changes, which can be exploited therapeutically. Important factors with relevance to the success of this strategy deserve more attention; leukemic vs. solid tumour (fluid phase vs. stroma), environment (hypoxia), epithelial-mesenchymal transition and related surface changes.

Complement activation may, however, also complicate therapy regimes; a recent study concluded that nanoparticles, which are polyethyleneglycol-grafted liposomes entrapping cytostatica, may activate complement on their surface, leading to unwanted systemic side effects (69). The extent of macrophage-mediated crosstalk of complement activation with TLR signalling may be decisive in determining the efficacy of therapeutic targeting of complement; in a murine study of photodynamic therapy of squamous cell carcinoma, TLR-mediated signalling led to an increase in transcription of complement genes (70).

There is a clear need for further systematic studies to elucidate mechanisms of complement-mediated control of growth and metastasis of tumours differing in their immuno-genicity. It is likely that tumours skew the type of immune responses (innate vs. adaptive), and success in therapeutically interfering with the innate and adaptive systems will depend on tumour immunogenicity. Mouse models ideally should cover all stages of tumour development; so far, only models of tumour escape have been investigated in complement-targeted mouse lines. Only one study has investigated prior stages in a transgenic model backcrossed to a C3 deficient background in which human keratin 14 promoter/enhancer controls the expression of early region genes of human papillomavirus type 16, providing a multistage epithelial carcinogenesis model, and found no role for C3 in neoplastic progression (71). Adjustment of observation times may be crucial as a study investigating the expressed serum proteome in murine bronchioalveolar carcinoma implies: properdin for example is elevated only at the late stage (14 months) (72). The design of the study does not allow for a conclusion as to the functional importance of this elevation. Use of laser capture micro-dissection and microarray analysis enables characterisation of gene expression in stroma vs. tumour (73), and may aid in the discovery of new functionalities. CD55 e.g. is more expressed in stroma than in colorectal tumour (74).

Modulation of complement activation and regulation has been a strategic approach in drug development with varying success (75). A greater understanding of the relationship of complement and the environment of relevance to tumour (hypoxia, modifications, binding partners and networks, enzymatic activity) is needed. In the majority of studies,

modulation of the complement system appears to act as an adjuvant to increase the efficiency of the primary treatment. This aspect will be elaborated hereafter.

The potential lies in combination therapy approaches. Promising results, favoring an approach to interfere with more than one system involved in the anti-tumour response, have been achieved using mouse models (31). Anti-VEGF (bevacizumab) with ß-glucan were effective in a mouse model of lung cancer (s.c. and orthotopic) (76), and anti-EGFR (cetuximab) combined with ß-glucan lead to significant regression non-small cell lung carcinoma (s.c.) and increase in survival in a mouse model (77). ß-glucans function by priming tumour effector cells; ß-glucans are taken up by macrophages, and ß-glucan fragments are released in the reticuloendothelial system and taken up via CR3 by phagocytic cells, which are then more likely to uptake antibody labelled tumour cells (78). The significance of the tumoricidal effect of ß-glucan has been shown *in vitro* (79) and *in vivo* (80). A triple treatment employing anti- CD55, ß-glucan, and trastuzumab (anti-Her-2/neu Ab) has been efficacious in a mouse xenograft model of ovarian carcinoma (81), yielding a targeted and local overactivation of complement.

Greater, tumour-specific knowledge of expression of regulatory complement proteins and their relation of clinical progression is needed in order to tailor combinatorial therapies (82). Further avenues to explore are the usefulness of combining PEG-liposomes (used for administration of the cytostatic doxorubicin, as Doxil®) with sCR1 or with C5aR antagonist (to block undesired, PEG-liposome-mediated, systemic complement activation, see ref. 69,83) and increasing the efficacy of IL-12 therapy of cancer (84) by co-administering C5aR blockade (to block C5a-mediated suppression of IL-12 by IFNγ primed cells, see ref. 85).

By contrast, the results of a most recent study favour as therapeutic approach a blockade of complement activation because C5a blocking peptide PMX-53 was beneficial to the outcome in a non-orthotopic mouse model over a period of at least 34 days (86).

Is this approach really in complete contrast to the therapeutic downregulation of complement regulators? These have been most successfully used together with tumour-specific antibodies, whose efficacy is directly dependent on complement activation via the classical pathway (30). The net result of their functions will be a considerable increase of C5a, while lesser C5a amounts are needed to exert chemotactic functions. This differential integration of C5a levels has been suggested in the literature and a model discussed, by which the strength of exposure to C5a and the presence of complement enhancers such as C3b, iC3b modulate the immune response (87). It is conceivable that tumour cells growing *in situ* and those that hematogenously spread generate different levels of C5a by the mere compartment they are in. This may indeed be a factor in the opposing conclusions on the role of complement reached in the two seminal pieces of work investigating epithelial cancer *in situ* (20) and metastasising bladder cancer (17) in mice of the same genetic background. In fact, by design of the mouse models that demonstrate the relevance of downregulated CD55 or CD59

in decreasing tumour burden, these are metastatic either by route of administration of manipulated tumour cells (58) or by the time of observation (s.c., 6 weeks) (52). Thus, it appears that adjuvant support of C5a may hold promise in limiting metastasis, thereby providing a unifying hypothesis as to the reason for significantly less tumour burden in wild-type compared to C3 deficient mice in a metastasising melanoma model (57), while the blockade of C5a leads to a reduced tumour burden in neoplastic growth models. It is unclear at present to which extent the C5a receptors, C5aR and C5L2 (a decoy receptor of C5aR with anti-inflammatory properties; ref. 88) are involved in the mediation of these effects. While the affinities for C5a are comparable between C5aR and C5L2, C5L2 binds C5adesarg with greater affinity than C5aR (89). It is intriguing to speculate that the seemingly opposing role of complement in tumour growth and metastasis could be due to a skewing in triggering C5aR/C5L2. Clearly, more, systematically conducted, research is needed to arrive at a unifying concept and constructively inform the design of therapeutics that target complement.

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