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review

Complement resistance impairs anti-tumour therapy

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Background. *Various studies during the last two decades clearly indicate that resistance of human tumour cells to autologous complement is mainly based on the expression of membrane-bound complement regulatory proteins (mCRP) like CD59, CD55 and CD46 with good evidence for a predominant role of CD59. Beyond these in vitro findings the importance of this phenomenon for the patients` outcome now becomes evident from first clinical studies. Overcoming complement resistance of tumour cells is therefore considered a promising way to improve therapeutic options and prognosis in a variety of cancer diseases. In this short review two feasible approaches are discussed in more detail: (1) neutralisation of mCRP by monoclonal or recombinant antibodies and (2) gene silencing strategies to down-regulate mCRP by blocking the expression of these proteins on the RNA level using siRNA.*

Conclusions. *As mCRP are also present on all normal tissues like endothelial cells, parenchymatous organs (liver, kidney etc.) or blood cells, mCRP blocking strategies have to be targeted selectively to malignant cells sparing the surrounding healthy tissues from the deleterious complement attack. Despite first encouraging results, translation of mCRP inhibition to improve antibody-based immunotherapy into the clinic is still a great challenge.*

Key words: neoplasms – drug therapy; immunology; complement inactivators

Introduction

The complement system is a cascade of serin proteases that plays an important role in the immune defense, linking innate and acquired immunity.[1] Activation of complement results in the release of highly potent proinflammatory molecules, the so-called anaphylatoxins, in the formation of the lytic membrane attack complex (MAC), C5b-9, as well as in the opsonisation of pathogens and immune complexes for efficient phagocytosis. To protect themselves from unrestricted complement attack, all cells exposed to complement express various membrane complement regulatory proteins (mCRP), such as membrane cofactor protein (MCP, CD46), decay accelerating factor (DAF, CD55) and CD59.[2] In the last years, multiple studies have shown that complement resistance of tumour cells is a widespread phenomenon that is based on various mechanisms like secretion of soluble complement inhibitors or soluble forms of mCRP, respectively, into the microenvironment[3-7], expression of sialic acid[8] or complement cleaving proteases.[9]

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Also rebinding of secreted soluble complement inhibitors to the tumour has been observed.[10,11] The most important mechanism, however, is the overexpression of one ore more of the membrane-bound complement regulatory proteins CD46, CD55 and CD59.[12,13] Although the influence of each mCRP varies between different tumour cell lines and has to be determined separately, there is strong evidence for an exceptional role of CD59, that blocks the assembly of the membrane attack complex (MAC) by interfering with the insertion of C9 thereby preventing the formation of the lytic pore. The functional importance of CD59 has been underlined by several approaches: whereas the mere number of mCRP only in part correlates with tumour cell resistance to complement-mediated lysis, transfection of CD59-negative tumour cells with CD59-cDNA increases their complement resistance considerably.[14-16] Moreover, many studies have demonstrated that neutralisation of CD59 but also of other mCRP by using monoclonal antibodies significantly increases the susceptibility of cancer cells to complement-mediated killing.[13]

Complement resistance as a prognostic factor?

There are only few clinical studies yet to underline the functional importance of complement resistance for tumour cell survival and disease progression.

Recently, Watson *et al.*[17] showed that expression of CD59 goes along with a deterioration of the patients' prognosis in colorectal carcinomas. Furthermore, the expression of CD59 correlated with local tumour progression and tissue dedifferentiation in prostate cancer.[18] High levels of CD59 are associated with an earlier biochemical relapse measured by increasing PSA levels after radical prostatectomy. However, contradicting data for other tumours do not allow to generalise about the potential impact of mCRP expression levels on disease prognosis. In a study with breast cancer patients, the loss of CD59 expression could be found to go along with a reduced over-all-survival.[19]

Also other mCRP and their association with the disease prognosis have been studied. The overexpression of CD55 seems to predict a poorer prognosis in patients with colorectal cancer.[20] The 7-year survival of patients with high expression levels of CD55 was remarkably lower than that of patients with low expression levels (24% vs. 50%). For breast tumours, Madjd *et al.*[21] found that overexpression of CD46 correlated with worse histological staging and a higher risk of tumour recurrence. Interestingly, in certain malignancies the loss of CD55 or CD59 may also result in more aggressive tumour growth (bigger tumour size, worse grading, higher rate of lymph node metastases) and a poorer prognosis.[19,22] For gastric carcinomas a correlation between CD97(EGF) and CD55, respectively, and tumour invasion into the surrounding tissue is reported.[23] High expression profiles of these two molecules go along with aggressive local tumour growth and a higher pathological and clinical staging.

All in all, overexpression of mCRP by cancer cells and its possible influence on patients' mortality seems to be rather heterogenous and has to be examined separately for each type of cancer.

Impact of complement resistance on immunotherapy

Complement resistance has gained significant importance with the introduction of anti-tumour immunotherapy. It not only influences the course of disease but also the patients' prognosis by impairing therapeutic options.

The rapid progress in molecular biology and recombinant antibody technology during the last two decades promoted immunotherapy of malignant diseases. Since then, anti-tumour antibodies successfully made their way from the laboratories to the clinic and meanwhile present a well-established adjuvant therapy regimen for a variety of cancer diseases (Table 1).[24]

Classical murine monoclonal antibodies derived from hybridomas according to Köhler and Milstein[25] could not succeed in clinical testing because of the risk of severe anaphylactic reactions and formation of neutralising human anti-mouse-antibodies (HAMA) with rapid loss of effector functions.[26] With the advent of recombinant technology, `designer' antibodies became a powerful tool in anti-cancer therapy. Beyond the well-known classical antibody effector functions such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC), there are additional effects on the target cells that rather depend on the epitope than on the antibody itself.[27] These so-called epitope-specific antibody effects can trigger apoptosis or can modulate the auto- and paracrine secretion of tumour cells, thus influencing the tumour's microenvironment.[28] It is often difficult to determine which effect is most important for the antibody's anti-tumour response.

Despite the great success of recombinant antibodies in cancer therapy, clinical oncologists and tumour immunologists are

Table 1. Anti-tumour antibodies in clinical use

MAb name	Trade name	Target	Type	Approval date	Used to treat
Rituximab	Rituxan	CD20	IgG1, Chimeric	1997	Non-Hodgkin lymphoma
Trastuzumab	Herceptin	$p185^{neu}$	IgG1, Humanised	1998	Breast cancer
Gemtuzumab-ozogamicin*	Mylotarg	CD33	IgG4, Humanised	2000	Acute myelogenous leukemia (AML)
Alemtuzumab	Campath	CD52	IgG1, Humanised	2001	Chronic lymphocytic leukemia (CLL)
In-111/Y-90-Ibritumomab-tiuxetan*	Zevalin	CD20	IgG1, Murine	2002	Non-Hodgkin lymphoma
Daclizumab	Zenapax	CD25	IgG1, Chimeric	2002	Acute and Chronic leukemia
I-131-Tositumomab*	Bexxar	CD20	IgG2, Murine	2003	Non-Hodgkin lymphoma
Bevacizumab	Avastin	VEGF	IgG1, Humanised	2004	Colorectal cancer
Cetuximab	Erbitux	EGFR	IgG1, Chimeric	2004	Colorectal cancer

* conjugated monoclonal antibodies

confronted with limitations of this approach. Similar to the well-known phenomenon of chemoresistance of tumours, *i.e*. the capacity of certain tumour cell clones to become refractory to cytostatic agents, there is also a phenomenon of resistance to antibodies.[29] After repetitive treatment cycles, tumour cells get resistant against further antibody therapy. Several mechanisms may lead to antibody resistance, *e.g* down-regulation of the target epitope or diminished effector functions. Various studies indicate that the up-regulation of mCRP, namely CD55 and CD59, is responsible for resistance against CD20 serotherapy with rituximab.[29-32] Blocking of these regulatory molecules can restore the tumour cells' susceptibility to rituximab *in vitro*.[30,31] The cytotoxic effects of the anti-her2/neu antibody used in the therapy of metastased breast tumours could be augmented by blocking of mCRP *in vitro*.[33]

From these data mCRP appear as interesting target epitopes for new adjuvant therapeutic regimen.

Strategies for tackling complement resistance on human tumours

The significance of complement resistance of human tumours became obvious through multiple experiments applying murine monoclonal antibodies that blocked mCRPs. [4,6,12,13,34,35]

However, translation of these findings into the clinic is hampered by two major obstacles: (1) to find the most effective and secure way of mCRP neutralisiation and (2) restriction of the potentially dangerous intervention to cancer cells.

Different to the benchside situation, a therapeutic strategy must be tolerable for the patient. Blocking mCRP by murine monoclonal antibodies is not appropriate (for reasons as discribed above). Two promising approaches have been developed for the future clinical application, which, however, still require comprehensive preclinical investigation.

Bispecific mCRP-blocking antibodies

For antibody-based immunotherapy the possibility to generate bispecific antibodies that can recognize two different epitopes by their two different antigen binding sites widens the scope and improves the chances to generate truly tumour-specific »magic bullets«.[36,37] Bispecific antibodies, which allow mCRP inhibition to be restricted to tumour cells *in vitro* have been produced by various means.[38-41] Harris *et al*.[40] generated chimeric anti-CD59 x anti-CD19 and anti-CD59 x anti-CD38 antibodies by chemical linkage. B cell specific binding and lysis could be observed while sparing surrounding bystander cells. Although this work served as »proof of principle«, the chemical synthesis of bispecific antibodies is a cumbersome procedure and inappropriate for clinical testing. Blok *et al*.[41] obtained murine bispecific anti-CD55 x anti-G250 antibodies applying classical hybridoma or quadroma technology with good activity against renal cell carcinomas *in vitro*. Recently, a bispecific monoclonal anti-CD55 x anti-MHC class I antibody proved its efficacy on human colorectal and cervix carcinoma cell lines resulting in elevated C3-deposition and augmentation of complement-mediated cell lysis.[39]

For therapeutic approaches the use of humanised or at least chimeric antibodies is mandatory. These bispecific antibodies are nowadays constructed by recombinant »antibody engineering«.[42]

However, despite all progress in the field of recombinant antibody technology it remains difficult to obtain continuously sufficient amounts of bispecific antibodies for *in vivo* testing in experimental animals or even clinical studies. The best established

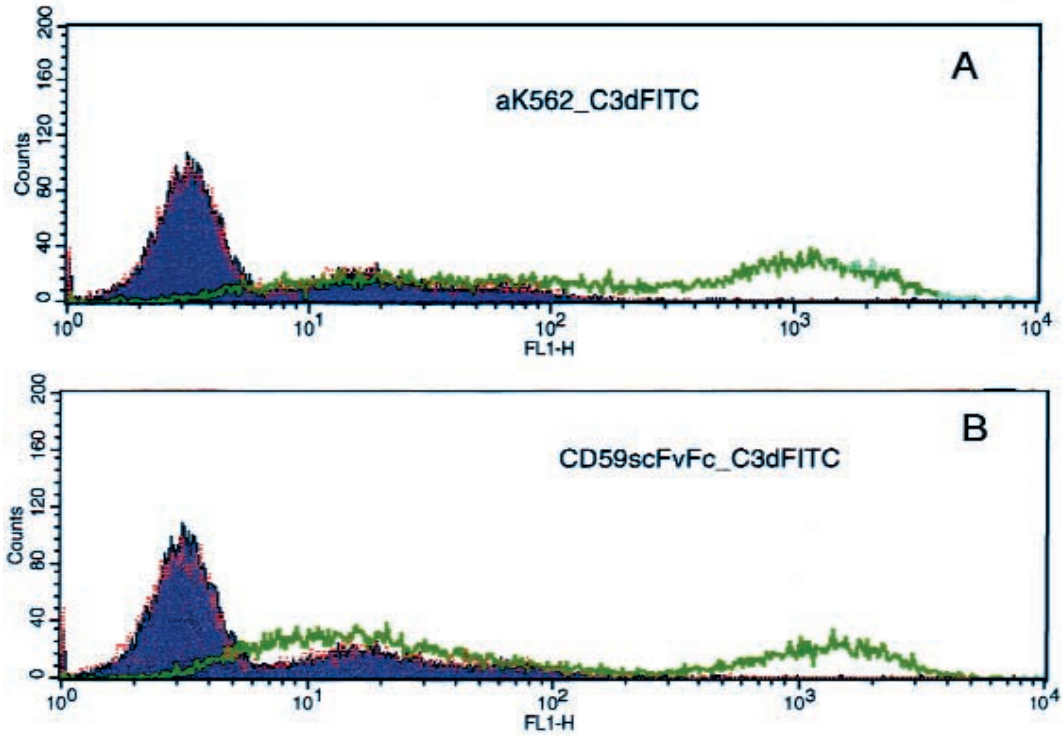

Figure 1. Blocking of CD59 augments tumour-directed complement activation: FACS-Scan for C3 (C3d) detection on human K562 erythroleukemic cell line after preincubation with polyclonal rabbit-anti-K562 or chimeric anti-CD59-miniantibody and pooled human serum as complement source. (A) Positive control with polyclonal rabbit-anti-K562 (green line), (B) Chimeric anti-CD59-scFv-Fc (green line). (Underlied curves each show two negative controls with heat inactivated serum or with irrelevant human IgG, respectively).

way to produce humanised bispecific antibodies takes advantage of expression vectors which contain the antibody genes. These vectors are commonly transfected into mammalian or insect cells that subsequently secrete the recombinant antibody into the cell culture supernatant. However, this technology still suffers from difficulties in achieving stably transfected clones, varying and vanishing protein production yields, a highly inefficient heterodimerisation of the different antibody chains, and problems with the purification of the heterodimeric bispecific antibodies. Despite the fact that there are several strategies which may help to overcome these difficulties, construction and expression of recombinant bispecific antibodies remains a »high risk challenge« far away from laboratory routine and with still unpredictable outcome.

We recently developed a chimeric mouse/human anti-CD59 miniantibody (scFv-Fc) from a murine hydridoma (MEM43) that was able to trigger C3-deposition on human tumour cells via the Fc-mediated classical pathway although it failed to significantly augment complement-dependent killing (Figure 1). [43]

Ziller *et al.*[44] generated humanised anti-CD59 and anti-CD55 miniantibodies, that were able to trigger complement-mediated lysis on human lymphoma cell lines. Furthermore, the lytic effect of rituximab could be augmented by these antibodies.

Silencing of mCRP genes

Another approach for tackling complement resistance of human tumours is RNA interference (RNAi). By using small interfering RNAs (siRNA) this technique offers great potential as a novel therapeutic strategy in tumour therapy but also in a wide field of other possible applications.

SiRNA technology, known since 2001, is based on short double-stranded RNA oligomers which cause highly specific and efficient silencing of target genes by posttranscriptional gene knockdown (Figure 2).[45] The antisense-strand of the siRNA molecule is complementary to the mRNA of the target protein.

SiRNAs induce the intracellular formation of a protein-complex, called „RNA-induced silencing complex (RISC)« consisting of helicase and nuclease-acitivity among others. The RISC-complex induces the separation of the sense and antisense strand, mediates the recognition of the target mRNA and catalyses the degradation of bound mRNA. The result is the specific inhibition of target-protein synthesis.

Although siRNA and its functionality in mammalian cells was detected just 5 years ago, plenty of studies demonstrating the therapeutic potential of siRNA have already been published. *In vivo* studies showed positive results applying siRNA for the therapy of neoplastic diseases[46-48], the treatment of sepsis[49] and the reduction of cholesterol levels.[50] Meanwhile the first clinical trial of siRNA therapy of the age-related macula degeneration (AMD) has been started.

To better exploit complement for cancer cell eradication, we tried to reduce complement-resistance of neoplastic cells by blocking mCRP function using siRNA-technology. SiRNAs targeting the mCRPs CD59, CD55 and CD46 were designed and tested concerning their downregulation efficiency *in vitro*. In this study siRNAs were either in-

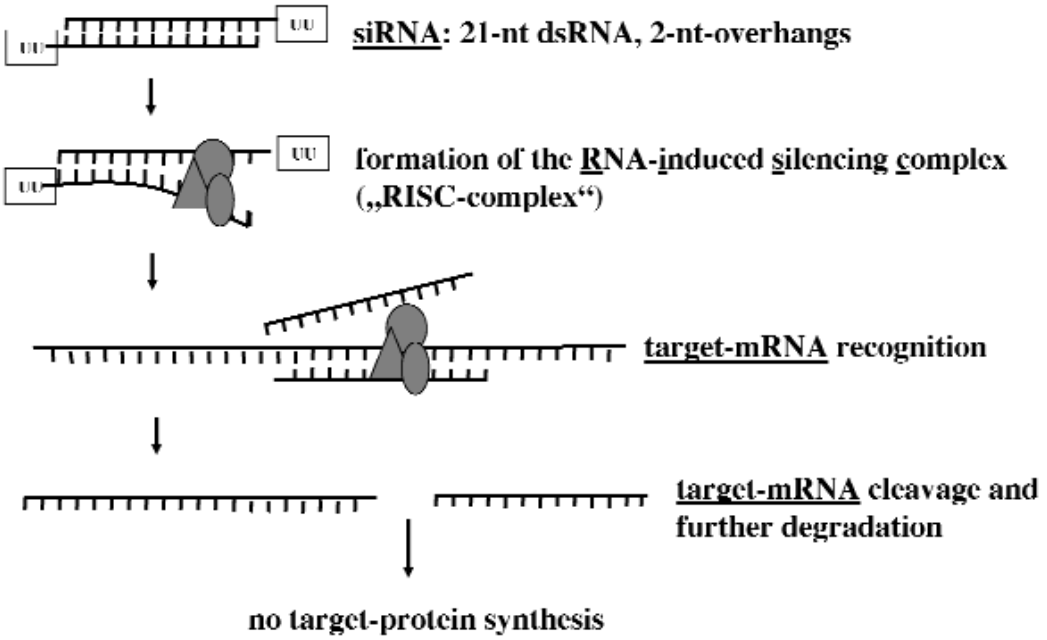

Figure 2. Schematic presentation of siRNA-induced silencing mechanism.

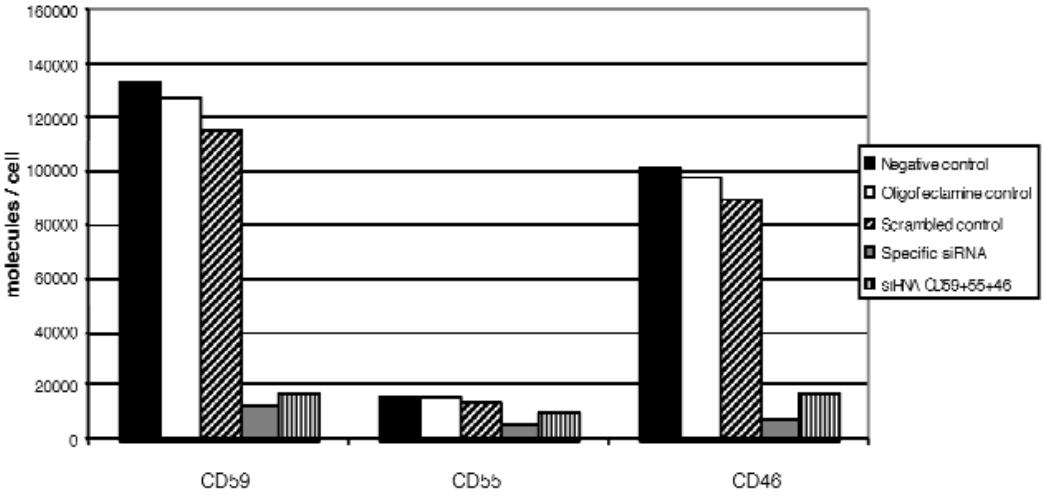

Figure 3. Cell surface expression of mCRPs CD59, CD55 and CD46 on BT-474 breast carcinoma cells after transfection of the corresponding anti-mCRP siRNA individually or in combination, respectively.

dividually or combined transfected into Du145 prostate carcinoma cells or BT474 breast carcinoma cells, respectively. The inhibition of target protein expression was analysed both on protein level by FACS analysis and on mRNA level by RT-PCR. Downregulation of mCRP up to 80% could be achieved (Figure 3). Complement-resistance of CD55-, CD46- and/or CD59-deficient tumour cells, subsequently evaluated by cytotoxicity assays and by analysis of C3 deposition, clearly indicated that siRNA-induced inhibition of mCRP expression sensitised tumour cells to complement attack.[51]

Despite these encouraging findings and the outstanding potency and selectivity of siRNA, promising to improve targeted cancer therapy, the systemic administration of aqueous siRNA, even chemically stabilized, is still limited by unspecific side effects and a lack of activity in the target tissue due to limited blood stability on the one hand and poor intracellular uptake on the other hand.[46,52,53]

The need for devices enabling systemic administration and targeted delivery to tumour tissue and disseminated metastatic lesions is obvious.

Strategies based on viral vector delivery would be a possible approach but for safety reasons they are hitherto only of limited clinical use. A feasible approach, providing tissue selectivity and safe systemic delivery is based on immunoliposome-technology.[54] Liposomes are widely investigated for their properties as site-specific drug carriers allowing higher drug doses due to fewer systemic side effects.[55,56] Liposomes are able to alter the pharmacokinetic profile of a drug, delivering the encapsulated agent preferentially to solid tumours, and acting as a slow-release depot for the drug in the diseased tissue.[57] These attributes often result in a more favourable toxicity profile and an improved therapeutic window for the use of the agent.

Though conventional liposomes allow passive tumour site targeting to some degree, the idea of conjugation of cell-specific antibodies to liposomes (immunoliposomes) has been studied for selective drug delivery.[58-60]

Tumour-associated antigens can be utilised as appropriate target molecules. Monoclonal antibodies against tumour-associated antigens have been successfully adopted for targeting to various types of cancer cells.[61]

Internalisation of immunoliposomes by receptor-mediated endocytosis into target-cells results in intracellular drug delivery.

A variety of cytotoxic drugs have been delivered to target cells *in vitro* by using immunoliposomes; *e.g.* doxorubicin, vinorelbine, methotrexate[62] and daunomycin.[63] Anti-HER2 immunoliposomal doxorubicin is awaiting Phase I clinical trials. Furthermore, immunoliposomes have been employed to deliver oligodeoxyribonucleotides (ODN) designed to specifically inhibit gene expression by blocking translation, splicing or transcription process *in vitro*, thereby providing powerful therapeutic tools against viral diseases and cancer.[64] Moreover, *in vivo* knockdown of gene expression with intravenous RNA interference (RNAi) using a small hairpin RNA (shRNA) expression plasmid encapsulated in immunoliposomes has been shown.[65]

To conclude, immunoliposomes containing siRNA combine specific antibody-mediated tumour recognition with gene-specific downregulation of target mRNAs.

Another promising approach of targeted siRNA delivery *in vivo* has been achieved by complexation of chemically unmodified siRNAs with polyethylenimine (PEI).[66,67] Self-assembling nanoparticles constructed with polyethylenimine were adapted for siRNA. Target-specific delivery can be achieved by attaching peptide ligands (*e.g.* to bind to integrins) to the nanoparticle.

Furthermore, a protamine-antibody fusion protein for systemic, cell-type specific, antibody-mediated siRNA delivery was developed recently.[68] This approach takes advantage of the non-covalent nucleic acid-binding properties of protamine, which originally nucleates DNA in sperm. In combination with the site-specific delivery properties of the antibody Fab fragment this fusion protein is a feasible device to administer siRNA systemically.

Conclusion

Complement resistance is a widespread and nowadays well examined mechanism that enables tumour cells to withstand autologous immune attack. A magnitude of *in vitro* and several *in vivo* studies support the notion that blocking of mCRP is a feasible approach for tackling cancer cells. By means of modern recombinant technologies humanised bispecific anti-mCRP-anti-tumour antibodies and siRNA based immunoliposomes for mCRP gene silencing are promising strategies that could allow transferring experimental complement research to clinical application. Encouraging results from *in vitro* and animal studies have to be reproduced and then could widen the scope of clinical anti-tumour therapy.

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