Hypoxia increases susceptibility of non-small cell lung cancer cells to complement attack.

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Abbreviations:
NSCLC: non-small lung cancer cells; FI: factor I; FH: factor H; XTT: 2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide; ROS: reactive oxygen species; NHS: normal human serum; C4BP: C4b-binding protein; DAF : decay-accelerating factor; MCP: membrane cofactor protein; CR1: complement receptor 1; MBL: mannan-binding lectin; VEGF: vascular endothelial growth factor; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; 7-AAD: 7-Amino-actinomycin D; MFI: mean fluorescence intensity

Abstract:

The complement system can be specifically targeted to tumor cells due to molecular changes on their surfaces that are recognized by complement directly or via naturally occurring antibodies. However, tumor cells often overexpress membrane-bound complement inhibitors protecting them from complement attack. We have previously shown that non-small cell lung cancer (NSCLC) cells, additionally to membrane-bound inhibitors, produce substantial amounts of soluble regulators such as factor I (FI) and factor H (FH). Since low oxygen concentration is associated with rapidly growing solid tumors, we studied how NSCLC cells protect themselves from complement attack under hypoxic conditions. Unexpectedly, mRNA levels and secretion of both FI and FH was significantly decreased already after 24 hour exposure to hypoxia while cell viability measured by XTT assay and annexin V/7-AAD staining was affected only marginally. Furthermore, we observed decrease of mRNA level and loss of membrane-bound complement inhibitor CD46 and increased deposition of early (C3b) and terminal (C9) complement components on hypoxic NSCLC cells. All three complement pathways (classical, lectin and alternative) were employed to deposit C3b on cell surface. Taken together, our results imply that under hypoxic conditions NSCLC give up some of their available defense mechanisms and become more prone to complement attack.

Introduction

It has been proposed that cells capable to form tumors appear with significant frequency but the vast majority of them never results in detectable tumors due to insufficient blood supply (1). Lack of a blood vessel network dense enough for proper delivery of oxygen and nutrients or exchange of metabolites is the limiting factor of tumor growth. Therefore, the capability of tumor cells to induce angiogenesis determines their invasive phenotype. However, in fast growing tumors there is usually a state of equilibrium between proliferating, well oxygenated cells, and not proliferating or dying, hypoxic parts. Whereas normal tissue exhibits oxygen tension between 30-70 mmHg, solid tumors contain areas where this value drops to less than 10 mmHg (2). Appearance of hypoxic areas within tumors can be explained by two major reasons. Rapidly expanding tumor cells consume large quantities of oxygen and in the meantime cells increase their distance from supplying blood vessels. Furthermore, new tumor-induced blood vessels are often blind-ended, have incomplete endothelial lining and basement membrane and have a tendency to collapse (3). Under certain conditions, severe ischemia can induce apoptosis (4), however low ATP concentration observed in hypoxic tumors disables the apoptotic cascade and leads to necrosis (5,6). Wild type tumor suppressor genes like p53 predispose to cell death when oxygen tension drops, so hypoxia selects cells with p53 mutations (7). If these cells survive with non-functional p53 they will likely accumulate further mutations, thus gaining a more aggressive phenotype. On the other hand, sudden restoration of oxygen to hypoxic cells (reoxygenation) can be also harmful, causing massive intracellular production of reactive oxygen species (ROS), which will further affect pivotal cell organelles like mitochondria or cellular membranes, leading to cell death (8).

The complement system is one part of the innate immunity targeting tumor cells since deposition of complement components has been shown in tumor tissues of various origins

(9). However, over-expression and shedding of membrane-bound complement inhibitors by many tumor cells limit their susceptibility to complement attack (10). Previously we have found that many non-small cell lung cancer (NSCLC) cell lines additionally produce soluble complement inhibitors such as factor I (FI), factor H (FH) and C4b-binding protein (C4BP) (11,12). Soluble complement inhibitors were shown to boost the level of NSCLC protection beyond the level attainable for membrane-bound inhibitors (12). Moreover, down-regulation of FH production by NSCLC cells resulted in reduced tumor growth in a mouse xenograft model (13). This is particularly important now as many anti-tumor therapies employ antibodies that exert a significant part of their action via activation of complement (14,15). Studies on endothelial cells have revealed that hypoxia and/or hypoxia-reoxygenation increased expression of membrane-bound complement inhibitors such as decay-accelerating factor (DAF, CD55), membrane-cofactor protein (MCP, CD46) and complement receptor 1 (CR1, CD35) (16,17). How tumors regulate expression of membrane-bound and soluble complement inhibitors under hypoxic conditions remains an open question. Upregulation of endogenous complement inhibitors by tumor cells under hypoxia could lead to increased survival, thus saving the reservoir of invasive cells. On the other hand, downregulation of complement inhibitors may provoke local inflammation, leading to indirect but beneficial effects such as induction of angiogenesis (18) or those resulting from accessory functions of infiltrating cells. In order to address this question we have used two NSCLC cell lines: H2087 and H358 as a model and challenged them with hypoxia and hypoxia/reoxygenation. Both of these cell lines correspond to lung adenocarcinoma, which is the most common histological type of lung cancer (19). Moreover, we previously showed that these cells are equipped with a functional set of both soluble and membrane complement inhibitors, which allow them to actively influence local complement activation (12).

Materials and Methods

Cells

H2087 (lung), H358 (lung), AGS (gastric) HT-29 (colorectal) and PC-3 (prostatic) adenocarcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). All cells were cultured in RPMI 1640 supplemented with L-glutamine, 10% fetal calf serum, streptomycin and penicillin (Invitrogen, Carlsbad, CA) and passaged by trypsinization. One day prior to the assays, cells were seeded into 96-well microtiter plates (Nunc, Glostrup, Denmark) at 10^5 cells per well to reach 100% confluency. Afterwards cells were washed with PBS and 100 μl of serum-free Optimem medium (Invitrogen) was added.

Hypoxic conditions

In order to mimic hypoxic conditions, cells were incubated at 37°C in a humidified hypoxia chamber (Hypoxia Workstation 400; Ruskinn Technology, Leeds, UK), connected to a Ruskinn gas mixer module supplying 94% N_2, 5% CO_2 and 1% O_2. Cells were either kept in hypoxic chamber for 48 hours (referred to as 48 h hypoxia) or for 24 h followed by 24 h at normal O_2 tension (referred to as 24 h hypoxia/reoxygenation). Control cells (normoxia) were cultured for 48 h under normal O_2 tension.

Real-time RT-PCR

Total RNA was isolated and reverse-transcribed using random primers with the SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR reactions were performed using the 7300 Real Time PCR System (Applied Biosystems, Foster City, CA) and the SYBR Green PCR Master Mix (Applied Biosystems) in a total volume of 25 µl containing 0.2 µl of cDNA with the following thermocycling steps: 50ºC for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s, and 60°C for 1 min. PCR efficiencies were calculated using the standard curve method in accordance with the supplier's recommendations. Relative levels of expression were assessed by the threshold cycle (Ct) values and expressed as a percentage relative to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA. Every assay was performed in triplicate. Primers used are listed in Table 1.

Measurement of CD46, CD55 and CD59

Expression of CD46, CD55 and CD59 was assessed with specific antibodies as described previously (12). Cells were examined by flow cytometry using a FACS Calibur (Beckton Dickinson, Franklin Lakes, NJ) and obtained data were analyzed with WinMDI 2.8 Software (freeware, © Joseph Trotter).

Ig binding and complement deposition assays

Cells were harvested with versene (Invitrogen), washed twice with (140 mM NaCl, 10 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.2) and incubated with 20% normal human serum (NHS) (Invitrogen) diluted in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ for 30 min at 37º C and finally washed with binding buffer. Deposition of IgG, IgM and IgA was measured by incubation with their class specific anti-Ig polyclonal antibodies (Dako) diluted 1:100, followed by incubation with FITC-conjugated goat-anti rabbit antibodies 1:1000 (Dako). Measurement of C3 deposited on NSCLC cells was performed as described previously (12). Deposition of C9 was detected with polyclonal goat anti-C9 (Complement Technology, Tyler, Texas) diluted 1:200 in binding buffer followed by FITC-conjugated anti-goat Ab (Dako Cytomation, Glostrup, Denmark) diluted 1:75. To determine which complement pathway was activated under hypoxic conditions, C3 deposition was measured after incubation with 20% factor B-depleted NHS (Quidel, San Diego, CA) in $DGVB^{2+}$ (2.5 mM veronal buffer, pH 7.3, 72 mM NaCl, 140 mM glucose, 0.1% gelatin, 1 mM $MgCl_2$, and 0.15 mM $CaCl_2$) allowing both classical (CP) and lectin pathway (LP) activation, or in Mg^{2+}-EGTA buffer (2.5 mM veronal buffer, pH 7.3, 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM $MgCl_2$, and 10 mM EGTA) allowing alternative pathway (AP) activation. To find out if both lectin and classical pathways were activated in $DGVB^{2+}$, C1q and MBL deposition was evaluated by flow cytometry. Cells were harvested, incubated with 20% NHS in $DGVB^{2+}$ and then stained with anti-MBL Ab (1:150) (HyCult Biotechnology, Uden, The Netherlands) or anti C1q (1:100) diluted in binding buffer (Dako Cytomation) followed by their respective FITC-conjugated secondary Abs.

Measurement of FH and FI secreted by NSCLC cells

Conditioned serum-free Optimem medium was collected, centrifuged to eliminate cell debris and the content of FH and FI was determined by ELISA, as described previously (12). Plasma-purified human FH and FI were used as standards.

XTT assay

In order to measure cell viability, 50 μl of a solution containing 1 mg/ml XTT (2,3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (Sigma, St. Louis, MO) and 25 μM phenazine methosulfate (PMS; Fluka, Buchs, Switzerland) diluted in Optimem were added to cells growing in wells and incubated for 40 minutes at room temperature. Optimem medium alone was used as a negative control and was used later for subtraction of the background value from experimental points. The absorbance was then measured with a microtiter plate reader (Bio-Tek Instruments, Winooski, VT) at the wavelength of 405 nm.

Annexin V/ 7-AAD staining

Double staining with annexin V and Via Probe (7-Amino-actinomycin D (7-AAD) - containing reagent; Beckton Dickinson) was used to determine the live, apoptotic and necrotic cells by flow cytometry. First, 10^5 cells per experimental point were detached with versene, washed twice with PBS, resuspended in 50 μl of binding buffer and stained for 30 minutes with each individual component (2.5 μl of annexin V or Via Probe, respectively) to set the compensation values. The same settings were later used for analysis of double stained cells. Data were preceded with WinMDI 2.8 Software and cells were categorized as live (annexin V and 7-AAD negative), apoptotic (annexin V positive, 7-AAD negative) or necrotic (annexin V and 7-AAD positive).

Measurement of complement-mediated lysis

For evaluation of lysis we used calcein release assay as previously described (12). Briefly, cells were first incubated with 2 mg/ml calcein AM (Molecular Probes, Carlsbad, CA) for 30 min at 37 °C, washed three times, and incubated with 50% NHS for 60 min at 37 °C. Released calcein was measured in a Wallac Victor 2 fluorescence reader (Wallac, Turku, Finland) using 485/535 nm filters. Full lysis (100%) was assessed by measurement of samples in which NHS was replaced with 1% Triton. Every readout was normalized to heat-inactivated NHS to eliminate complement-independent lysis.

Results

Hypoxia results in time-dependent loss of viability of NSCLC cells.

Survival capability of the cells challenged by hypoxia or hypoxia/reoxygenation was measured by two independent methods: by XTT assay based on mitochondrial enzymatic activity (20) and by annexin V/7-AAD staining, which allows to distinguish between live, apoptotic and necrotic cells. Hypoxia *in vivo* is associated with dense foci of tumor cells, proliferation of which is limited because of lack of nutrients and growth factors due to inefficient blood supply. To model such conditions we cultured NSCLC cells at 100% confluency and in a serum-free medium, thus limiting their proliferative potential. Another reason for full confluency at the starting point was to limit the differences in

total (live and dead) cell number between normoxic and hypoxic or reoxygenated cells at the time of analysis (especially for the analyses of soluble complement inhibitors' concentrations presented below). Importantly, none of the cell groups appeared as multilayers when examined under the microscope thus fulfilling our experimental assumption (data not shown). When compared to normoxia, 24 h hypoxia/reoxygenation did not cause a significant drop of survival in the two tested cell lines (H2087 and H358) while 48 h hypoxia resulted in a significant decrease of survival by approximately 30%, as measured by the XTT assay (Table 2). According to annexin V/7-AAD staining, 24 h hypoxia/reoxygenation did not cause significant increase in numbers of apoptotic and necrotic cells while 48 h hypoxia resulted in significant, over 40-50% decrease of live cells, depending on the cell line tested. Of the detected 40-50% dying cells, 10% were defined as apoptotic (annexin V-positive, 7-AAD negative) while 30-40% were necrotic (positive for both annexin V-positive and 7-AAD; Fig. 1). Small differences between the two assays could be explained by lower sensitivity of the XTT assay, which relates to metabolic processes but also produces positive readout from apoptotic bodies or subcellular fragments containing active enzymes.

Hypoxia reduces the expression of complement inhibitors

Previously we have shown that H2087 and H358 cells express membrane-bound complement inhibitors: CD46, CD55 and CD59, but not CD35 (12). Herein we investigated how hypoxic conditions influence mRNA expression of CD46, CD55 and CD59. We incubated the cells in a hypoxic chamber for 48 hours (hypoxia) or for 24 h followed by 24 h at normal O_2 tension (hypoxia/reoxygenation). Control cells (normoxia) were cultured for 48 h under normal O_2 tension. Additionally we also studied cells exposed to 6 h hypoxia in order to find out how fast hypoxic conditions influence expression of complement inhibitors. Semiquantitative real time RT-PCR showed a significant reduction of expression of soluble and membrane inhibitors in both cell lines, except for CD59 in H2087 cells (Fig. 2). Interestingly, reduction of mRNA levels for soluble inhibitors was obvious already after 6 hours of hypoxia and decreased further while the drop of mRNA for membrane inhibitors was noticed after 24 hours. We next evaluated protein expression levels. While no significant differences were found for expression of CD55 (except for a slight but significant decrease in reoxygenated H358 cells) and CD59, substantial loss of CD46 was observed after 24 h hypoxia/reoxygenation and intensified after 48 h hypoxia (Fig. 2). We have previously reported substantial secretion of FI and FH by some NSCLC cell lines (12). In relation to protein secretion, both hypoxia/reoxygenation and hypoxia decreased protein levels in the conditioned medium in comparison to normoxia: FI was reduced 3-fold (H2087 cells) and 4-fold (H358) whereas secretion of FH was decreased 2.5 and 3-fold in these cell lines, respectively (Table 3). It is worth underlining that cells diminished secretion in both conditions tested in a similar manner, irrespective of previously found differences in cell survival. Levels of FI and FH in the conditioned media after 6 h incubation were close to the background and, therefore, changes in their expression after hypoxic conditions could not be determined.

Hypoxia induces deposition of early and late complement components on NSCLC cells
Since hypoxic conditions decreased expression of several complement inhibitors in the two tested cell lines we wanted to determine if this affected how the cells were opsonized by complement factors. Therefore, we measured deposition of C3 and C9 on the surface of H2087 and H358 cells subjected to hypoxic conditions. When incubated with 20% NHS in PBS supplemented with calcium and magnesium, hypoxic H2087 and H358 cells showed significantly higher deposition of both C3 (Fig 3A and B) and C9 (Fig 3E) than normoxic cells. However, substantial deposition of early (C3) and late (C9) complement components was not sufficient to cause complement-mediated lysis, as showed by the calcein release assay (Fig 3H).

Hypoxia causes activation of all three complement pathways in NSCLC cells.
PBS supplemented with calcium and magnesium, which was used in our experimental conditions, allows activation of classical, lectin and alternative pathways. To find out which pathway was activated by hypoxia, we employed experimental conditions to promote exclusively the activation of the alternative pathway (buffer with Mg^{2+} and EGTA) or the classical and lectin pathways (factor B-depleted serum in $DGVB^{2+}$ buffer). We detected statistically significant levels of complement activation in both conditions, both for H2087 and H358 cells. Alternative pathway was not evidently activated in reoxygenated H358 cells but obvious activation was seen after 48 hour hypoxia (Fig. 4A and B). To further assess whether both lectin and classical pathways were activated, we determined the deposition of their initiating molecules, MBL and C1q, respectively. Both molecules were bound significantly to cell surfaces with hypoxia and hypoxia/reoxygenation, with exception of MBL in reoxygenated cells (Fig 3C and D). These results suggest that under hypoxic conditions NSCLC cells activate all three complement pathways. Deposition of C1q on hypoxic cells was correlated with increased binding of antibodies naturally occurring in NHS (Fig 3F and G). We also checked whether hypoxic cells bind serum properdin, an enhancer and initiator of the alternative pathway, but we did not find any differences between normoxic and hypoxic or reoxygenated cells (data not shown).

Hypoxia causes loss of CD46 but not increased C3 deposition in adenocarcinomas from origins other than NSCLC.
To extend our findings established in NSCLC cell lines to other adenocarcinomas, we determined levels of membrane-bound complement inhibitors present on the surface of AGS (gastric), HT29 (colorectal) and PC3 (prostatic) cancer cells exposed to 24 h hypoxia/reoxygenation or 48 h hypoxia. Neither of these cells expressed detectable amounts of CD55 (data not shown). Loss of CD46 was apparent after 48 h hypoxia in the three cell lines tested and after 24 h hypoxia/reoxyganation in HT29 cells, whereas only hypoxic HT29 cells lost CD59. However, we did not see any significant increase of C3 deposition in any of these cell lines (Table 4).

Discussion

Both hypoxia and reoxygenation occur in many common clinical conditions such as myocardial ischemia, stroke and organ transplantation (reviewed in (21)) therefore

playing an important role in human pathophysiology. Several reports support a major role of complement in hypoxia/reperfusion injury in vascular endothelium (16). It is not fully understood yet, how hypoxic conditions can lead to complement activation. One possible explanation employs reactive oxygen species generated by cytosolic and mitochondrial enzymes, which could react with proteins and lipid membranes changing their antigen determinants (21). Another theory involves carbohydrate patterns on cell surfaces, which could be changed due to selective activation of certain sugar metabolism genes during hypoxia (22). Accordingly, we found that NSCLC cells subjected to hypoxia bound both MBL and C1q. Finally, complement can be activated by apoptotic and necrotic events (23-25), which take place secondary to hypoxia. Opsonization of apoptotic and necrotic cells by early complement components will likely play a role in safe and efficient removal of these unwanted and potentially dangerous cells by attracted phagocytes (26). In such case complement activation is beneficial up to the level of C3b because it allows interaction with complement receptors that are present on phagocytes, and thereby enhances phagocytosis. If proceeding further, release of anaphylatoxin C5a and cell lysis induced by MAC will evoke local inflammation. Furthermore, rapid loss of CD46 but not CD55 and CD59 was observed in apoptotic cells of different origins (25) suggesting that CD46 may in fact act as "do not eat me signal" and its removal by itself enhances apoptosis. On the other hand, in the case of endothelial cells challenged by hypoxia, expression of membrane-bound complement inhibitors increased, suggesting that these cells actively opposed complement-mediated damage (16,17). In our experiments we also observed substantial loss of CD46 protein, but reoxygenated cells lost more than would be proportionally expected from the increase of apoptotic/necrotic cell population. The loss of CD46 on hypoxic cells was accompanied by increased deposition of C3. In spite of retaining membrane-bound inhibitors acting at later stages of the complement cascade, the terminal complement pathway was also activated as measured by C9 deposition onto hypoxic cells. We found previously that blocking of CD46 on H358 cells only slightly (30%) increased complement-mediated lysis whereas blocking of CD59 resulted in 2-fold increase in lysis (12). Therefore, loss of CD46 cannot be the only factor responsible for the observed increase in C9 deposition. Furthermore, the fact that some cells were rendered apoptotic/necrotic cannot be the only mechanism behind increased complement attack on hypoxic cells. Next, we found that production of soluble complement inhibitors was almost equally inhibited in hypoxic and reoxygenated cells. In accordance with studies at protein level, mRNA synthesis for FH, FI and CD46 was significantly decreased in hypoxic conditions. In contrast to the protein studies, CD55 mRNA was downregulated in H358 cells, and a slight reduction of CD59 mRNA was observed in H358 cells. These differences between mRNA and protein levels may be due to the fast turnover of CD46 on the cell surface, while CD55 and CD59 may be retained longer. Indeed, CD46 was found previously to be constantly shed from the surface of tumor cells (27) and we also noticed this fact in H2087 and H358 cells (12). Previously we showed that FI and FH could diminish complement activation and complement-mediated lysis of NSCLC cells already expressing membrane-bound inhibitors (12). Moreover, NSCLC cells with silenced FH expression injected into athymic mice produced smaller tumors than the same cells producing FH (13). Our present experiments cannot assess the influence of decreased secretion of soluble complement inhibitors on complement activation by the studied cells as the complement deposition assays were performed in the

absence of conditioned medium. However, we argue that FI and FH may play an important role in the defense of NSCLC cells from complement attack. To conclude, NSCLC cells exposed to hypoxia activated complement and seem not to use their available, diverse mechanisms of protection from complement and actively provoke complement deposition onto their surfaces. Loss of both CD46 and soluble complement inhibitors precedes cell death measured by 7-AAD or XTT assay. For instance, the other tested adenocarcinoma cell lines, which are not comparatively equipped with complement inhibitors, also lost CD46 upon hypoxia and hypoxia/reoxygenation but did not show significant complement deposition.

In the current study we examined two potentially harmful conditions for cells: hypoxia (metabolic changes) and hypoxia/reoxygenation (oxidative shock). Time points of 24 h hypoxia followed by reoxygenation or another hypoxic period were selected based on the previous studies (16), which reported that endothelial cells show differences in complement inhibitors and deposition of complement components in such conditions. However, it was also possible that reoxygenation could reverse the hypoxia-mediated damage. To answer this, we added two additional experimental points- first: 24 h hypoxia compared to 24 h normoxia and second- 48 h hypoxia followed by 24 h reoxygenation compared to 72 h normoxia, where FI and FH secretion, CD46 expression and survival (XTT and annexin V/ 7-AAD) were investigated (data not shown). We found that 24 h hypoxia did not alter survival substantially but there was already a significant drop in secretion of soluble complement inhibitors as well as CD46 expression. These changes were less pronounced than in 24 h hypoxia/reoxygenated cells. Therefore, the changes regarding expression of complement inhibitors appeared relatively early and reoxygenation after 24 h of hypoxia did not rescue the cells from unfavorable changes. On the other hand, reoxygenation after 48 h hypoxia causes cells to collapse as their viability decrease to 30-50 % of that of normoxic cells and complement inhibitors drop substantially. Previously, alternative (28), lectin (29) and classical (9) complement pathway activation by tumor cells has been described. Herein we demonstrate that activation of all these pathways is enhanced by hypoxic conditions.

Studies using animal tumor models, in which complement inhibitors are blocked (30) or silenced (31) showed that loss of complement-inhibitory function causes growth inhibition and renders tumors vulnerable to anti-cancer therapies. One may wonder why do hypoxic NSCLC cells purposely give up some of the available inhibitors and allow activation of MAC. Activation of the complement cascade up to the MAC level causes opsonization, generation of anaphylatoxins, release of cellular content and finally results in local inflammation. However, there is a growing number of evidence suggesting that inflammation can fuel tumorigenic processes in breast, lung, liver, ovarian, prostate, skin and colon cancers (32). A possible explanation involves the induction of tumor neovascularization by immune cells attracted to inflammatory sites. They secrete cytokines responsible for production of cyclooxygenase 2, which in turn upregulates vascular endothelial growth factor secretion by tumor cells (18,32,33). Additionally, macrophages recruited into hypoxic parts of tumors may inhibit the presentation of tumor antigens to T cells and secrete growth and angiogenic factors (2,34). It is therefore possible that complement components activated during hypoxia drive inflammatory processes leading to tumor progression. Thus in the first stages of neoplasia it may be advantageous for cancer cells to inhibit complement in order to survive, and at the later

hypoxic stages it may be more advantageous to activate complement in order to stimulate neovascularization. Interestingly, in our experimental model we could not observe complement-mediated lysis even at 50% serum concentration in spite of massive C9 deposition. One possible explanation is the presence of CD59, which is retained by hypoxic cells. Previously we showed that blocking of CD59 on both cell lines tested here increased complement-mediated lysis up to 3 times (12). It is worth noting that we achieved complement-mediated lysis at 20% serum only when NSCLC were first sensitized with tumor-specific antibodies. Without sensitization (current experiments) complement activation takes place but it is apparently not sufficient to lyse tumor cells. In such situation NSCLC cells would probably activate macrophages and cause anaphylatoxin release, taking all putative benefits from local inflammation while avoiding being damaged by MAC.

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Table 1 Sequences of primers used in real-time PCR

protein	sense	antisense
CD46	5′TGCTGCTCCAGAGTGTAAAGTG3′	5′CGCTGCCATCGAGGTAAA3′
CD55	5′CCACAAAAACCACCACACC3’	5′GCCCAGATAGAAGACGGGTAGTA3′
CD59	5′GGAATCCAAGGAGGGTCTGT3′	5′CAGTCAGCAGTTGGGTTAGGA3′
FH	5′GAAGGCACCCAGGCTATCTA3′	5′ATCTCCAGGATGTCCACAGG3′
FI	5′GAGGAAAGCGAGCACAACTG3′	5′GTCGGGGTGTATCCAGTCTACTA3′
GAPDH	5′GAAGGTGAAGGTCGGAGTC3′	5′GAAGATGGTGATGGGATTTC3′

Table 2 Survival of NSCLC cells under hypoxic conditions measured by XTT assay

cell line	hypoxia 24 h/reoxygenation	hypoxia 48 h
H2087	87.3 % (±11.2)	69.1 % (±9.5) *
H358	94.5 % (±5.3)	74 % (±10.3) *

Data were collected from 3 independent experiments performed in triplicates. Results were normalized to the readout of normoxic cells in each experiment, referred to as 100%. Statistical significance was calculated by Student’s T-test for unpaired data where ** and *** denote $P<0.01$ and $P<0.001$, respectively.

Table 3 Secretion of FI and FH under hypoxic conditions

cell line	**conditions**	**FI concentration (ng / ml)**	**FH concentration (ng / ml)**
H2087	normoxia 48 h	140.9 (±5.1)	8.81 (±1.8)
	hypoxia 24 h/reoxygenation	49.2 (±15.9) **	3.36 (±0.04) **
	hypoxia 48 h	50.9 (±11.9) ***	3.17 (±0.88) **
H358	normoxia 48 h	59.39 (±10.1)	83.58 (±12.71)
	hypoxia 24 h/reoxygenation	15.01 (±7.5) *	29.3 (±2.3) *
	hypoxia 48 h	12.39 (±5.3) ***	24.89 (±3.18) **

Concentrations of FI and FH were measured by ELISA. Data were collected from 3 independent experiments performed in quadruplicates. Statistical significance was calculated by ANOVA with Tukey post-test, where *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$, respectively.

Table 4
Membrane-bound complement inhibitors and C3 deposition on adenocarcinoma cells under hypoxic conditions.

Cell line	protein expression, % of normoxia			C3 deposition, % of normoxia	
AGS	CD46	Hypoxia / reoxygenation	96.8 (± 31.5)	Hypoxia / reoxygenation	118.1 (± 51.2)
		Hypoxia	37.3 * (± 26.0)		
	CD59	Hypoxia / reoxygenation	116.3 (± 20.2)	Hypoxia	100.6 (± 17.8)
		Hypoxia	65.7 (± 26.7)		
HT29	CD46	Hypoxia / reoxygenation	29.5 *** (± 12.4)	Hypoxia / reoxygenation	186.2 (± 120.9)
		Hypoxia	16.5 *** (± 10.3)		
	CD59	Hypoxia / reoxygenation	61.2 * (± 15.4)	Hypoxia	129.8 (± 46.2)
		Hypoxia	55.8 * (± 14.8)		
PC3	CD46	Hypoxia / reoxygenation	99.7 (± 22.6)	Hypoxia / reoxygenation	121.6 (± 89.0)
		Hypoxia	69.4 ** (± 12.1)		
	CD59	Hypoxia / reoxygenation	103.0 (± 29.1)	Hypoxia	111.9 (± 28.8)
		Hypoxia	79.2 (± 17.0)		

Values are given as means (± SD). Significance levels: * - $p< 0.05$, ** - $p <0.01$, *** - $p<0.001$ according to ANOVA with Tukey post-test.

Figure legends

Fig. 1 Viability of NSCLC cells exposed to hypoxia assessed by flow cytometry
Graphs show representative (out of 3 independent experiments) dot plots of NSCLC cells subjected to 48h hypoxia or 24 h hypoxia followed by 24 h reoxygenation and stained with annexin V and 7-AAD. Percentage of live (lower left quadrant), apoptotic (lower right quadrant) and necrotic (upper right quadrant) cells is given next to each analyzed quadrant. * - $p<0.05$, ** - $p<0.01$, *** - $p< 0.001$ according to ANOVA with Tukey post-test, when compared to respective quadrant for normoxic cells.

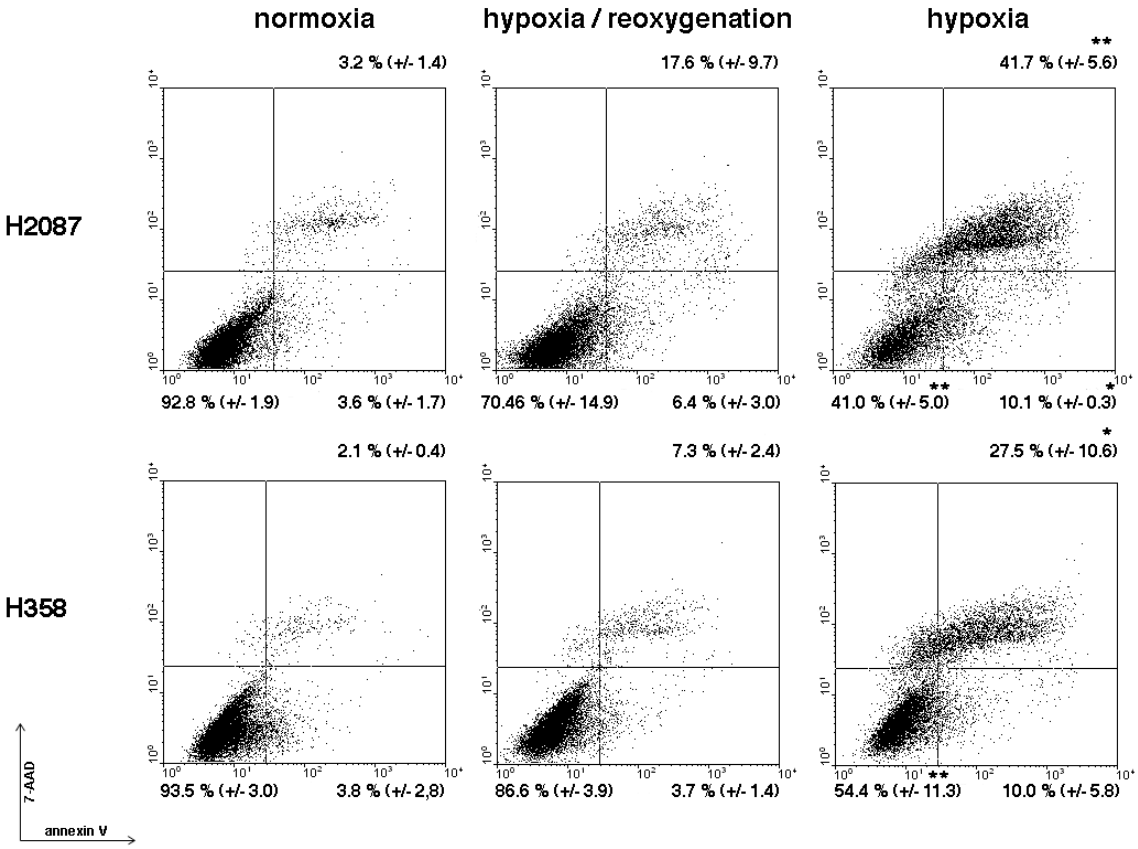
Fig. 2 Expression of complement inhibitors by hypoxic NSCLC cells
A) Semiquantitative mRNA quantification of complement inhibitors in hypoxic conditions. mRNA expression of each complement inhibitor studied was calculated as percentage relative to the GAPDH mRNA. Expression in normoxic cells was set as 100% (indicated by a dotted line). Data are collected from 5 independent experiments and shown as means ± standard deviation (SD). * - $p<0.05$, ** - $p<0.01$, *** - $p< 0.001$ according to ANOVA with Tukey post-test. B) Presence of membrane-bound complement inhibitors on NSCLC cells exposed to hypoxia. Particular membrane inhibitors were measured by flow cytometry and data from all experiments are summarized in the graphs and expressed as percent of mean fluorescence intensity (MFI) shown as means ± SD. Expression in normoxic cells was set as 100% and is indicated by a dotted line. * - $p<0.05$, ** - $p<0.01$, *** - $p< 0.001$ according to ANOVA with Tukey post-test, when compared to normoxic cells.

Fig. 3 Complement deposition on NSCLC cells exposed to hypoxia
Graphs show summarized (from at least 3 independent experiments for each cell line) flow cytometry data of NSCLC incubated with 20% NHS and stained for C3 (A, B), MBL (C), C1q (D) C9 (E) or immunoglobulin (F, G) deposition. In A and B three different buffers were used: PBS + Ca^{2+}/Mg^{2+} allowing activation of all pathways, $DGVB^{2+}$ together with factor B-depleted serum allowing classical pathway (CP) and lectin pathway (LP) or Mg^{2+}-EGTA buffer allowing the alternative pathway (AP). Results are expressed as percent of MFI and shown as means ± SD. H) Complement-mediated lysis measured by calcein release. * - $p<0.05$, ** - $p<0.01$, *** - $p< 0.001$ according to ANOVA with Tukey post-test, when compared to normoxic cells.

References

1. Folkman, J. (1974) *Adv Cancer Res* **19**(0), 331-358
2. Murdoch, C., Giannoudis, A., and Lewis, C. E. (2004) *Blood* **104**(8), 2224-2234
3. Brown, J. M., and Giaccia, A. J. (1998) *Cancer Res* **58**(7), 1408-1416
4. Greijer, A. E., and van der Wall, E. (2004) *J Clin Pathol* **57**(10), 1009-1014
5. Saikumar, P., Dong, Z., Patel, Y., Hall, K., Hopfer, U., Weinberg, J. M., and Venkatachalam, M. A. (1998) *Oncogene* **17**(26), 3401-3415
6. Swinson, D. E., Jones, J. L., Richardson, D., Cox, G., Edwards, J. G., and O'Byrne, K. J. (2002) *Lung Cancer* **37**(3), 235-240
7. Graeber, T. G., Osmanian, C., Jacks, T., Housman, D. E., Koch, C. J., Lowe, S. W., and Giaccia, A. J. (1996) *Nature* **379**(6560), 88-91
8. Webster, K. A. (2007) *Antioxid Redox Signal* **9**(9), 1303-1307
9. Jurianz, K., Ziegler, S., Garcia-Schuler, H., Kraus, S., Bohana-Kashtan, O., Fishelson, Z., and Kirschfink, M. (1999) *Mol. Immunol.* **36**(13-14), 929-939
10. Gorter, A., Meri, S. (1999) *Immunol Today* **20**, 576-582
11. Ajona, D., Castano, Z., Garayoa, M., Zudaire, E., Pajares, M. J., Martinez, A., Cuttitta, F., Montuenga, L. M., and Pio, R. (2004) *Cancer Research* **64**, 6310-6318
12. Okroj, M., Hsu, Y. F., Ajona, D., Pio, R., and Blom, A. M. (2008) *Mol Immunol* **45**(1), 169-179
13. Ajona, D., Hsu, Y. F., Corrales, L., Montuenga, L. M., and Pio, R. (2007) *J Immunol* **178**(9), 5991-5998
14. Manches, O., Lui, G., Chaperot, L., Gressin, R., Molens, J. P., Jacob, M. C., Sotto, J. J., Leroux, D., Bensa, J. C., and Plumas, J. (2003) *Blood* **101**(3), 949-954
15. Stern, M., and Herrmann, R. (2005) *Crit Rev Oncol Hematol* **54**(1), 11-29
16. Collard, C. D., Vakeva, A., Bukusoglu, C., Zund, G., Sperati, C. J., Colgan, S. P., and Stahl, G. L. (1997) *Circulation* **96**(1), 326-333
17. Collard, C. D., Bukusoglu, C., Agah, A., Colgan, S. P., Reenstra, W. R., Morgan, B. P., and Stahl, G. L. (1999) *Am J Physiol* **276**(2 Pt 1), C450-458
18. Sharma, R. A., Dalgleish, A. G., Steward, W. P., and O'Byrne, K. J. (2003) *Oncol Rep* **10**(5), 1625-1631
19. Gabrielson, E. (2006) *Respirology* **11**(5), 533-538
20. Scudiero, D. A., Shoemaker, R. H., Paull, K. D., Monks, A., Tierney, S., Nofziger, T. H., Currens, M. J., Seniff, D., and Boyd, M. R. (1988) *Cancer Res* **48**(17), 4827-4833
21. Li, C., and Jackson, R. M. (2002) *Am J Physiol Cell Physiol* **282**(2), C227-241
22. Kannagi, R. (2007) *Chang Gung Med J* **30**(3), 189-209
23. Ciurana, C. L., Zwart, B., van Mierlo, G., and Hack, C. E. (2004) *Eur J Immunol* **34**(9), 2609-2619
24. Walport, M. J. (2001) *N. Engl. J. Med.* **344**, 1140-1144
25. Elward, K., Griffiths, M., Mizuno, M., Harris, C. L., Neal, J. W., Morgan, B. P., and Gasque, P. (2005) *J Biol Chem* **280**(43), 36342-36354
26. Trouw, L. A., Nilsson, S. C., Goncalves, I., Landberg, G., and Blom, A. M. (2005) *J Exp Med* **201**(12), 1937-1948

27. Hakulinen, J., Junnikkala, S., Sorsa, T., and Meri, S. (2004) *Eur J Immunol* **34**(9), 2620-2629
28. Budzko, D. B., Lachmann, P. J., and McConnell, I. (1976) *Cell Immunol* **22**(1), 98-109
29. Fujita, T., Taira, S., Kodama, N., Matsushita, M., and Fujita, T. (1995) *Jpn J Cancer Res* **86**(2), 187-192
30. Gelderman, K. A., Kuppen, P. J., Okada, N., Fleuren, G. J., and Gorter, A. (2004) *Cancer Res* **64**(12), 4366-4372
31. Varela, J. C., Imai, M., Atkinson, C., Ohta, R., Rapisardo, M., and Tomlinson, S. (2008) *Cancer Res* **68**(16), 6734-6742
32. Angelo, L. S., and Kurzrock, R. (2007) *Clin Cancer Res* **13**(10), 2825-2830
33. Gately, S., and Li, W. W. (2004) *Semin Oncol* **31**(2 Suppl 7), 2-11
34. Leek, R. D., and Harris, A. L. (2002) *J Mammary Gland Biol Neoplasia* **7**(2), 177-189


normoxia
hypoxia / reoxygenation
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H2087
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3.2 % (+/- 1.4)
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92.8 % (+/- 1.9)
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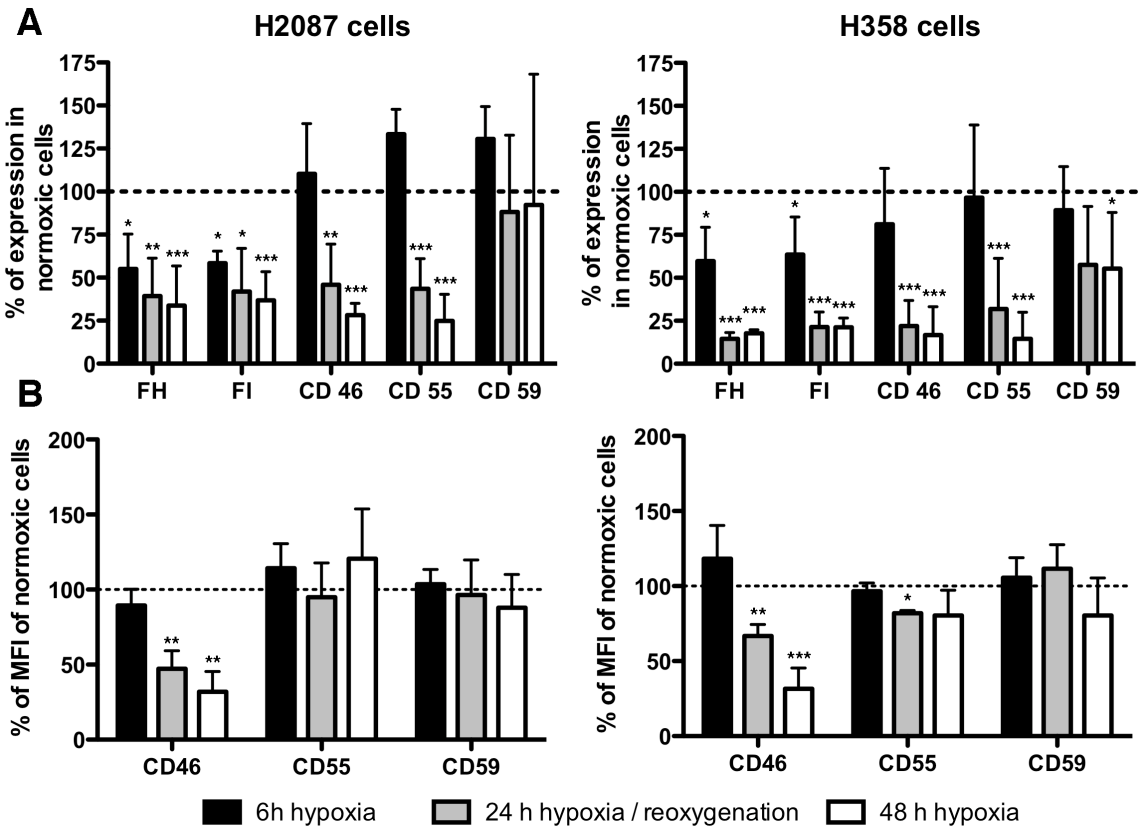
A
H2087 cells
H358 cells
% of expression in normoxic cells
% of expression in normoxic cells
175
150
125
100
75
50
25
0
FH
FI
CD 46
CD 55
CD 59
B
% of MFI of normoxic cells
% of MFI of normoxic cells
200
150
100
50
0
CD46
CD55
CD59
6h hypoxia
24 h hypoxia / reoxygenation
48 h hypoxia

Fig 4
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