Down-Regulation of Human Complement Factor H Sensitizes Non-Small Cell Lung Cancer Cells to Complement Attack and Reduces In Vivo Tumor Growth[1]

Daniel Ajona,* Yi-Fan Hsu,* Leticia Corrales,* Luis M. Montuenga,[2]*† and Ruben Pio[2]*‡

Malignant cells are often resistant to complement activation through the enhanced expression of complement inhibitors. In this work, we examined the protective role of factor H, CD46, CD55, and CD59 in two non-small cell lung cancer cell lines, H1264 and A549, upon activation of the classical pathway of complement. Complement was activated with polyclonal Abs raised against each cell line. After blocking factor H activity with a neutralizing Ab, C3 deposition and C5a release were more efficient. Besides, a combined inhibition of factor H and CD59 significantly increased complement-mediated lysis. CD46 and CD55 did not show any effect in the control of complement activation. Factor H expression was knockdown on A549 cells using small interfering RNA. In vivo growth of factor H-deficient cells in athymic mice was significantly reduced. C3 immunocytochemistry on explanted xenografts showed an enhanced activation of complement in these cells. Besides, when mice were depleted of complement with cobra venom factor, growth was recovered, providing further evidence that complement was important in the reduction of in vivo growth. In conclusion, we show that expression of the complement inhibitor factor H by lung cancer cells can prevent complement activation and improve tumor development in vivo. This may have important consequences in the efficiency of complement-mediated immunotherapies. *The Journal of Immunology,* **2007, 178: 5991–5998.**

There are important features that distinguish cancer cells from their normal counterparts, making them recognizable by the immune system. In fact, cancer cells must develop mechanisms to avoid immune recognition or activation (1). The elucidation of these mechanisms may provide ways to improve cancer immunotherapy. Many mAbs against cancer-associated Ags are also able to activate the complement system (2). Chimerized or humanized mouse mAbs containing the human IgG1 Fc region are examples of complement-activating mAbs (3). The Fc regions of membrane-bound Abs interact with the heterooligomeric complex C1q and activate the classical pathway. Complement activation results in the deposition of C3b, which leads to the formation of the cytolytic membrane attack complex (MAC)[3] (3) in a process known as complement-dependent cytotoxicity. Complement-dependent cellular cytotoxicity also happens when C3b is converted to iC3b, which interacts with CR3 (CD11b/CD18) on mononuclear phagocytes (4), NK cells (5), and lymphocytes (6). Finally, the complement cascade of proteolytic enzymes releases anaphylatoxins C4a, C3a, and C5a which mediate proinflammatory responses. Despite these powerful effector mechanisms, tumor cells are usually resistant to complement attack through a variety of protective strategies (7). Membrane-bound complement regulatory proteins (mCRPs), CD55 (decay-accelerating factor), CD46 (membrane cofactor protein), and CD59 contribute to the protection of several tumor cells (8–10). A role for soluble complement inhibitors, such as factor H, has also been suggested (10–14). Factor H is a 150-kDa glycoprotein present in human plasma, which inhibits the formation and activity of the C3 convertase (15–17). Besides, alternative splicing of factor H mRNA yields a 42-kDa protein, named factor H-like protein 1 (FHL-1), which shares the complement inhibitory activities of factor H (18, 19). Expression of factor H and/or FHL-1 has been described in primary tumors and cell lines from different origins (13, 20–25). We have recently demonstrated that factor H is frequently expressed in non-small cell lung cancer (NSCLC). Factor H is also secreted to the extracellular milieu and is able to bind to lung tumor cell surfaces, inhibiting the activation of the alternative pathway of complement (14). In the present work, we demonstrate that factor H expression is important for the control of complement after activation of the classical pathway in lung cancer cells. First, we show that two lung cancer cell lines are able to resist the activation of complement in vitro by the expression of factor H, but not by the expression of the mCRP CD46 and CD55. Second, using a nude mouse xenograft model, we show that factor H down-regulation sensitizes tumor cells to complement-mediated attack and reduces tumor growth in vivo.

Materials and Methods

Lung cancer cell lines

H1264 (lung adenocarcinoma) and A549 (bronchoalveolar lung carcinoma) cell lines were obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 with L-Glutamax (Invitrogen Life Technologies) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

*Division of Oncology, Center for Applied Medical Research, †Department of Histology and Pathology, and ‡Department of Biochemistry, School of Medicine, University of Navarra, Pamplona, Spain

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[2] Address correspondence and reprint requests to Dr. Ruben Pio or Dr. Luis M Montuenga, Oncology Division, CIMA Building, Pio XII, 55, Pamplona 31008, Spain. E-mail address: rpio@unav.es or lmontuenga@unav.es

[3] Abbreviations used in this paper: MAC, membrane attack complex; mCRP, membrane-bound complement regulatory protein; FHL-1, factor H-like protein 1; NSCLC, non-small cell lung cancer; NHS, normal human serum; HI-NHS, heat-inactivated NHS; siRNA, small interfering RNA; CVF, cobra venom factor.

Sera

Normal human serum (NHS) was used as the source of complement. A pool of sera from 12 healthy donors was prepared. Heat-inactivated NHS (HI-NHS) was obtained by incubation of the serum at 56°C for 30 min.

Antibodies

Mouse anti-human factor H mAb OX-24 was prepared and purified as previously described (14). Mouse anti-human CD46 mAb GB-24 was a gift from Dr. J. Atkinson (Washington University, St. Louis, MO). Mouse anti-human CD55 mAbs BRIC 110 and BRIC 216 were purchased from IBGRL. Rat anti-human CD59 mAb YTH53.1 was purchased from Serotec. Isotype controls MOPC-21 (mouse IgG1) and YTH53.1 (rat IgG2b) were purchased from Sigma-Aldrich and Abcam, respectively. Polyclonal antisera against H1264 and A549 cells were prepared by immunizing female New Zealand White rabbits (Harlan) with whole-cell lysates from each cell line. The experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Navarra. Immunization was performed by three injections of 10^7 cells each at 15-day intervals. For the first injection, cells were resuspended in 0.5 ml of PBS (10 mM phosphate and 150 mM NaCl, pH 7.4) and mixed with CFA (Difco). The mixture was injected intradermally on the back of the rabbits. Subsequent s.c. and i.m. booster injections were conducted with cells mixed with IFA (Difco). Sera obtained from the rabbits were incubated at 56°C for 30 min to inactivate complement activity. Antiserum immunoreactivity against each cell line was confirmed by flow cytometry using a FACSCalibur from BD Biosciences. CellQuest Pro software (BD Biosciences) was used for data acquisition and analysis.

Expression of mCRPs

Cells were detached from the culture dishes with 1 mM EDTA, washed once, and resuspended in binding buffer (PBS containing 1% BSA and 0.1% sodium azide). Cells (1×10^5) in 50 μl of binding buffer were incubated with the primary mAb for 30 min at 4°C. After three washings, cells were incubated for 30 min at 4°C with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) or FITC-conjugated rabbit anti-rat IgG (Serotec) diluted 1/100 in a total volume of 50 μl. Cells were washed three times and analyzed by flow cytometry after the addition of propidium iodide. Data were collected as mean fluorescence intensity. Cells incubated only with the anti-mouse secondary Ab were used as negative control.

Deposition of C3-related fragments

Cells were detached from the culture dishes with 1 mM EDTA, washed once, and resuspended in veronal buffer (1.8 mM barbital, 3.1 mM barbituric acid, 141 mM NaCl, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$, pH 7.4). Cells (2×10^5) were incubated for 30 min at 4°C with the specific rabbit antisera diluted 1/50 in a total volume of 100 μl. After three washes, cells were again resuspended in veronal buffer (75 μl) and mixed with 125 μl of NHS diluted 1/5. Cells were incubated in the presence of NHS (final dilution, 1/8) for 30 min at 37°C. Deposition of C3 or related fragments was determined as described previously (14). Briefly, cells were incubated for 30 min at 4°C with a FITC-conjugated goat anti-human complement C3 Ab (ICN Biomedicals) and analyzed by flow cytometry after the addition of propidium iodide. To block mCRPs, cells were preincubated, before the addition of the rabbit antiserum, with the blocking Abs (GB-24 or BRIC 110/BRIC 216) at saturating concentrations (49.2, 7.2, and 2.3 μg/ml, respectively) during 30 min at 4°C. To block factor H, NHS (diluted 1/5) was preincubated for 30 min at 4°C with OX-24 at 0.16 mg/ml.

C5a release

Cells were processed and treated as described in the previous paragraph. Release of C5a was quantified in the supernatants using the Human C5a ELISA Kit (BD Biosciences) according to the manufacturer's instructions.

Complement-mediated cytotoxicity

Cell lysis was evaluated using the calcein release assay as previously described, with slight modifications (26). In brief, 4×10^6 cells were resuspended in 2 ml of veronal buffer containing 2 μM calcein-AM (Molecular Probes). Cells were loaded with calcein at 37°C for 1 h and washed once with veronal buffer. Aliquots of 2×10^5 cells were treated with the antisera and neutralizing Abs as described in the previous section. Besides, the Ab YTH53.1 was used to block CD59 activity at a saturating concentration of 60 μg/ml. After incubation with NHS, cells were pelleted by centrifugation and supernatants were transferred to a 96-well plate (plate 1). Pelleted cells were lysed in 55 μl of 0.1% Triton X-100 and transferred to a 96-well plate (plate 2). Fluorescence was measured with excitation at 485 nm and emission at 520 nm. Calcein release (percent) was calculated as follows: (plate 1 value) $\times$ 100/(plate 1 value + plate 2 value). The specific release (complement-mediated cytotoxicity) was the calcein release calculated above minus the calcein release in cells not exposed to complement (cells treated with HI-NHS).

Vector-based small interfering RNA (siRNA)

Factor H oligonucleotides for siRNA were cloned in the pSHH vector using the GeneSilencer system (Imgenex). Oligonucleotides were designed against positions 808–828 of factor H mRNA (G.I. 31964). A plasmid containing an irrelevant siRNA with a scramble sequence (AATTCTC CGAACGTGTCACGT) was used as control. For transfection, A549 cells (10^6) were grown in a 100-mm^2 culture plate with DMEM supplemented with 10% FBS during 48 h. Transfection was performed using a mixture of 10 μg of siRNA plasmid and 10 μl of Lipofectamine 2000 (Invitrogen Life Technologies) in 1 ml of αMEM. Cells were incubated at 37°C with the DNA-Lipofectamine mixture and after 4 h were diluted twice with DMEM containing 20% FBS. Stable clones were selected in RPMI 1640 supplemented with 10% FBS and 0.5 mg/ml geneticin (Invitrogen Life Technologies).

Factor H quantification

A polystyrene 96-well plate was coated with 50 ng/well of the anti-factor H mAb OX-24 (in 50 μl of 50 mM sodium bicarbonate, pH 8.3) during 1 h at room temperature. After washings, the plate was blocked overnight at 4°C with blocking buffer: TBS (25 mM Tris and 150 mM NaCl, pH 7.4) with 1% BSA and 0.1% Tween 20. A volume of 50 μl of samples (supernatants of cells grown in RPMI 1640 without FBS for 48 h) or standards (factor H ranging from 1.5 to 200 ng/ml) was added and the plate was incubated for 2 h at room temperature. Human factor H was obtained from Sigma-Aldrich. After washings, a rabbit anti-factor H Ab (1/1000; Serotec) was added, and after a 30-min incubation at room temperature the assay was developed using a donkey anti-rabbit Ab coupled to HRP (1/2000; Amersham Biosciences) and *o*-phenylenediamine dihydrochloride (Sigma-Aldrich). The plate was read at 450 nm.

Western blotting

Supernatants of cells grown in RPMI 1640 without FBS for 48 h were concentrated, and factor H expression was analyzed as described previously (14).

Northern blotting

RNA purification was achieved with the Ultraspec Total RNA Isolation Reagent (Biotecx) according to the manufacturer's instructions. Analysis for factor H mRNA expression by Northern blotting was performed as described previously (14).

Cell proliferation assay

Stable A549 siRNA cells (750 cells/well) were seeded in 96-well plates and cultured in 100 μl of RPMI 1640 supplemented with 10% FBS and 0.5 mg/ml geneticin. During 5 days, cell proliferation was determined daily using a MTT assay (Roche) per the manufacturer's instructions.

In vivo xenograft studies

A549 cells stably transfected with the siRNA vector (at 80% confluence) were trypsinized and washed twice with PBS. Six million cells were resuspended in 150 μl of PBS and injected s.c. on the right flank of 4- to 6-wk-old female athymic nude mice (Harlan). Tumor development was monitored for ~6 wk. Tumors were measured with a caliper and tumor volumes (V) were calculated using the formula: $V\,(mm^3) = L \times W^2$, where L is the length and W is the width of the tumor. In a subset of cases, cells were first preincubated in 3 ml of PBS with 15 μl of anti-A549 antiserum or 15 μl of preimmune serum and washed three times before injection. In these cases, continuous stimulation of complement was performed by intratumoral injection of antiserum 1/5 (or preimmune serum) in 75 μl of PBS every 3 days.

Depletion of complement

Depletion of complement in nude mice was achieved by i.p. injection of 5 μg of cobra venom factor (CVF; Aczon) in 100 μl of PBS at 28, 24, and 4 h before injection of tumor cells. This regimen of injections has been previously described (27). In control mice, i.p. injections of 100 μl of PBS were performed. To avoid complement recovery during the experiment, periodically 5 μg of CVF injections was administered every 3 days. Complement depletion was monitored by quantification of C3 in mouse sera.

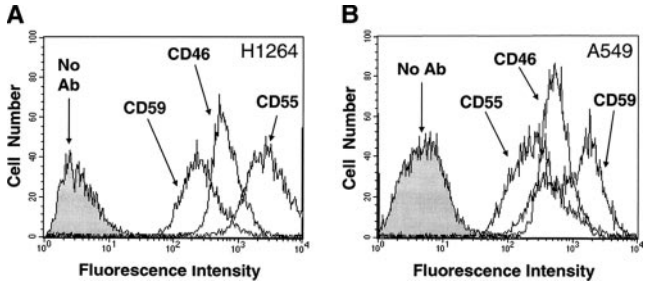

FIGURE 1. Cell membrane expression of mCRPs CD46, CD55, and CD59 in H1264 (*A*) and A549 (*B*) lung cancer cell lines assessed by flow cytometry and measured as the increase in intensity in the green channel. Incubation of cells without the primary Ab was used as negative control.

Quantification of C3 was conducted following the protocol described above for factor H. Plates were coated with goat anti-mouse C3 (1/1000; Cappel Laboratories), serum samples were loaded at different dilutions, and the assay was developed using a goat anti-mouse C3 coupled to HRP (1/10000, Cappel Laboratories). Percentage of C3 depletion was calculated using the levels of C3 in serum before treatment with CVF as reference.

Statistical analysis

Data were analyzed by Student's *t* test. A $p < 0.05$ was considered to be statistically significant.

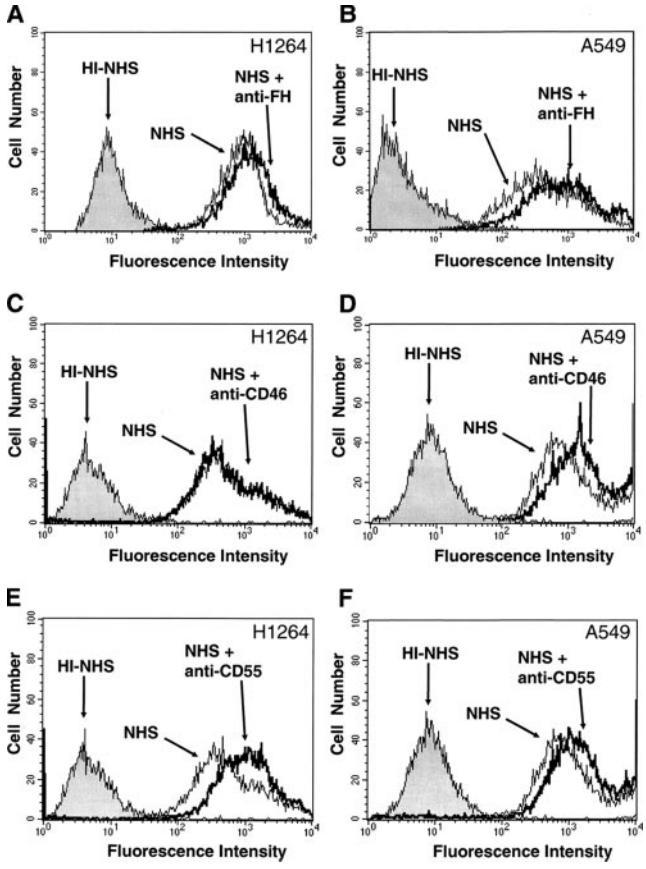

FIGURE 2. Deposition of C3 and C3-related fragments after stimulation of the classical pathway of complement in H1264 (*A*, *C*, and *E*) and A549 cells (*B*, *D*, and *F*). C3 deposition was determined by flow cytometry using a polyclonal Ab that recognizes C3 and C3-related fragments. Deposition was analyzed after incubation of the cells with NHS (diluted 1/8), HI-NHS, or NHS after blocking factor H (*A* and *B*), CD46 (*C* and *D*), or CD55 (*E* and *F*) with the corresponding neutralizing Abs (OX-24, GB-24, or BRIC110/226, respectively). Deposition is evidenced by an increase in intensity in the green channel.

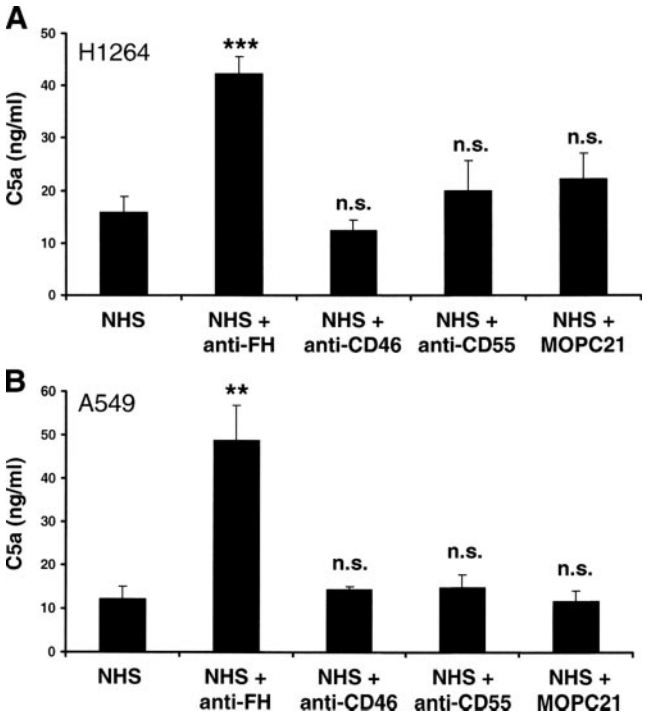

FIGURE 3. Anaphylatoxin C5a release (nanograms per milliliter) after stimulation of the classical pathway of complement in H1264 (*A*) and A549 cells (*B*). C5a was measured in the medium by ELISA after incubation of the cells with NHS (diluted 1/8) or NHS after blocking factor H, CD46, or CD55 with specific neutralizing Abs (OX-24, GB-24, or BRIC110/226, respectively). The graphs show mean and SD of three independent experiments. Incubation with MOPC-21 was used as an isotype control for the neutralizing Abs. Statistical significance of each treatment when compared with NHS is indicated. **, $p < 0.01$; ***, $p < 0.001$; n.s., Not significant.

Results

Expression of mCRPs in H1264 and A549 lung cancer cells

In a previous study, we have shown that H1264 and A549 cells are able to produce and bind the complement regulator factor H (14). We now studied the presence of the membrane-bound complement inhibitors CD46, CD55, and CD59 in both lung cancer cell lines. Flow cytometry analysis revealed the expression of the three complement regulators in the two cell lines. Expression of CD46 was similar in H1264 and A549 cells, while expression of CD55 was higher in H1264 cells and expression of CD59 was higher in A549 cells (Fig. 1).

Activation of the classical pathway of complement on H1264 and A549 cells

Human NSCLC cells are highly resistant to the activation of the classical pathway of complement as compared with normal cells (28). Factor H, CD46, and CD55 are complement regulators that inhibit C3 convertases, key enzymes in the activation cascade of complement. We evaluated whether the resistance on H1264 and A549 NSCLC cells was related to the activity of any of these regulators. The classical pathway of complement was activated with specific antisera generated in rabbits against whole-cell extracts of both cell lines. The activity of each regulator was blocked with specific mAbs (OX-24 for factor H/FHL-1, GB-24 for CD46, and BRIC 110/216 for CD55). C3 convertase activity was evaluated by flow cytometry with an Ab that recognizes C3 and C3-derived fragments. Fig. 2 shows C3 deposition after activation of the classical pathway with NHS (diluted 1/8) in the presence or

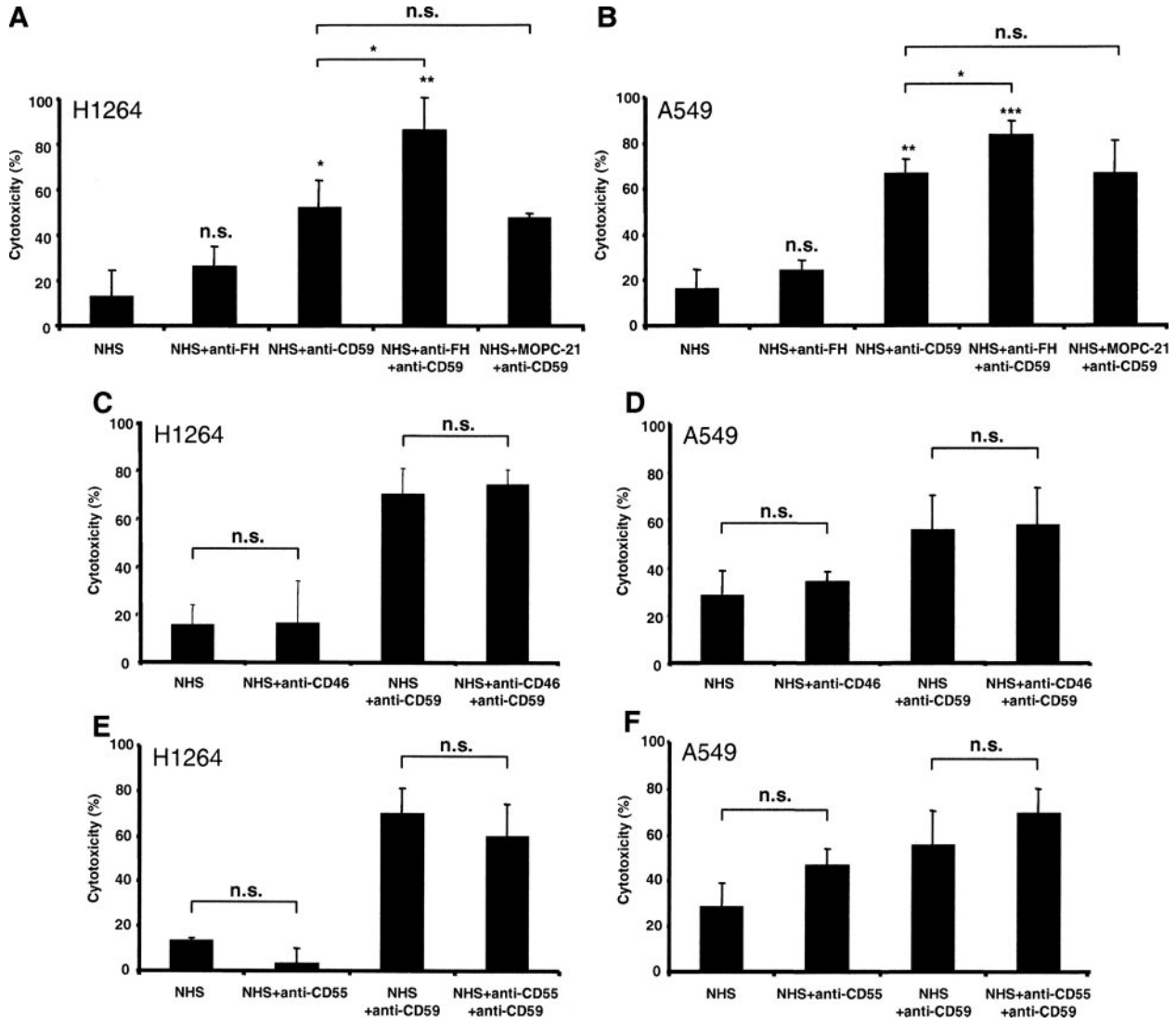

FIGURE 4. Complement-mediated cytotoxicity after stimulation of the classical pathway of complement in H1264 (*A*, *C*, and *E*) and A549 (*B*, *D*, and *F*) cells. Cells were incubated with NHS (diluted 1/8), HI-NHS, or NHS after blocking factor H (*A* and *B*), CD46 (*C* and *D*), CD55 (*E* and *F*), or CD59 (*A–F*) with neutralizing Abs (OX-24, GB-24, BRIC110/226, or YTH53.1, respectively). MOPC-21 was used as an isotype control. Lysis was measured with a calcein release assay and percentage of complement-mediated lysis was calculated by subtracting the percentage of lysis obtained after incubation with HI-NHS. Graphs show mean and SD of three independent experiments. *A* and *B*, If not indicated, statistical analysis was performed between the corresponding treatment and the treatment with NHS alone. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., Not significant.

absence of neutralizing Abs. Blockade of either factor H or CD55 slightly increased C3 deposition on both H1264 and A549 cell membranes, suggesting that the activities of factor H and CD55 protect these lung cancer cells against the deposition of C3-related fragments. Inhibition of CD46 increased C3 deposition on A549 cells, but not on H1264 cells. In the activation cascade, C3 convertase activity leads to the formation and activation of C5 convertases, which cleave C5 in two fragments: cell-bound C5b and anaphylatoxin C5a. We evaluated the ability of factor H, CD46, and CD55 to control the C5a production on H1264 and A549 lung cancer cells upon activation of the classical pathway of complement. Incubation of H1264 and A549 cells in the presence of NHS (1/8) produced a moderate increase in the release of C5a (Fig. 3). Inhibition of factor H by the neutralizing mAb OX-24 increased significantly the release of C5a in H1264 (from 15.8 ± 3.2 ng/ml to 42.3 ± 3.2 ng/ml, $p < 0.001$) and A549 cells (from 12.3 ± 2.8 ng/ml to 48.7 ± 8.1 ng/ml, $p = 0.002$) (Fig. 3). When the same experiment was conducted in the presence of MOPC-21, an isotype control, C5a release was not affected. Neither neutralization of CD46 nor neutralization of CD55 affected C5a release after activation of the classical pathway of complement. Therefore, the three complement regulators are able to control the deposition of C3-related fragments on H1264 and A549 cell membranes, but only neutralization of factor H triggers an increase in C5 convertase activity. These data suggest that factor H protects H1264 and A549 cells after activation of the classical pathway of complement.

Complement-mediated cytotoxicity on H1264 and A549 cells

Among other immunological implications, activation of the classical pathway of complement may ultimately lead to complement-mediated cytotoxicity. We tested whether the inhibition of factor H, CD46, or CD55 may increase this cytotoxicity. H1264 and A549 cells were incubated in the presence of NHS with and without neutralizing Abs against the three complement regulators. Cytotoxicity was determined as calcein release using HI-NHS to differentiate complement-mediated release from nonspecific release. After activation of the classical pathway, blockade of factor H increased cytotoxicity, although this effect did not reach statistical

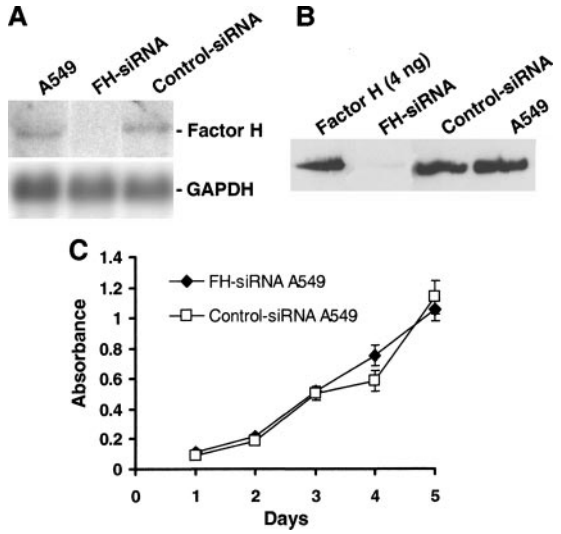

FIGURE 5. Down-regulation of factor H expression in A549 cells after stable transfection with factor H siRNA molecules. The expression of factor H was evaluated in A549 cells, a stable clone transfected with factor H siRNA (FH-siRNA A549), and a stable clone transfected with a scramble siRNA (control-siRNA A549). *A*, Northern blot analysis was conducted with 15 ng of total RNA per sample. Detection of GAPDH mRNA was used to ensure equal loading and RNA integrity. *B*, Western blot analysis was performed with serum-free conditioned medium (10 μg of total protein). Four nanograms of purified factor H was used as positive control. *C*, FH-siRNA A549 cells and control-siRNA A549 cells showed the same growth rate in vitro as determined by a MTT assay. The graph shows mean and SD for an experiment conducted in sixtuplicate and corresponds to an example of two independent experiments.

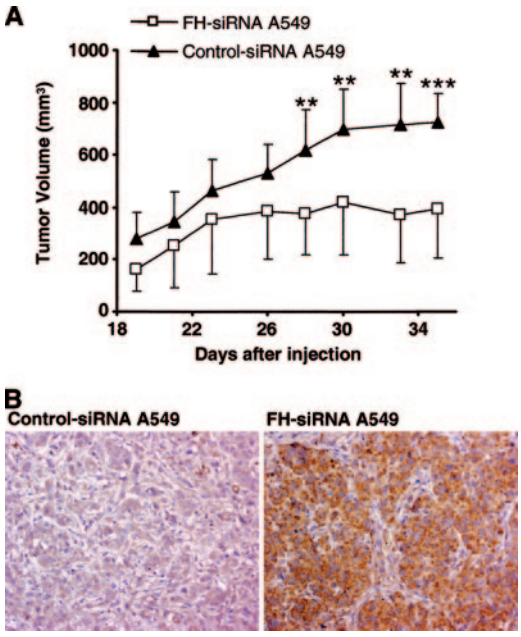

FIGURE 6. Effect of factor H expression on A549 in vivo xenograft growth. *A*, Tumor volume of A549 cells stably transfected with a specific FH-siRNA ($n = 10$) or a control-siRNA ($n = 9$) grown on athymic mice. Tumor volume is expressed as mean ± SD. After 26 days postinjection, significant differences were observed between both groups. **, $p < 0.01$; ***, $p < 0.001$. *B*, Representative examples of C3 deposition on xenograft tumors from A549 cells stably transfected with a FH-siRNA or a control-siRNA. C3 deposition was determined by immunocytochemistry using an anti-mouse C3 Ab.

significance (Fig. 4, *A* and *B*). In contrast, neutralization of CD59, with mAb YTH53.1, significantly increased complement-mediated cytotoxicity in both H1264 (from 13.1% ± 11.5 to 52.2% ± 11.8; $p = 0.015$) and A549 cells (from 16.0% ± 8.6 to 66.5% ± 6.2; $p = 0.001$) (Fig. 4, *A* and *B*). CD59 is a complement regulator that prevents the formation of the lytic membrane attack complex (MAC). Interestingly, simultaneous neutralization of factor H and CD59 increased lysis to 85.7 ± 14.6% in H1264 cells and to 83.4 ± 6.0% in A549 cells (Fig. 4, *A* and *B*). In both cell lines, cytotoxicity was significantly higher than that obtained by neutralization of CD59 alone (H1264: $p = 0.036$; A549: $p = 0.028$). Isotype controls for OX-24 and YTH53.1 did not affect cytotoxicity. YTH53.1 did not induced C5a release, ruling out an activation of complement by this Ab that may account for the increase in lysis. Our data suggest that there is an increase of the activity of the classical pathway of complement in H1264 and A549 cells after neutralization of factor H, but this does not trigger cell lysis due to the presence of CD59. Neither inhibition of CD46 nor inhibition of CD55, alone or in combination with inhibition of CD59, had any effect on cell lysis after activation of the classical pathway of complement (Fig. 4, *C–F*).

Role of factor H in the protection of lung cancer cells in vivo

Neutralization of factor H increases C3 fragment deposition and C5a release after complement activation. Therefore, factor H activity may be relevant for the growth of tumors in vivo. To evaluate this hypothesis, we blocked the expression of factor H in A549 cells and grew them in athymic mice as s.c. xenografts. Athymic mice are immunodeficient and cannot develop a complete adaptive immune response, but have normal complement activity.

First, we generated an A549 stable clone in which expression of factor H/FHL-1 was inhibited by siRNA (clone named FH-siRNA A549). A549 cells stably transfected with an irrelevant siRNA were used as control (clone named control-siRNA A549). The gene expression knockdown was confirmed by Northern blot and Western blot analyses (Fig. 5, *A* and *B*). More than 90% inhibition, measured by ELISA in the serum-free conditioned medium, was achieved (24.2 ± 0.8 pg/μl in FH-siRNA A549 vs 365.0 ± 101.5 pg/μl in control-siRNA A549). Control-siRNA and FH-siRNA A549 cells showed identical proliferation rates in vitro, ruling out any effect of factor H down-regulation on in vitro growth (Fig. 5*C*). Xenograft tumor growth in vivo was then evaluated in athymic mice. We injected 10 mice with FH-siRNA cells and 10 mice with control-siRNA A549 cells. Tumor growth was monitored at least twice a week, starting at day 19 postinjection. One mouse in the control group had to be euthanized at the beginning of the experiment due to its rapid weight loss and debilitation. After 28 days postinjection, xenografts formed by FH-siRNA A549 cells were significantly smaller than those formed by control-siRNA A549 cells (Fig. 6*A*). At day 35, mean tumor volumes were 722 ± 110 mm^3 for control cells and 393 ± 185 mm^3 for factor H-deficient cells ($p < 0.001$; Fig. 6*A*). The experiment was repeated once with very similar results. To verify the role of complement in this growth inhibition, deposition of mouse C3 fragments on explanted xenograft tumors was evaluated by immunohistochemical analysis. In all cases, FH-siRNA tumor cells showed higher levels of C3 staining when compared with those of control-siRNA tumor cells (Fig. 6*B*). The contribution of complement in the reduction of tumor growth in vivo was further confirmed by the generation of complement-deficient mice with i.p. injections of CVF. CVF is a protein that forms stable C3/C5 convertases in mammalian serum with an elevated half-life (29). The activity of these convertases triggers an uncontrollable activation of the complement system, resulting in complement depletion. In our experimental conditions,

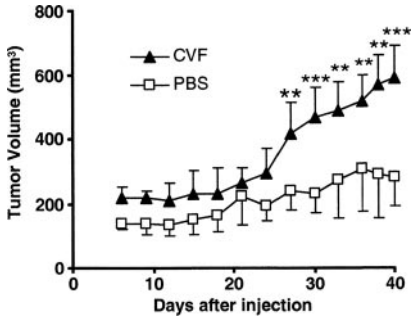

FIGURE 7. Effect of the down-regulation of factor H expression on the growth of A549 xenograft tumors on athymic mice depleted of serum C3. Ten mice were treated with CVF before inoculation of FH-siRNA A549 cells and every 3 days throughout the experiment. Cells grown in 10 mice treated with PBS were used as control. Tumor volume is expressed as mean ± SD. From day 24 postinjection, significant differences were observed between both animal groups. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

serum C3 levels were reduced to <10% and, if CVF was not injected periodically, C3 recovered to 50 and 100% after 5 and 7 days, respectively (data not shown). To maintain a continuous depletion of serum in our experiments, CVF was injected every 3 days throughout the experiment. We inoculated FH-siRNA A549 cells in both PBS- and CVF-treated athymic mice (nine mice per group). Tumor volume was monitored twice or three times a week for 40 days. Significant differences were observed between CVF-treated mice and control mice from day 24 to the end of the experiment (Fig. 7). At day 40 after injection, the mean tumor volume was 591 ± 100 mm^3 in mice treated with CVF and 282 ± 89 mm^3 in mice treated with PBS ($p < 0.001$). This experiment was repeated once with similar results. Therefore, the growth rate of FH-siRNA A549 cells in complement-deficient mice is recovered, which strongly supports the role of complement activation in the reduction of FH-siRNA A549 cell growth.

Finally, to strengthen the activation of the classical pathway of complement (athymic mice would likely be unable to elicit an appropriate Ab-mediated response), FH-siRNA A549 cells pretreated with anti-A549 antiserum were used. We injected these cells into four mice and monitored xenograft growth for 46 days. Continuous stimulation of the classical pathway of complement was achieved by intratumoral injection of antiserum every 3 days. Tumor growth was measured twice or three times a week. Four mice in which FH-siRNA A549 cells were treated with the corresponding rabbit preimmune serum were used as control. From the beginning of the experiment, xenografts of FH-siRNA A549 cells treated with antiserum had a significantly smaller volume than FH-siRNA A549 cells treated with preimmune serum (Fig. 8). At day 46 postinjection, mean tumor volumes of FH-siRNA A549 cells treated with antiserum or treated with preimmune serum were 108 ± 42 mm^3 and 422 ± 220 mm^3, respectively ($p = 0.03$). When the same experiment was conducted with control-siRNA A549 cells, no differences in growth were observed between cells treated with antiserum and cells treated with preimmune serum (data not shown). All of these data suggest that factor H protects A549 lung tumor xenografts against complement activation in vivo and inhibition of factor H expression increases complement response against these cells.

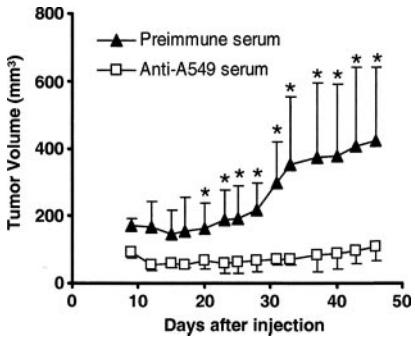

FIGURE 8. Effect of the down-regulation of factor H expression on the growth of A549 xenograft tumors after activation of the classical pathway of complement. The graph represents tumor volume (mean ± SD) of FH-siRNA A549 cells injected into athymic mice after preincubation of the cells with A549 antiserum or preimmune serum (four mice per group). Intratumoral injections of specific antiserum or preimmune serum were administered every 3 days throughout the experiment. *, $p < 0.05$; n.s., Not significant.

Discussion

Lung cancer is the leading cause of cancer death worldwide. Despite the important advances in surgery, radiotherapy, and chemotherapy, the 5-year survival rates for lung cancer are <15% and have not changed significantly over the past two decades. New anticancer treatments have been recently proposed that are based on mAbs targeted to tumor-associated Ags. These Abs, among other mechanisms, can initiate complement-dependent cell lysis (2). To make the most of this strategy, the interaction between the complement system and the tumor cell needs to be clarified. Upon activation of the classical pathway of complement, lung cancer cells show a high resistance to complement-mediated cytotoxicity compared with normal respiratory epithelial cells (28). Immunohistochemical analysis has also revealed that lung tumors have minimal deposition of C3b and apparently lack activation of the lytic membrane attack complex (30). In the present study, we demonstrate that the complement inhibitor factor H, and/or its alternative splice form FHL-1, plays an important role in the resistance of H1264 and A549 lung cancer cells against activation of the classical pathway of complement. Both H1264 and A549 cells express factor H, secrete this protein to the extracellular milieu, and are able to bind it to their cell membranes (14).

In our work, we have first confirmed that NSCLC cells are highly resistant to complement. The antisera against H1264 and A549 cells were highly immunoreactive and allowed a powerful stimulation of the classical pathway of complement. In fact, we observed extensive C3 deposition in both H1264 and A549 cell lines after the stimulation. However, despite the strong initial complement activation, the percentage of complement-mediated lysis remained low (~15%), suggesting the presence of highly efficient complement inhibitors. This fact was previously observed by Varsano et al. (28) using lung cancer cells ChaGo K-1 and H596 preincubated with anti-carcinoembryonic Abs. Intense C3 deposition in the absence of subsequent complement activation may indicate the presence of inactive C3 fragments on the cell membrane due to the action of C3 convertase inhibitors, such as the mCRPs CD46 and CD55. However, Varsano et al. (28) observed that neutralizing Abs anti-CD46 and anti-CD55 were entirely ineffective in increasing the susceptibility of the lung cancer cells to complement. Interestingly, the neutralization of the same inhibitors increased significantly the level of complement-mediated lysis in normal nasal epithelial cells (28), showing a different behavior from malignant cells. Our present data suggest that factor H may be responsible for this inhibitory activity in lung cancer cells. Neutralization of factor H increased C3 deposition moderately and triggered a significant increase of the C5 convertase activity, as determined by C5a release. These data suggest a real augment of active C3b deposition when factor H is blocked. In contrast, we

have also confirmed that neither CD46 nor CD55 has any effect on the control of complement activation. The expression of these inhibitors in H1264 and A549 cells was high, but their neutralization moderately increased C3 deposition and had no effect on C5 convertase activity and complement-mediated cytotoxicity. Interestingly, despite the increase of complement activity after factor H inhibition, complement-mediated cytotoxicity did not augment significantly. Only when we neutralized simultaneously factor H and CD59, an inhibitor of the MAC formation, cell lysis increased, both in H1264 and A549 cells. As previously reported, blockade of CD59 alone also increased complement-mediated cytotoxicity (28). We conclude that both factor H and CD59 play a major role in the protection of H1264 and A549 lung cancer cells against complement activity.

More importantly, we show that expression of factor H by A549 lung cancer cells is critical for A549 tumor growth in vivo. The resistance against complement activation in vivo confirms the in vitro results and underlines the importance of modulating complement activity on lung cancer cells to improve Ab-based immunotherapies. The in vivo experiments were conducted with A549 cells stably transfected with a siRNA specific for factor H or an irrelevant siRNA. Xenografts from factor H-deficient cells were significantly smaller than those from control cells. We ruled out a direct effect of factor H on A549 cell growth rate in vitro. Our results with CVF, along with the high C3 deposition observed in xenografts from factor H-deficient A549 cells, strongly suggests that the effect on tumor growth was mediated by an increase in complement activation on the lung cancer cells. Besides, in complement-depleted mice, factor H-deficient A549 cell growth was comparable to that of normal A549 cells, suggesting that the complement response played a critical role in the tumor growth reduction. This may be considered surprising since factor H inhibition in vitro was not able to increase significantly complement-mediated lysis. However, it has to be remembered that, upon activation, the complement system has several potential effects. First, deposition of C3b on the cell surface leads to the formation of MAC, disrupting the membrane's integrity and causing lysis. Second, deposition of complement components has an important role in fostering opsonization. Finally, during complement activation, powerful anaphylatoxins are released which promote inflammation by stimulating histamine release and attracting phagocytic cells to the area of activation (31). In an in vivo setting, the control of C3 deposition by factor H may prevent the establishment of relevant immune responses against the tumor, independent of the formation of MAC. In a syngeneic mouse model of metastatic lymphoma, inhibition of MAC-mediated lysis by expression of CD59 did not hinder the efficacy of Ig2a and IgM mAbs specific for the ganglioside GD2, indicating that both complement-dependent cellular cytotoxicity and Ab-dependent cellular cytotoxicity operate in vivo (32). It is also important to note that human factor H and murine factor H share high structural and functional similarities and that short consensus repeats 18–20 of human factor H are able to bind to murine C3b (33). These results suggest that human factor H should be able to interact with murine complement. Using factor H-deficient mouse serum, we have observed a reduction in the deposition of murine C3 on human H1264 and A549 cells when increasing concentrations of human factor H were added (our unpublished data). Therefore, human factor H is able to interact with murine complement and inhibit its activity. This is not the case for CD59, since human CD59 is not able to regulate the murine complement system (34). The incapacity of human CD59 to inhibit the formation of MAC on A549 cells injected into mice may also help to explain the profound impact of factor H in the in vivo model. A question that arises from the in vivo experiments is the relevance of the endogenous murine factor H in the protection against complement by human lung cancer cells. Factor H is produced at high levels in mice and should be able to interact with tumor cells in a similar way to endogenously produced human factor H. However, based on our results, we can conclude that the expression of human factor H, but not the mere presence of murine factor H, protects human tumor cells against complement activation. It has been previously suggested that factor H/FHL-1 production by tumor cells causes a high accumulation of this protein in the tumor microenvironment, which would favor its protected role (25). Further investigation is warranted to clarify this interesting observation.

Given the numerous genetic and epigenetic changes associated with carcinogenesis, it is clear that tumor cells express many neoantigens that may be recognized by the immune system (1). This is the basis of the immune surveillance hypothesis, which proposes that the immune system surveys the body for these tumor-associated Ags, eliminating many or most tumors. A corollary to this hypothesis is that tumor cells in progressive cancers develop active mechanisms to escape immune recognition or resist immune attack. Although there is not irrefutable evidence for the existence of an effective immune surveillance, a wealth of published data support the role of the immune system as a primary defense against neoplasia and the importance of the protective mechanisms developed by the tumors (35, 36). Based on these results and on our previous studies (14), we propose that lung cancer cells may develop a protective mechanism against complement attack by expressing and binding factor H to their cell membranes. Several studies have also suggested the importance of factor H in the protection of other tumor cells against complement activation (12, 13, 25, 37). For the first time, we demonstrate the importance of factor H expression for the protection of cancer cells in an in vivo model. Hopefully, these results will help to elucidate the mechanisms used by lung tumor cells to avoid complement activity and will assist in the design of more efficient complement-mediated immunotherapies.

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Disclosures

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