[CANCER RESEARCH 64, 6310–6318, September 1, 2004]

Expression of Complement Factor H by Lung Cancer Cells: Effects on the Activation of the Alternative Pathway of Complement

Daniel Ajona,[1] Zafira Castaño,[1] Mercedes Garayoa,[1,3] Enrique Zudaire,[4] Maria J. Pajares,[1,3] Alfredo Martinez,[4] Frank Cuttitta,[4] Luis M. Montuenga,[1,3] and Ruben Pio[1,2]

[1]Division of Oncology, Center for Applied Medical Research, Pamplona, Spain; [2]Departments of Biochemistry and [3]Histology and Pathology, School of Medicine, University of Navarra, Pamplona, Spain; and [4]Cell and Cancer Biology Branch, National Cancer Institute, NIH, Bethesda, Maryland

ABSTRACT

The complement system is important in immunosurveillance against tumors. However, malignant cells are usually resistant to complement-mediated lysis. In this study, we examine the expression of factor H, an inhibitor of complement activation, and factor H-like protein 1 (FHL-1), its alternatively spliced form, in lung cancer. We also evaluate the potential effect of factor H/FHL-1 in the protection of lung cancer cells against the activation of the complement cascade. By Northern blot analysis we demonstrate a high expression of factor H and FHL-1 in most non-small cell lung cancer cell lines, although neuroendocrine pulmonary tumors (small cell lung carcinoma and carcinoid cell lines) had undetectable levels. Western blot analysis of conditioned medium showed the active secretion of factor H and FHL-1 by cells that were positive by Northern blot. Expression of factor H/FHL-1 mRNA was also shown in a series of non-small cell lung cancer biopsies by *in situ* hybridization. Interestingly, many cultured lung cancer cells were able to bind fluorescence-labeled factor H to their surfaces. Deposition of C3 fragments from normal human serum on H1264, a lung adenocarcinoma cell line, was more efficient when factor H/FHL-1 activity was blocked by specific antibodies. Blocking factor H/FHL-1 activity also enhanced the release of anaphylatoxin C5a and moderately increased the susceptibility of these cells to complement-mediated cytotoxicity. In summary, we demonstrate the expression of factor H and FHL-1 by some lung cancer cells and analyze the contribution of these proteins to the protection against complement activation.

INTRODUCTION

Lung cancer is the leading cause of cancer deaths throughout the world (1). The 5-year survival rates for lung cancer are $<15\%$ in all developed countries and $\leq 5\%$ in many developing countries. These poor survival rates demand new strategies for early detection and major improvements in therapy. New biological and molecular knowledge of lung carcinogenesis has led to the proposal of new therapeutic strategies against lung cancer. Some of these strategies are based on monoclonal antibodies targeted to tumor-associated antigens that, among other mechanisms, can initiate complement-dependent cell lysis (2, 3). However, tumor cells seem to have mechanisms to circumvent this complement-mediated immune response (4). It has been suggested that human non-small cell lung cancer cells are especially resistant to complement-mediated lysis as compared with normal cells (5).

The complement system consists of a cascade of functionally related proteins capable of causing cell lysis. This system plays a key role in the elimination of non-self cells and the initiation of inflammatory response (6). The activation of the complement cascade is highly controlled by several regulatory proteins that prevent complement activation. The alternative pathway of complement is spontaneously activated and requires specific protection mechanisms to restrict the destructive effects of an uncontrolled activated system (7). Complement factor H is a key inhibitor of the activation of the alternative pathway. Factor H is a 150-kDa glycoprotein present in human plasma, which is constitutively produced by the liver but is also synthesized extrahepatically by mononuclear phagocytes, fibroblasts, endothelial cells, mesangial cells, astrocytes, oligodendrocytes, and neurons (8–10). Factor H is composed of 20 repetitive domains termed short consensus repeats, each ~60 amino acids in length. Additionally, a 42-kDa protein produced by alternative splicing from the factor H mRNA has also been found. This protein, named factor H-like protein 1 (FHL-1), contains the first 7 domains of factor H and 4 additional amino acids at the COOH-terminal end (11). FHL-1 shares the complement regulatory functions of factor H. Both factor H and FHL-1 bind to C3b, the key component for complement activation, destroying the C3 convertase. Factor H is also a necessary cofactor for the inactivation of C3b by factor I. The ultimate result of factor H activity is the inhibition of the alternative pathway of complement (12–14). In addition to its regulation of complement activation, other functions have been found for factor H. It is a ligand for L-selectin (15) and also binds to the integrin Mac-1 (CD11b/CD18), enhancing the activation response of human neutrophils (16). Factor H also induces the secretion of interleukin 1β by monocytes (17) and acts as a chemotactic protein for these cells (18). Factor H binds to cell surface components of several pathogens (19–23), inhibiting the alternative pathway of complement and thus enhancing their pathogenicity. Finally, factor H binds and increases the receptor-mediated activity of adrenomedullin, a vasodilator and tumor-promoting peptide (24).

Expression of factor H and FHL-1 has been demonstrated in cell lines from several malignancies: glioblastomas, myosarcomas, neuroblastomas, and carcinomas from nasopharynge, ovary, cervix, bladder, and kidney (25–30). Ovarian tumors also produce factor H and FHL-1 (29). Finally, a clinically approved immunoassay for the detection of bladder cancer in urine is based in the quantification of factor H or a factor H-related molecule (30).

The objective of our study was to determine whether factor H is expressed in lung cancer, and if so, to investigate the contribution of factor H to the resistance of the lung cancer cells to complement-mediated cytotoxicity. We found that factor H and FHL-1 are expressed in many non-small cell lung cancer cell lines and non-small cell lung cancer tumors. Factor H is also secreted to the extracellular milieu and is able to bind to the tumor cell surface. Our results also demonstrate that factor H and/or FHL-1 limit the activation of complement on lung cancer cell membranes, although, in our experimental conditions, not to the extent to fully justify the resistance observed in lung cancer cells to complement-mediated lysis.

Received 7/29/03; revised 5/17/04; accepted 6/24/04.

Grant support: Funded through the "UTE project CIMA," and partially supported by Red Temática de Investigación Cooperativa de Centros de Cáncer del Ministerio de Sanidad y Consumo (#C03/10). R. Pio was the recipient of the 2004–2006 AACR-Cancer Research and Prevention Foundation Career Development Award in Translational Lung Cancer Research.

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Requests for reprints: Ruben Pio, Department of Biochemistry, School of Medicine, University of Navarra, Irunlarrea 1, Pamplona 31080, Spain. Phone: 34-948-425600; Fax: 34-948-425649; E-mail: rpio@unav.es.

MATERIALS AND METHODS

Lung Cancer Cell Lines and Biopsies. A range of lung cancer cell lines from the American Type Culture Collection (Manassas, VA) was used. Cells were grown in culture flasks (75 cm^2) using RPMI 1640 with L-Glutamax (446 mg/ml) supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Invitrogen, Carlsbad, CA). Cell lines included in the study were: small cell lung carcinomas (H69, H209, H345, N417, H510, H82, and H187), adenocarcinomas (H23, H1264, and H2087), bronchioloalveolar carcinomas (A549, H358, and H820), large cell carcinomas (H460, H661, H1155, and H1385), squamous cell carcinomas (H157 and H226), and carcinoids (H720 and H727). Lung carcinoma biopsies were obtained at the Clínica Universitaria de Navarra (Pamplona, Spain) under an institution-approved human tissue procurement protocol. Tumors consisted of 3 squamous cell carcinomas and 5 adenocarcinomas (including 3 bronchioloalveolar carcinomas). Samples were immersed in buffered formalin within 20 min from surgical resection. All of the samples were removed from the fixative solution after 24 h. Tissues were embedded in paraffin and sectioned (3 μm thick).

Sera. Normal human serum, used as a source of complement, was freshly prepared from healthy donors. Heat inactivated-normal human serum was obtained by incubating normal human serum at 56°C for 30 min.

Factor H Purification and Labeling. Human complement factor H was purified from plasma by liquid chromatography as described previously (31). Final concentration was 0.9 mg/ml in PBS. Factor H was labeled using the Alexa Fluor 488 Protein Labeling kit (Molecular Probes, Eugene, OR). Final concentration of labeled factor H in PBS was 420 μg/ml, and the labeling ratio was 14.4 moles of dye per mol of factor H.

Production and Purification of Antifactor H Antibodies. Mouse hybridoma lines OX-23 and OX-24 (European Collection of Cell Cultures, Center for Applied Microbiology and Research, Salisbury, United Kingdom) were maintained in RPMI 1640 supplemented with 10% FBS and penicillin-streptomycin. For production of OX-23 and OX-24 IgG1 monoclonal antibodies, hybridomas were grown in a protein-free growth medium (CD Hybridoma Medium, Invitrogen) from which antibodies were purified by ammonium sulfate precipitation followed by affinity chromatography (HiTrap Protein G MP, Amersham Pharmacia, Piscataway, NJ). Final concentration of both monoclonal antibodies was 1.2 mg/ml in PBS. In addition, rabbit antifactor H antibodies (Serotec, Raleigh, NC) were purified from sera by affinity chromatography after coupling factor H to the column gel (UltraLink Biosupport Medium, Pierce, Rockford, IL). Affinity purified polyclonal antibodies were stored in PBS at 1 mg/ml. Immunoreactivity of both monoclonal and polyclonal antibodies was determined by ELISA and purity confirmed by SDS-PAGE and Coomassie staining.

Northern Blotting. Total RNA was isolated from the cell lines using the guanidine isothiocyanate and cesium chloride method (32). Fifteen micrograms of RNA were loaded per lane, run in 1% agarose gels containing 2.2 mol/L formaldehyde, blotted onto nitrocellulose membranes, and baked for 2 h at 80°C. Equal loading and integrity of RNA were monitored by ethidium bromide staining of the 28 S and 18 S subunits of rRNA. A PCR product (854 bp) of the human factor H cDNA (GenBank accession no. Y00716) was generated using primers 5′-GACACGGATGCATCTGGGAGTA-3′ (sense) and 5′-CAATGGAACCAGATCGGGAATA-3′ (antisense) and cloned into the pCRII vector (Invitrogen). The insert was sequenced to validate the integrity of the probe. The human factor H probe was generated from the pCRII vector by digestion with *Eco*RI. Probe was labeled with [α-32 P]dCTP (3000 Ci/mmol; NEN Life Science Products, Boston, MA) using the Prime-it RmT Random Primer Labeling kit (Stratagene, La Jolla, CA). Unincorporated nucleotides were removed by MicroSpin G-50 Columns (Amersham Pharmacia Biotech). Hybridization was carried out overnight at 42°C in a hybridization buffer containing 40% formamide (32). After stringency washes, blots were exposed to X-ray films.

Western Blotting. Supernatants from cells grown in 100-mm cell culture dishes (Corning Incorporated, Corning, NY) using 12 ml of RPMI 1640 without FBS for 24 h were concentrated 60-fold using centrifugal filter units (Centricon YM-10, Millipore Corporation, Billerica, MA). Ten micrograms of total protein were electrophoretically fractionated on 4–12% Bis-Tris gel (Novex, San Diego, CA) under nonreducing conditions, transferred to a 0.45 μm nitrocellulose membrane, and blocked with 5% fat-free dry milk in PBS. The membrane was then incubated with antifactor H monoclonal antibody OX-24 (1:2,000) and developed using the Lumi-Light Western Blotting kit (Roche). Factor H and FHL-1 were used as positive controls. Recombinant FHL-1 was kindly provided to F. C. by Dr. Peter Zipfel (Hans Knoell Institute for Natural Products Research, Jena, Germany).

Factor H/FHL-1 Quantitation. A polystyrene 96-well plate (Corning) was coated with 200 ng/well of purified rabbit antifactor H antibodies [in 50 μl of 50 mM sodium bicarbonate (pH 8.3)] during 1 h at room temperature. The plate was blocked overnight at 4°C with Tris-buffered saline, 1% bovine serum albumin, and 0.1% Tween 20 (pH 7.4). After washing, 100 μl of supernatants (from cells grown in RPMI 1640 without FBS for 24 h) or standards (factor H ranging from 1.5 to 200 ng/ml) were added in blocking buffer and incubated for 2 hours at room temperature. After washing, 100 μl of OX-23 monoclonal antifactor H antibody (1:200) were added, and after 2-hour incubation at room temperature the assay was developed using a goat antimouse antibody coupled to horseradish peroxidase (1:1,000, Sigma-Aldrich, St. Louis, MO) and *O*-phenylenediamine dihydrochloride (Sigma-Aldrich). The plate was read at 450 nm.

***In situ* Hybridization.** The pCRII vector containing factor H cDNA was linearized with *Sac*I and *Xba*I and used as a template to synthesize digoxigenin-labeled sense and antisense riboprobes. After proteinase K treatment and acetylation, hybridization was performed in a moist chamber at 58°C for 18 hours. After stringency washes, visualization of digoxigenin was performed using an alkaline phosphatase-labeled antidigoxigenin antibody and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate (Roche, Indianapolis, IN). Sense probe was used as negative control.

Binding of Factor H to Lung Tumor Cells. Cells were detached from culture dishes with 1 mM EDTA, washed once, and resuspended in binding buffer (PBS, 1% bovine serum albumin, and 0.1% sodium azide). Cells (1×10^5 in 50 μl of binding buffer) were incubated with 2 μl of labeled human factor H for 30 min at 4°C. Cells were washed three times and analyzed on a flow cytometer (FACSCalibur, Becton Dickinson) after the addition of propidium iodide. Cell Quest Pro software was used for data acquisition and analysis. Data were collected as mean fluorescence intensity. For competition, 10 μl of unlabeled human factor H or 10 μl of polyclonal goat-antihuman factor H serum (Quidel, San Diego, CA) were also added to the incubation.

Deposition of C3-Related Fragments. Cells were detached from culture dishes with 1 mM EDTA, washed once, and resuspended in veronal buffer [1.8 mM barbital, 3.1 mM barbituric acid, 141 mM sodium chloride, and 0.5 mM MgCl (pH 7.4)]. Cells (2×10^5 in 100 μl) were incubated for 30 min at 37°C with 100 μl of normal human serum (diluted 1:8) or 100 μl of normal human serum (diluted 1:8) preincubated for 30 min at 4°C with monoclonal antibody OX-23 or OX-24 at 0.6 mg/ml. After washing twice with binding buffer, cells were incubated for 30 min at 4°C with a fluorescein isothiocyanate-conjugated goat antihuman-complement C3 antibody (ICN Biomedicals Inc., Aurora, OH) diluted 1:100 in a total volume of 50 μl. Cells were washed twice and analyzed by flow cytometry after the addition of propidium iodide. Data were collected as mean fluorescence intensity.

Determination of C5a Production. Cells were processed and treated as described in the previous paragraph. Generation of C5a was quantified in supernatants after incubation with normal human serum, heat inactivated-normal human serum, or normal human serum with monoclonal antibodies OX-23 and OX-24. Quantitation was performed using the Human C5a ELISA kit (BD Biosciences PharMingen, San Diego, CA) following the manufacturer's instructions.

Complement-Mediated Cytotoxicity. When a 1:16 final dilution of serum was tested, cells were processed and treated as described above. For experiments with serum diluted 1:8, a double concentration of serum and monoclonal antibodies was used. Cells were analyzed by flow cytometry after the addition of propidium iodide. Data were collected as mean fluorescence intensity.

Statistical Analysis. Data obtained from C3 deposition, C5a quantitation, and complement-mediated cytotoxicity were analyze by Student's t test. A $P < 0.05$ was considered statistically significant.

RESULTS

Factor H and FHL-1 Expression in Lung Cancer. We first compared the amount of factor H and FHL-1 mRNA in lung cancer cell lines with that in cancer cell lines from other origins: breast,

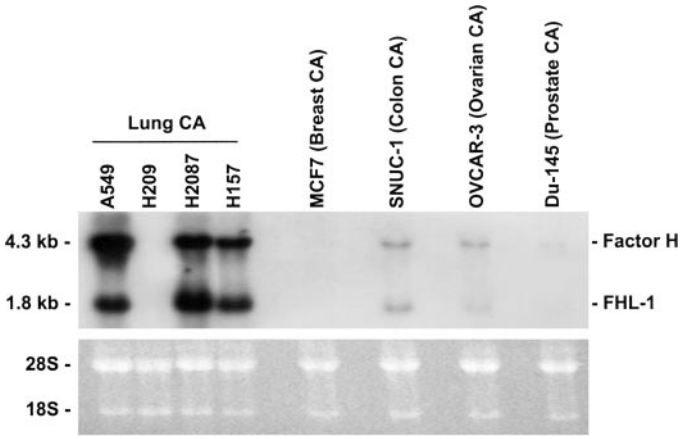

Fig. 1. Northern blot analysis showing the expression of factor H and FHL-1 in lung cancer cell lines and cancer cell lines from other tissue origins. Fifteen micrograms of total RNA were loaded per lane, and ethidium bromide staining of 18S and 28S rRNA was used to ensure equal loading and RNA integrity.

colon, prostate, and ovary. Northern blot analysis revealed that factor H and FHL-1 mRNA expression was much higher in lung tumor cell lines than in cell lines from other origins (Fig. 1), although one of the lung cancer cell lines (H209) was negative. We next examined mRNA levels in a panel of 18 lung tumor cell lines from the main types of lung cancer (Fig. 2*A*). Many of them expressed factor H and FHL-1 mRNA, although the amount of mRNA was highly variable between cell lines. In particular, we did not detect any factor H or FHL-1 mRNA in any small cell lung carcinoma cell line. To confirm the production of factor H and FHL-1 by lung cancer cell lines, we analyzed the presence of both proteins in whole cell lysates. We used most of the cells present in the Northern blot analysis and included some additional cell lines. In these extracts, we were not able to detect factor H or FHL-1 by Western blotting (data not shown). We then evaluated the possibility that both proteins were secreted to the extracellular medium. To avoid interferences with the presence of factor H in FBS, media were obtained from cells cultured during 24 h in serum-free RPMI 1640. By Western blot analysis we were able to detect factor H and FHL-1 in the serum-free conditioned media of many non-small cell lung cancer cell lines, confirming that these cells produce and secrete factor H and FHL-1. Fig. 2*B* shows a representative Western blot in which expression of factor H can be observed in most non-small cell lung cancer cell lines. FHL-1 was observed in some non-small cell lung cancer cell lines (Fig. 2*B*). Longer exposure times of the blot also revealed FHL-1 in cell lines H1264, H157, and H226 (data not shown). In contrast, factor H and FHL-1 were undetectable in small cell lung carcinoma cells. Similar findings were observed for the neuroendocrine-like carcinoid tumor cell lines H720 and H727. By ELISA, we quantified the levels of factor H/FHL-1 secreted by these cells (Table 1). Globally, there was a high concordance between factor H and FHL-1 mRNA and protein levels for each cell line. These data suggest that factor H and FHL-1 are expressed at high levels by many non-small cell lung cancer cell lines but are not stored intracellularly. Rather, they are efficiently secreted to the extracellular medium.

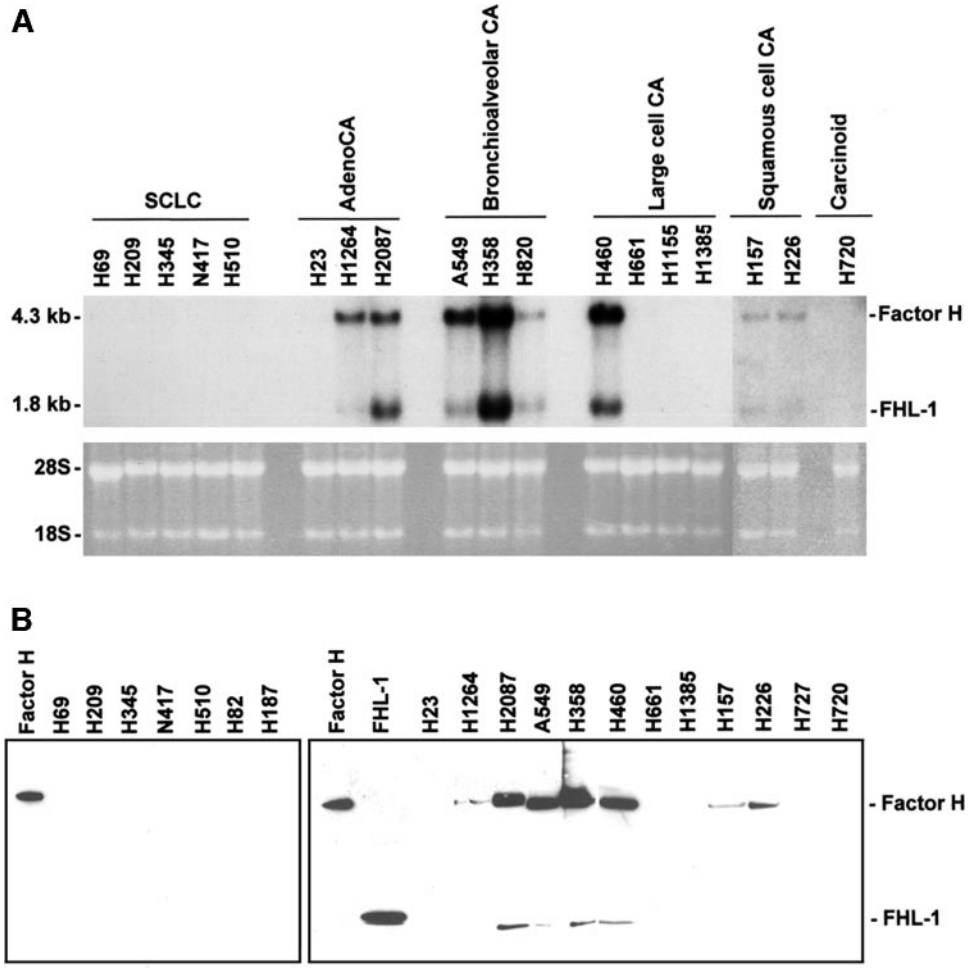

Fig. 2. Expression of complement regulators factor H and FHL-1 in small cell lung carcinoma (*SCLC*) and non-small cell lung carcinoma cell lines. *A*, Northern blot analysis in which 15 μg of total RNA were loaded per lane. Ethidium bromide staining of 18S and 28S rRNA was used to ensure equal loading and RNA integrity. *B*, Western blot analysis of the presence of factor H and FHL-1 proteins in the extracellular medium. Samples are serum-free conditioned media (10 μg of total protein) from lung cancer cell lines that had been conditioned for 24 hours. Four nanograms of purified factor H and recombinant FHL-1 were used as positive controls.

Table 1 *Expression of factor H/FHL-1 by lung cancer cell lines*

Cell line*	Conditioned media (pg/μg total protein)
H1264	7 ± 1.7†
H2087	52.2 ± 2.9
A549	38.7 ± 11
H358	312.3 ± 71.5
H460	50.6 ± 6.2
H157	8.1 ± 3.4
H226	4.7 ± 2.3

* Small cell lung carcinoma cell lines, H23, H661, H1385, H727, and H720 had undetectable levels.

† Data represent mean ± SD ($n = 2$).

We then determined the expression of factor H and/or FHL-1 in lung tumors *in vivo*. Considering that factor H and FHL-1 are rapidly secreted to the extracellular medium and no detectable amounts of the proteins are stored into the cultured lung cancer cells, we decided to use *in situ* hybridization to localize the mRNA of these factors in lung tumor biopsies. We analyzed paraffin-embedded lung tumors, adenocarcinomas, and squamous cell carcinomas using an RNA probe that recognizes both factor H and FHL-1 mRNA. In agreement with our previous data with cell lines, we observed cytoplasmic accumulation of factor H and/or FHL-1 mRNA in most lung cancer tumors (6 of 8). mRNA levels varied between positive tumors (Fig. 3, *A*, *D*, and *G*). In all of the cases the sense probe was used as a negative control (Fig. 3, *B*, *E*, and *H*).

Binding of Factor H to Lung Tumor Cell Lines. Because factor H and FHL-1 are actively secreted to the extracellular medium, we wanted to determine whether lung tumor cells were able to bind factor H to their surfaces. Cells were incubated with Alexa Fluor 488-labeled factor H. After thorough washes to remove the unbound protein, flow cytometry analysis showed marked binding of fluorescent factor H to many cell lines (H69, H209, H82, H187, H23, H1264, A549, and H460). As a representative example, Fig. 4 shows the binding of labeled factor H to H1264 cell surface. Specificity of the binding was demonstrated by competition with unlabeled factor H or an antifactor H polyclonal antiserum. We obtained a complete competition when the antiserum was used (Fig. 4*B*), whereas unlabeled factor H did not completely compete the binding (Fig. 4*A*). This suggests the presence of both specific and nonspecific binding to the cell surface. In our analysis we also found cell lines with very little and probably nonspecific binding of factor H (H345, H661, and H727). These results suggest that some lung cancer cells that are able to produce and secrete factor H have also the ability to bind soluble factor H from the fluid phase. Additionally, other lung cancer cell lines that do not express factor H are also able to bind it to their surface (i.e. H82). H1264, a cell line that both produces and binds factor H, was chosen to additionally analyze the activation of the alternative pathway of complement on lung cancer cells.

Deposition of C3-Related Fragments on the Surface of H1264 Lung Cancer Cells. In view of our results showing the ability of cell line H1264 to bind factor H, we evaluated whether the recruitment of factor H by these lung cancer cells affects the activation of complement. A key event in the activation cascade of complement is the deposition of C3b on the cell surface. Factor H causes the degradation of C3b and, therefore, the inactivation of the C3 convertase C3bBb. The inactivation of C3b prevents the deposition of additional C3b molecules in the cell membrane, which is mediated by this convertase. For that reason, the deposition of C3 fragments on the cell surface is a good indicator of activation of the alternative pathway of complement. In our study, we used a polyclonal antibody that recognizes C3 and C3 fragments such as C3b and inactive C3b, one of the products from C3b cleavage. The minimal activation of complement by lung cancer cells has already been described (5). We confirmed this observation with some of our lung cancer cell lines. In the presence of normal human serum diluted 1:16 very little C3-fragment deposition by the alternative pathway was observed in all of the lung cancer cell lines tested (H1264, A549, H82, and H661). Fig. 5, *A* and *C*, shows

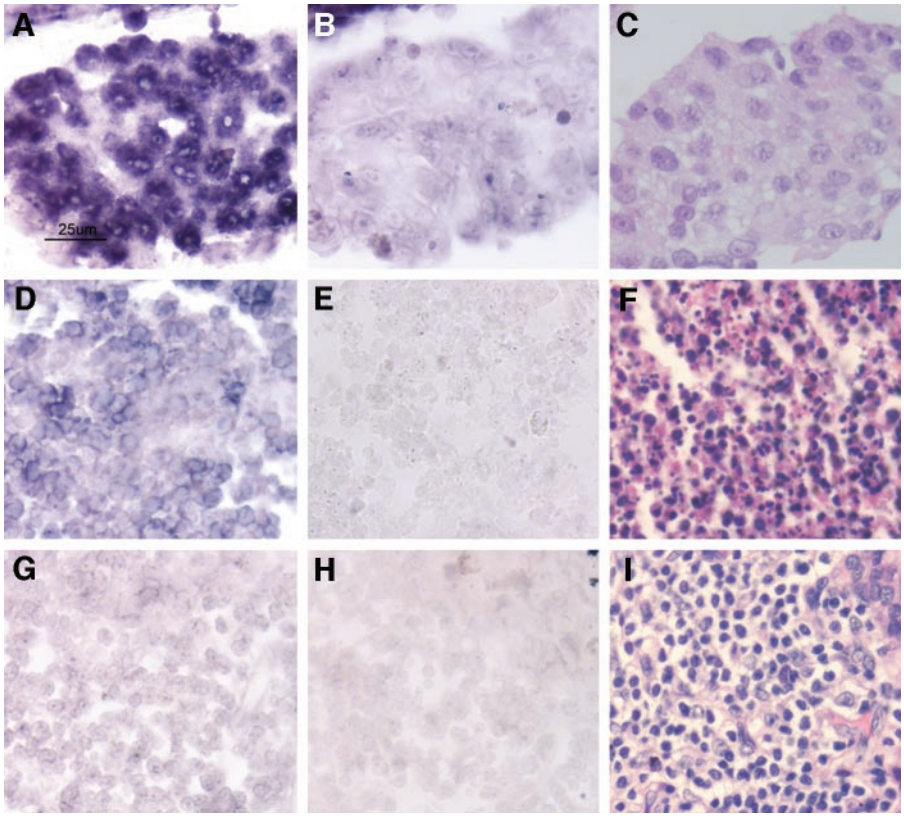

Fig. 3. *In situ* hybridization detecting the presence of factor H mRNA in sections of non-small cell lung carcinoma biopsies. A digoxigenin-labeled antisense probe was used to detect the expression of factor H/FHL-1 mRNA (*A*, *D*, and *G*). The figure shows examples of tumors with different levels of factor H/FHL-1 expression. *A*, squamous cell carcinoma with a strong cytoplasmic expression. *D*, adenocarcinoma with moderate labeling. *G*, squamous cell carcinoma with very low expression. The sense probe was used in serial sections as a negative control (*B*, *E*, and *H*). Hematoxylin and eosin staining is also shown for each tumor to assess their morphological features (*C*, *F*, and *I*).

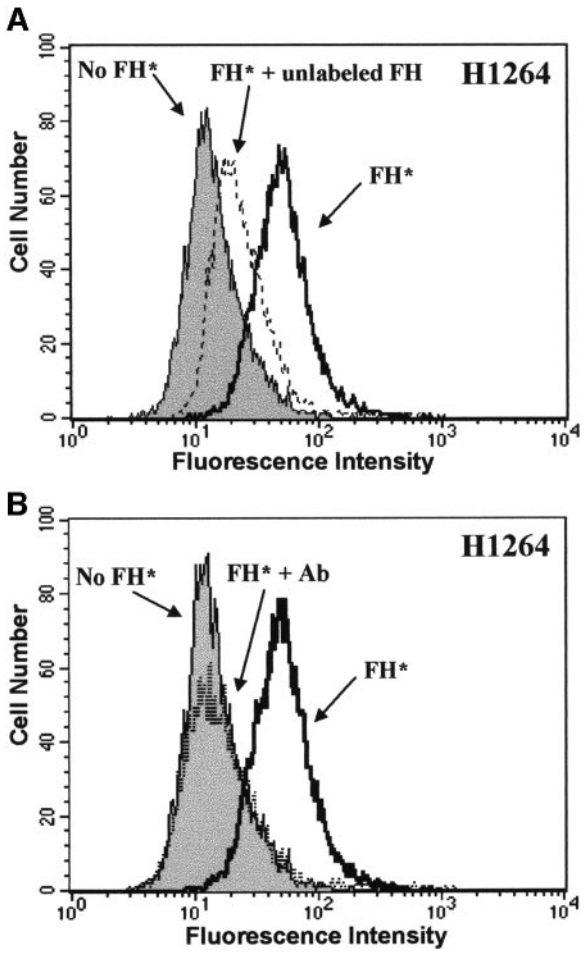

Fig. 4. Flow cytometry analysis of the binding of fluorescent factor H (*FH**) to H1264 cell surface. Specificity in the binding of labeled factor H to H1264 cells is shown by competition with 10 μl of unlabeled factor H (*A*) or 10 μl of antiserum against factor H (*B*). Factor H binding is indicated by an increase in intensity in the green channel.

the deposition of C3-fragments in H1264 cells in the presence of normal human serum (diluted 1:16) when compared with the deposition in the absence of serum or in the presence of heat inactivated-normal human serum. To study the effect of the inhibition of factor H activity on this deposition, we used the monoclonal antibodies OX-24 and OX23. OX24 is an antibody that inhibits the binding of factor H and FHL-1 to surface-bound C3b (33). In contrast, OX-23 is an antibody against factor H and FHL-1 that binds to a different epitope from OX-24 and does not affect the binding of factor H and FHL-1 to surface-bound C3b (33). When H1264 cells were incubated with normal human serum in the presence of OX-24, the deposition of C3 or related fragments increased significantly ($P = 0.021$; Fig. 5, *B* and *C*). When the same experimental conditions were carried out in the presence of OX-23 antibody no changes were observed when compared with normal human serum alone ($P = 0.458$). The difference in C3 deposition between the treatment with OX-24 and OX-23 also reached statistical significance ($P = 0.027$). These data suggest that the activities of factor H and/or FHL-1 protect these lung cancer cells against the deposition of C3-related fragments. When the same experiments were carried out with cell line H661, which does not show a significant binding of factor H, we observed a minimal increase of the deposition of C3-fragments in the presence of normal human serum (diluted 1:16) when compared with the deposition in the absence of serum or in the presence of heat inactivated-normal human serum. However, neither OX-24 nor OX-23 affected this deposition (data not shown), suggesting that in cell lines unable to bind factor H, blockade of factor H activity does not modify C3 deposition on their membranes.

Control of Anaphylatoxin C5a Production by Factor H on H1264 Lung Cancer Cells. C3 convertase activity leads to the formation and activation of C5 convertases, which cleave C5 in two fragments: cell-bound C5b and C5a. C5a, a potent anaphylatoxin and chemotactic factor, is important for initiation and maintenance of inflammatory responses upon complement activation. We have shown that factor H blockade by the monoclonal antibody OX-24 augments the deposition of C3-related fragments and that this deposition may be the result of an increase in C3 convertase activity. Therefore, we were interested in evaluating whether this increase in C3 convertase activity was followed by an increase in C5 convertase activity and a release of C5a. For that purpose we determined the concentrations of C5a released after activation of the alternative pathway of complement on H1264 cells. As expected, incubation of H1264 cells in the presence of normal human serum produced a moderate increase of C5a levels in the medium: 22.8 $\pm$ 2.6 pg/ml *versus* 13.3 $\pm$ 0.6 pg/ml in the medium from cells incubated with heat inactivated-normal human serum. When cells were incubated with normal human serum in the presence of OX-24 the release of C5a increased significantly (52.8 $\pm$ 3.2 pg/ml; $P = 0.0003$). When the same experimental conditions were carried out in the presence of OX-23 antibody no significant changes were observed when compared with normal human serum alone (32.5 $\pm$ 3.4 pg/ml; $P = 0.063$). These results suggest that the presence of factor H and/or FHL-1 inhibits the release of C5a after activation of the complement alternative pathway.

Complement-Mediated Cytotoxicity on H1264 Lung Cancer Cells. We have demonstrated that the binding of factor H and FHL-1 to lung cancer cells inhibits the deposition of C3 fragments. Among other immunological implications, this deposition should affect the susceptibility of these lung cancer cells to complement-mediated cytotoxicity. To confirm this hypothesis, we analyzed the effect of factor H neutralization on complement-mediated cytotoxicity of H1264 cells. Factor H activities are primarily associated with the inhibition of the alternative pathway of complement (12–14). Therefore, we set up the conditions for the evaluation of the cytotoxic effect of the alternative pathway of complement on H1264 cells. All of the experiments were conducted in the presence of Mg^{2+} and in the absence of Ca^{2+} to avoid the activation of the classical pathway. Complement-mediated cytotoxicity was determined by flow cytometry using propidium iodide uptake as an indicator of damaged cells. To distinguish between nonspecific and complement-mediated cell deaths we used heat inactivated-normal human serum as a control. The number of propidium iodide-positive cells in the presence of normal human serum minus the number of positive cells in the presence of heat inactivated-normal human serum was considered the number of cells specifically attacked by complement. When treated with normal human serum diluted 1:16, ~5% of H1264 cells were killed by complement, indicating that these cells are highly resistant to complement-mediated cytotoxicity. When factor H/FHL-1 were neutralized by monoclonal antibody OX-24 the sensitivity of H1264 cells to complement-mediated cytotoxicity increased moderately when compared with an incubation with normal human serum alone or normal human serum in the presence of OX-23 ($P = 0.031$ and 0.004, respectively; Fig. 6). The treatment with the monoclonal antibody OX-23 did not modify significantly the viability of the cells ($P = 0.058$), although a small increase in viable cells was observed. The same set of experiments was carried out with serum diluted 1:8. In these conditions, approximately the same number of cell was killed by complement. When factor H and FHL-1 were neutralized by monoclonal antibody OX-24 the cytotoxicity increased almost 3-fold

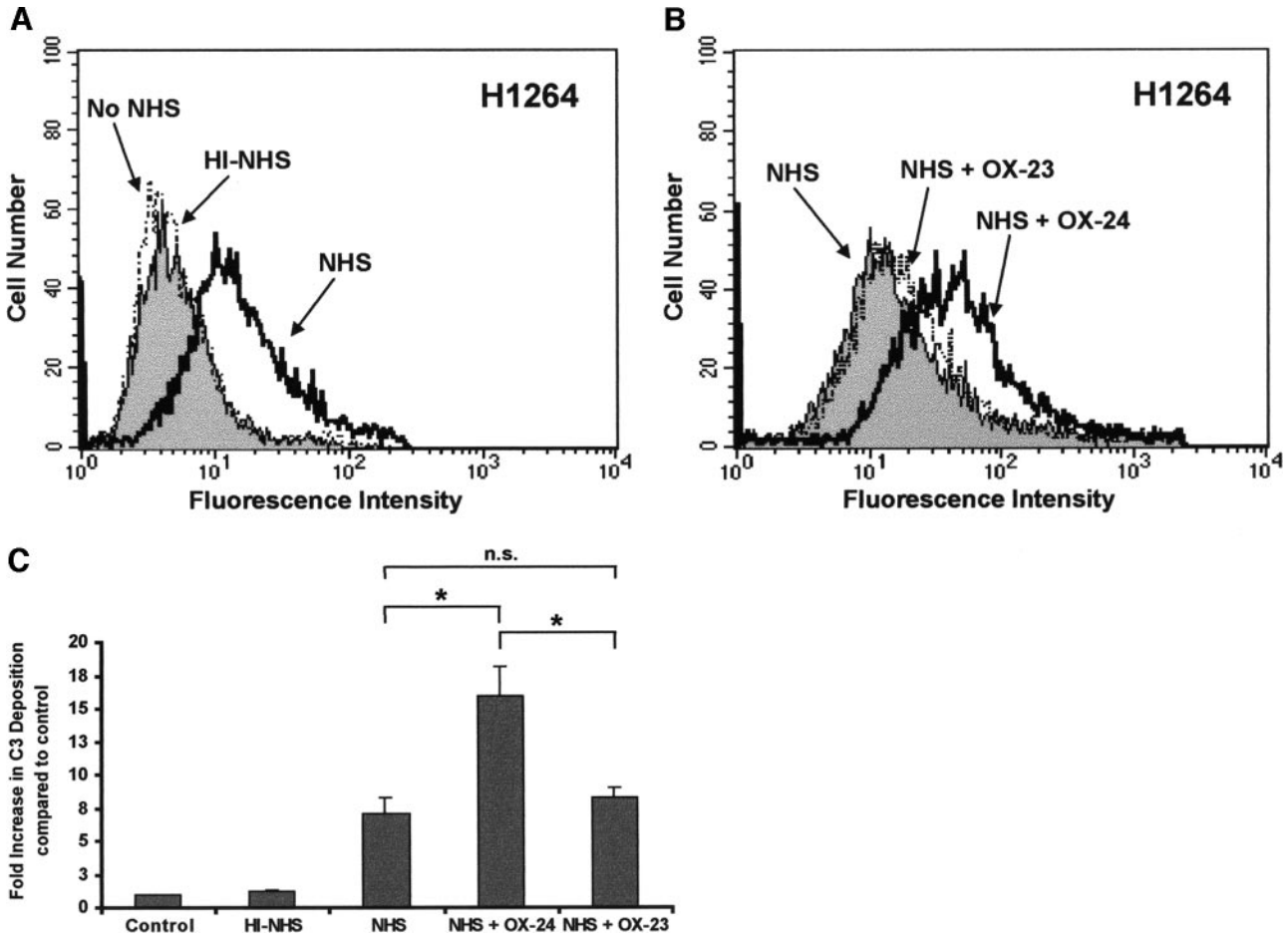

Fig. 5. Effect of factor H and/or FHL-1 in the deposition of C3-related fragments. C3 deposition was determined by flow cytometry using a polyclonal antibody that recognizes C3 and C3 fragments and is indicated by an increase in intensity in the green channel. Graphs in *A* and *B* are representative examples of the repeated experiments. *A*, deposition of C3-related fragments on H1264 cells incubated with normal human serum (*NHS*) diluted 1/16, or heat-inactivated normal human serum (HI-NHS) at the same dilution. Incubation without NHS is used as control. *B*, deposition of C3-related fragments on H1264 cells incubated with NHS (diluted 1/16) in the presence of antifactor H antibodies OX-24 and OX-23. *C*, graph representing mean from the results obtained in three independent experiments; *bars*, ±SE. Data were analyzed as the ratio between the mean fluorescence intensity for treatments and the mean fluorescence intensity for the control (incubation of the cells without NHS). *, $P < 0.05$; *n.s.*, not significant.

when compared with an incubation with normal human serum alone or normal human serum in the presence of OX-23 ($P = 0.012$ and 0.023, respectively; Fig. 6). Therefore, in these conditions, the blockage of factor H activity had a more pronounced effect on cytotoxicity than when serum diluted 1:16 was used. The treatment with the monoclonal antibody OX-23 did not modify the viability of the cells ($P = 0.904$).

DISCUSSION

Alterations on membrane proteins associated to cellular transformation mark tumor cells for immune recognition. Transformed cells can escape immune surveillance by developing inhibitory mechanisms that provide resistance to immunological recognition and subsequent attack. Both experimental and clinical studies indicate that complement activation can play an important role in the immune surveillance against tumors. However, tumor cells exploit several strategies to circumvent the immune response (4). One of the better understood protective mechanisms against immune surveillance is the expression of membrane-associated complement regulatory proteins such as CD55 (decay-accelerating factor), CD46 (membrane cofactor protein), and CD59. Many current hypotheses propose that expression of these proteins on the neoplastic cell membrane protects tumors from complement activation (34–37).

In lung carcinomas, immunohistochemical analysis has revealed expression of CD55, CD46, and CD59 (38). Additionally, lung tumors show minimal deposition of C3b and no activation of the lytic membrane attack complex. *In vitro* studies have confirmed that lung cancer cell lines are extremely resistant to complement-mediated lysis, and this resistance is much higher than that observed in normal cells such as human nasal epithelium primary cell cultures (5, 39). Besides, lung cancer cell lines have the ability of limiting the activation of complement in their cell membrane and express high levels of cell membrane complement inhibitory proteins (5). For these reasons, it was suggested that membrane-associated complement regulatory proteins may act as mediators of lung cancer resistance to complement activation (38). However, neutralizing antibodies against CD46 and CD59 are not effective in increasing the susceptibility to complement-mediated lysis (5), whereas the same antibodies are very effective in facilitating complement-mediated lysis of noncancer nasal epithelial cells (39). This suggests the existence of alternative mechanisms that would explain the resistance to complement activation in lung cancer. In our study, we show the expression and binding of factor H to some lung cancer cells and analyze its implications in the control of complement activation and the protection from complement-mediated lysis.

To date, little evidence for the role of factor H in the resistance of

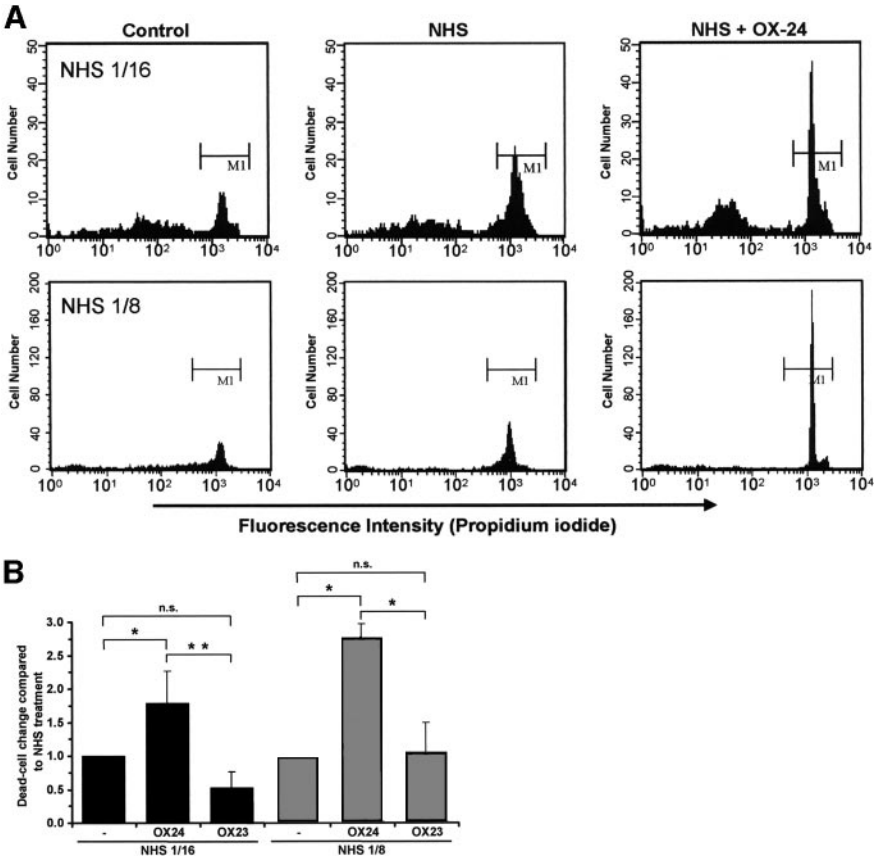

Fig. 6. Susceptibility of H1264 lung cancer cell lines to complement mediated cytotoxicity after neutralization of factor H/FHL-1 activity. H1264 cells were incubated with normal human serum (*NHS*) or heat-inactivated normal human serum (*HI-NHS*; control), and the number of dead cells was determined as propidium iodide-positive cells found in NHS-treated cells minus the ones found in HI-NHS-treated cells. A gate was established for the specific selection of these cells. *A*, representative examples of the analysis of cells damaged by complement, using NHS diluted 1/16 or 1/8, in the absence or presence of the antifactor H neutralizing antibody OX-24 (note the different scales on the *Y* axis of the two examples). *B*, graphs representing the effect of the neutralization of factor H/FHL-1 on complement-mediated cytotoxicity. Data are presented as fold change in the number of dead cells using NHS as the reference value. Each value represents mean from at least three independent determinations; *bars*, ±SE. *, $P < 0.05$; **, $P < 0.001$; *n.s.*, not significant.

tumor cells against complement damage has been reported. H2 glioblastoma cells are exceptionally resistant to complement-mediated lysis but, although these cells strongly expressed CD59, CD46, and CD55, a combined neutralization of these molecules does not increase their sensitivity to complement killing (40, 41). It has been demonstrated that H2 cells also produce factor H and FHL-1 and are able to bind them, promoting C3b cleavage. Antifactor H monoclonal antibodies enhance cell death (from 5% to 15%), confirming that factor H (or FHL-1) is involved in the complement resistance of this cancer cell line (40). In SK-MEL-93–2, a human melanoma cell line, factor H is the dominant factor regulating the inactivation of cell-bound C3b and is involved in the control of the classical pathway of complement (42). An antifactor H antibody also enhances complement-mediated killing of Raji cells, a cell line obtained from a Burkitt's lymphoma (43). Members of the SIBLING family can protect murine erythroleukemia and human myeloma and breast cancer cells from complement attack, likely by sequestration of factor H to the cell surface (44, 45). Factor H and FHL-1 are highly expressed by ovarian carcinomas, and both proteins are abundantly present in ascites from these tumors (29). By *in situ* hybridization, we have now demonstrated that non-small cell lung cancer primary tumors also express complement factor H mRNA.

In the present study, we have also shown that most non-small cell lung cancer cell lines constitutively produce complement factor H and FHL-1. Immunoblotting analysis has shown that these non-small cell lung cancer cell lines also secrete factor H and FHL-1 into the medium. Factor H production exceeded that of FHL-1 at both mRNA and protein levels. This is different from what has been described in normal lung, where there exists a predominant expression of the 1.8-kb mRNA (FHL-1) over the 4.3-kb mRNA (factor H), but it is similar to what is observed in the liver, in which the 4.3-kb species is more abundant (46, 47). Another interesting observation from our study is the absence of factor H/FHL-1 mRNA and protein expression in small cell lung carcinoma cell lines. In parallel to our findings with small cell lung carcinoma cell lines, the two carcinoid cell lines included in our study (H720 and H727) also lack factor H/FHL-1 expression, suggesting a phenotypic correlation between the expression of these proteins and the neuroendocrine differentiation of the tumors. Consistent with this proposed relationship, two of the four non-small cell lung cancer cell lines that lack factor H/FHL-1 expression (H1155 and H1385) have neuroendocrine features as well (48).

Complement can be activated via three different pathways: the classical, the alternative, and the lectin pathway. Activation of C3 by cleavage to C3b is a critical event in all three of the complement fixation processes. In the alternative pathway, spontaneously generated C3b molecules attach to cell surface molecules and generate a C3 convertase, a complex that catalyzes the cleavage of C3 to C3b. As the convertase progressively releases increasing amounts of C3b, more C3 convertase can be assembled, and, thus, an amplification loop is established. This leads to the accumulation of C3b on the cell membrane, which initiates the complement cascade and ultimately ends in cell lysis. This cascade of complement activation is tightly regulated. In many cells, complement inhibitors promote the dissociation of the convertase and/or the cleavage of C3b to inactive fragments such as inactive C3b. Our study demonstrates that in the presence of normal human serum there is a limited deposition of C3 fragments in lung cancer cell membranes, a deposition insufficient to trigger cell death. These results are in agreement with the minimal deposition of C3b and the absence of activation of the lytic membrane attack complex that has been reported for several types of lung carcinomas (38). As demonstrated in our studies, factor H is involved in the control of the amount of C3 deposited on the cell. Our results suggest that in some malignant lung cells there is a spontaneous deposition of C3 that is rapidly degraded to inactive C3b due in part to the presence of factor

H on the cell membrane. When factor H activity is impaired by a specific neutralizing antibody, C3b formed by spontaneous low-level activation of C3 is not cleaved, and the amplification loop is initiated. This can be detected as a higher deposition of C3-related fragments on the cell membrane. Some experimental data suggest that in lung cancer this effect is not carried out by membrane-associated complement regulatory proteins (CD46, CD55, and CD59), because cell membrane adsorption of C3-fragments is not affected by neutralization of these proteins (5). However, more work is needed to confirm this point, especially considering that the protection mediated by factor H would only be possible in cells that are capable of binding this protein.

In our experiments, it is not possible to determine whether the observed effects are due to factor H, FHL-1, or both. The two proteins are highly expressed by some lung cancer cells, and the neutralizing antibody used to analyze this protective effect may block the activities of both proteins. As indicated previously, the expression of FHL-1 mRNA in normal lung is higher than that of factor H mRNA (46, 47), whereas factor H is more abundant in lung tumor cell lines. The preferential presence of factor H over FHL-1 in lung cancer differs from what has been observed in glioblastoma and ovarian tumors, where the predominant form is FHL-1 (29, 40). Our data also show that factor H can bind to the cell membrane of some lung cancer cells, but nothing is known about the binding properties of FHL-1.

Although we have observed an increase in C3 deposition and C5a release after blocking factor H, in our studies, the activation of the complement cascade led to a modest increase in complement-mediated cytotoxicity. This paradox may be explained by several reasons. On the one hand, our results may reflect a limited role of factor H in regulating complement-mediated cytotoxicity on lung cancer cells, suggesting that other inhibitory molecules are involved in resistance to complement attack. However, on the other hand, our experimental design may mask some of the potential effects of factor H on cytotoxicity. In our study we have only focused on the alternative pathway of complement fixation, whereas in the *in vivo* setting of human carcinogenesis, factor H and FHL-1 could potentially block all three of the complement pathways. Additionally, even in the absence of lysis, an increase of C3 fragments in the cell surface can enhance the sensitivity of the tumor cells to be attacked by activated macrophages (49), natural killer cells (50), and lymphocytes (51). Finally, upon activation of C3 in our cells, the complement cascade of proteolytic enzymes releases the anaphylatoxin C5a, and likely C3a and C4a. These fragments exert various biological functions important to the initiation and maintenance of an inflammatory process. In conclusion, in a biological scenario, the control of C3 deposition, in this case by factor H, may prevent the establishment of an important immune response against the tumor. Taking this into account, it is still premature to state the real implication of this protein in the ability of human lung tumors to evade *in vivo* immune surveillance.

It should also be considered that we have focused our functional studies in the implication of factor H in the resistance to complement activation and more particularly on the control of the alternative pathway of complement. However, overexpression of factor H by some lung cancer cells may also have a profound impact on tumor growth through other additional mechanisms. For example, overexpression of factor H may have consequences in the regulation of the activities of the tumor growth factor adrenomedullin. Adrenomedullin is a 52 amino acid peptide with important tumor-promoting activities (52, 53). Adrenomedullin, together with its receptor, is expressed by lung carcinomas (54, 55) and mediates lung cancer cell growth (56), angiogenesis, tumor cell migration, and survival (57). Factor H binds to adrenomedullin and is able to increase its receptor-mediated activity (24). In this context, the expression of factor H by lung tumors may regulate adrenomedullin tumor survival capabilities, thus augmenting cancer progression.

In conclusion, in this study we show that several human non-small cell lung cancer cells constitutively express and secrete complement factor H and FHL-1. In addition, our results show that tumor cells that bind factor H to their surface can prevent C3b accumulation upon their cell membranes, although in our experimental conditions this effect seems to be insufficient to fully explain the high resistance of these cells to complement-mediated lysis. These results warrant additional studies to evaluate the relevance of these and other complement regulatory proteins in *in vivo* immune surveillance. The elucidation of these mechanisms would undoubtedly help to design efficient complement-mediated immunotherapies against human cancer.

ACKNOWLEDGMENTS

We thank Amaya Lavin for technical assistance. We also thank Dr. Wenceslao Torre, Dr. Javier Zulueta, and Usua Montes for their help in collecting the non-small cell lung cancer biopsies. We thank Dr. Peter F. Zipfel (Department of Biological Infection, Hans Knoell Institute for Natural Products Research, Jena, Germany) for gracious contribution of recombinant FHL-1.

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