Down-Regulation and Antiproliferative Role of C/EBPα in Lung Cancer

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ABSTRACT

The transcription factor, CCAAT/enhancer binding protein α (C/EBPα) is important in the terminal differentiation of granulocytes, hepatocytes, and adipocytes, and recurrent mutations of C/EBPα were described in acute myeloid leukemia. In the lung, C/EBPα is expressed in bronchial cells and type II pneumocytes. Abnormal proliferation of the latter cell type was reported in C/EBPα knockout mice. We determined the expression of C/EBPα by Northern blot analysis in 30 lung cancer cell lines and found significant down-regulation in 24 cell lines. Immunohistochemical study of primary tumor specimens showed undetectable or low expression of C/EBPα in 23 of 53 specimens. Its expression was more frequently down-regulated in adenocarcinoma and poorly differentiated cancer specimens than in squamous cell cancers. A higher frequency of reduced expression was found in more advanced stages. To investigate the consequences of C/EBPα expression in lung cancer cells, we stably transfected two cell lines that do not express the gene (Calu1 and H358) with a plasmid allowing for induction of C/EBPα protein expression. Induction of C/EBPα led to significant growth reduction attributable to proliferation arrest, morphological changes characteristic of differentiation, and apoptosis. These results suggest that C/EBPα is down-regulated in a large proportion of lung cancers and that it has growth-inhibitory properties in airway epithelial cells. Genetic analysis of the C/EBPα gene is in progress to fully evaluate its role as a novel tumor suppressor in lung cancer.

INTRODUCTION

There will be an estimated 164,000 new cases of lung cancer diagnosed in 2001 in the United States, and lung cancer will remain the leading cause of cancer deaths (1). The present treatment of locally advanced and metastatic lung cancer is very unsatisfactory, and the overall long-term survival of patients diagnosed with lung cancer is only ~13%. Our understanding of the genetic abnormalities underlying the development of lung cancer remains quite limited (2, 3). No specific abnormalities in lung-specific, growth-regulatory pathways have been described to date. A recent high-frequency allelotyping study demonstrated that in individual lung cancers, as many as 15–22 areas of loss of heterozygosity can be detected, suggesting that a large number of tumor suppressor genes remain unidentified (4).

C/EBPs are members of the basic leucine zipper super family of transcription factors. The gene of C/EBPα is located on chromosome 19q13.1; it is intronless, and two isoforms are generated from trans-splicing of two in-frame AUG codons, An 42,000 and 30,000 protein. The C/EBPα protein consists of two transactivation domains and a leucine-rich bZip dimerization domain. C/EBPα was shown to play a major role in the terminal differentiation of myeloid cells, hepatocytes, and adipocytes (5, 6). C/EBPα also has prominent antiproliferative activity, the mechanism of which could involve up-regulation of p21 in hepatocytes and/or the interaction of C/EBPα with the retinoblastoma/E2F protein complex in adipocytes and granulocytic cells (7–9).

In previous studies, we have demonstrated that C/EBPα is critical for normal myeloid differentiation and regulates the expression of important myeloid genes, such as the G-CSF and interleukin-6 receptors (5, 10). In C/EBPα knockout mice, a block in myeloid differentiation is observed with accumulation of immature myeloid cells. These findings led us to search for abnormalities in this myeloid-specific differentiation pathway in acute myeloid leukemia, and we have identified specific abnormalities in C/EBPα (mutations, decreased expression, and abrogation of DNA binding) in subtypes of acute myelogenous leukemia (11, 12). In particular, C/EBPα expression is reduced in bone marrow cells of patients with M2 subtype of this leukemia who carry the t(8;21) translocation (13). In addition, ~25% of M2 patients with a normal karyotype have mutations in the coding region of C/EBPα, and most of these mutations seem to act as dominant-negative mutants suppressing the function of the normal protein (11). Furthermore, C/EBPα is an important target of the promyelocytic leukemia/retinoic acid receptor α fusion protein (12) in acute promyelocytic leukemia associated with t(15;17), and it appears to play a major role in the all-trans retinoic acid-induced differentiation of myeloid cell lines.

In concordance with a dominant antiproliferative role in hepatocytes, possibly through up-regulation of p21, expression of C/EBPα was found uniformly reduced in specimens of hepatocellular cancer (14). Reinstatement of expression led to impaired proliferation and tumorigenicity in cell lines (15, 16). Similarly, C/EBPα also emerged as a critical protein in adipocyte differentiation. Its expression, along with that of peroxisome proliferator-activated receptor gamma, regulates the differentiation of preadipocytes to adipocytes and also causes growth arrest (17). On the basis of this and others’ work, the concept emerged that C/EBPα acts as a differentiation switch in several cell types, where its expression is strictly regulated, leading to lineage commitment of tissue-specific stem cells and growth arrest, along with expression of genes characteristic of a terminally differentiated, metabolically active phenotype (18). The role of C/EBPα in epithelial tissues has not been carefully investigated, although it is expressed in a number of epithelia, including the respiratory epithelium, breast, colon, and prostate (19).

The role of C/EBPα in lung development and airway epithelial cell differentiation is poorly understood. At least three transcription factors, thyroid transcription factor-1 (TTF-1), hepatocyte nuclear factor 3 β (HNF3β), and C/EBPα, appear to play a significant role in this process, but their particular role, especially in specialized cell compartments, remains largely unknown (20). C/EBPα regulates the expression of several genes directly or indirectly during lung differentiation, including surfactant B and urokinase (21, 22). Because C/EBPα is strongly expressed in the lung and specific lung abnormalities, such as an abnormal proliferation of type II pneumocytes, were described in C/EBPα−/− knockout mice (23, 24), we hypothe-
esized that C/EBPα may play a significant role not just in airway epithelial cell differentiation but also in lung cancer development. Abnormalities of the transcriptional control pathways governed by C/EBPα could be involved in both the development of lung cancer, as well as in the maintenance of the undifferentiated, fully neoplastic phenotype.

We have analyzed the expression of C/EBPα in both established lung cancer cell lines, as well as primary lung cancer specimens, and found significant down-regulation of C/EBPα. We also show that reestablishment of C/EBPα expression in non-small cell lung cancer cell lines leads to dramatic growth reduction, proliferation arrest, differentiation, and, ultimately, apoptosis. Our results suggest that C/EBPα is a novel candidate tumor suppressor gene in lung cancer.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The lung cancer cell lines used in our study were kind gifts from Drs. Mary E. Sunday (Department of Pathology, Brigham and Women’s Hospital, Boston, MA) and Benjamin G. Neel (Department of Hematology/Oncology, Beth Israel Deaconess Medical Center, Boston, MA). The following cell lines were used in our study: squamous cell cancer: Calu-1, SK-MES-1, H157, H520, SW900, and U1752; adenocarcinoma: A427, SK-LU-1, Calu-3, H23, and H441; adenocarcinoma, bronchoalveolar type: H358, A549, and H322; adenosquamous cancer: H125, H292, and H596; large cell cancer: H460 and H661; small cell lung cancer: H526, H187, H69, H345, H211, H60, H82, N417, H128, and UMC19. All non-small cell lung cancer cell lines were grown in RPMI 1640 supplemented with 10% fetal bovine serum, whereas all small cell lung cancer cell lines were grown in RPMI 1640 supplemented by HITES medium [final concentration of 2.5% fetal bovine serum, 2.8 mM glutamine, 10^-8 M hydrocortisone, 10^-8 M β-estradiol, and 1% insulin/transferrin/Na-selenite (Sigma Chemical Co., St. Louis, MO)].

Patient Material. Patients were identified through our Thoracic Oncology Database, and paraffin-embedded tissue specimens were obtained from the Department of Pathology, Beth Israel Deaconess Medical Center. These studies were approved by the Institutional Review Board of Beth Israel Deaconess Medical Center.
Table 1 Subtype distribution of C/EBPα expression by immunohistochemistry

<table>
<thead>
<tr>
<th>Histological type</th>
<th>Absent or low expressiona</th>
<th>Normal expressionb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>Bronchoalveolar carcinoma</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Large cell cancer</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Squamous cell cancer</td>
<td>2</td>
<td>9</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Small cell cancer</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>All</td>
<td>23 (43%)</td>
<td>30 (57%)</td>
</tr>
</tbody>
</table>

a Absent or low expression: 0 or 1+ scoring.

b Normal expression: 2+ or 3+ scoring (normal expression in basal bronchial layer equaling 3+).

Immunohistochemistry. Immunohistochemical studies were performed on formalin-fixed, paraffin-embedded tissue specimens using citrate-microwave antigen retrieval. A dilution (1:500) of a polyclonal rabbit anti-C/EBPα antibody (200 μg/0.1 ml; Santa Cruz Biotechnology, Santa Cruz, CA) was used. Specificity of staining was confirmed by the concomitant use of a specific blocking peptide (1:100 dilution, 100 μg/0.5 ml; Santa Cruz Biotechnology). Immunohistochemistry was performed usingVectastain ABC kits (Vector Laboratories, Burlingame, CA). Positive staining was visualized by incubating the slides with diaminobenzidine. Scoring of specimens was performed by an experienced lung pathologist (O. K.) comparing tumor staining to the staining of basal bronchial cells (3+).

Generation of Stable Lines. Stable transfectants were isolated after Lipofectamine transfection (Lipofectamine PLUS; Invitrogen Life Technologies, Inc., Carlsbad, CA) according to the manufacturer’s instructions. Confluent cells (70%) were transfected with the previously described, linearized ppc18 and ppc22 plasmids (7 and 14 μg; Ref. 15). Clones were selected on the basis of the G418 resistance and isolated by either isolation of single colonies or limited dilution.

Western Blotting. Whole cell lysates were isolated using radioimmuno precipitation assay lysis buffer and protease inhibitors (aprotinin, phenylmethylsulfonyl fluoride, pepstatin, and leupeptin; Ref. 25), and 20 μg of protein were electrophoresed in 10 or 12% polyacrylamide minigels. A dilution (1:2000) of a polyclonal rabbit anti-C/EBPα antibody (Santa Cruz Biotechnology) was used. Detection was performed using enhanced chemiluminescence (Amersham Life Science, Piscataway, NJ).

Northern Blotting. Total cellular RNA from cell lines was isolated by the guanidinium thiocyanate extraction followed by cesium chloride gradient purification. RNA (20 μg) per lane was separated on 1% agarose/4-morpholinepropanesulfonic acid/formaldehyde gels and transferred to MagnaGraph membranes (Osmonics, Westborough, MA; Ref. 26). The 700-bp EcoRI-HindIII of the 3′-untranslated region of C/EBPα labeled with [32P]dCTP served as the probe for human C/EBPα (10). Quantitation on scanned images was performed using ImageQuant 3.3 software (Molecular Dynamics, Sunnyvale, CA).

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assays were performed using standard protocols as suggested by the manufacturer. In brief, 50–70% confluent cells were grown for 45 min in the presence of 10 μM BrdUrd (Sigma Chemical Co.), the cells were fixed in 70% ethanol, denatured in 2 M HCl, stained with 50 μl (0.5 μg of antibody) of anti-BrdUrd-FLUOS antibody (Boehringer-Mannheim, Indianapolis, IN) for 45 min, counterstained with 1 μg/μl propidium iodide (Sigma Chemical Co.), and then analyzed on a fluorescence-activated cell scan flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Annexin/Propidium Iodide Apoptosis Assay. Cells were collected after trypsinization, washed with PBS, and stained with annexin/propidium iodide according to the manufacturer’s recommendations (Roche Diagnostics, Mannheim, Germany). Samples were analyzed on a fluorescence-activated cell scan cytometer (Becton Dickinson).

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assays were performed as described previously (27). Oligonucleotides spanning the C/EBPα binding site of the G-CSF promoter (position −57 to −38, sense sequence: AAGGTTGTTGCAATCCCCAGC) were annealed and [32P]dATP-labeled. Nuclear extract (10 μg) per sample was used. For competition experiments, a 50-fold excess of unlabeled competitor oligonucleotide was added before the addition of the labeled oligonucleotide. For supershift assay, 1.5 μl of the C/EBPα supershift antibody (200 μg/0.1 ml; Santa Cruz Biotechnology) was added.

RESULTS

C/EBPα mRNA Expression Is Down-Regulated in the Majority of Lung Cancer Cell Lines. To establish the expression pattern of C/EBPα in lung cancer, we have isolated RNA from 30 established lung cancer cell lines and performed Northern blots with a fragment of the 3′-untranslated region of C/EBPα (representative blot shown in Fig. 1a). Expression was quantified based on relative expression to that in normal lung (Lane 1) normalized to 28S RNA. We found that the expression of C/EBPα is reduced (<50% of normal lung) or is completely absent in 24 of 30 (80%) lung cancer cell lines. Whereas the expression was uniformly low in all adenocarcinoma, squamous cell cancer, large cell cancer, and poorly differentiated cell lines examined (17 of 17 altogether), it was widely variable in the small cell and bronchoalveolar lung cancer cell lines examined (subtype distribution shown in Fig. 1b).

Electron Microscopy. Cells were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C. The cells were pelleted into 5% agarose and refixed in the above fixative for 2 h. This was followed by postfixation in 1% osmium tetroxide in 0.1 M cacodylate buffer (1 h, 4°C). The cells were subsequently dehydrated in ascending alcohols, cleared with propylene oxide, and infiltrated with a mixture of epon resin and propylene oxide overnight. They were next infiltrated with pure epon resin and polymerized at 60°C for 48 h. The hardened blocks were sectioned to 70-nm thickness on a Reichert-Ultratcut E ultra microtome. The sections were placed on nickel grids and stained for contrast with uranyl acetate and lead citrate. They were viewed and photographed on a JEOL 100CX electron microscope.
addition of 100 μM ZnSO4 (Fig. 3a). The most highly inducible clones showed ~3-fold (Calu1) to 10-fold (H358) inducibility over baseline. Interestingly, whereas a number of Calu1 clones showed leakiness of the promoter, i.e., baseline C/EBPα expression without induction similar to our experience with myeloid cells (10), none of the isolated H358 transfectants showed significant baseline expression. This suggests that H358 cells may have a selective growth disadvantage even at low levels of baseline C/EBPα expression.

Time course experiments showed that the addition of zinc to the medium led to the induction of C/EBPα expression as early as in 3 h. This expression was maintained for ≥72 h. We performed gel-shift experiments using the C/EBPα-binding site of the G-CSF promoter to show that the produced C/EBPα is functional and able to bind to a well-characterized C/EBPα binding site. No binding was observed in mock-transfected clones or in noninduced ppc22-transfected H358 cells, whereas very strong binding was observed in nuclear extracts obtained after 24-h zinc induction (Fig. 3b). This binding could be competed by excess cold oligonucleotide, and a supershift was observed in the presence of C/EBPα antibody confirming specificity.

Expression of C/EBPα Leads to Morphological Changes Suggestive of Differentiation. Induction of C/EBPα expression led to morphological changes suggestive of differentiation, including cell spreading, axonal outgrowths in Calu-1 (appearing around day 10), and cytoplasmic granule and vacuole formation in H358 cells (starting around day 3; Fig. 4a). Electron microscopy performed on transfected H358 cells induced with zinc for 5 days demonstrated marked changes, including a highly increased number of small dense granules, the appearance of numerous fibrolamellar bodies, and large lipid vacuoles (Fig. 4b). Fibrolamellar bodies are found in type II pne-

Immunohistochemical Analysis of C/EBPα Protein Expression in Primary Lung Cancer Samples. To further demonstrate down-regulation of C/EBPα in primary lung cancer cells, we have also performed an immunohistochemical analysis of C/EBPα protein expression on 53 paraffin-embedded tissue samples of lung cancers of all subtypes. In normal lung, strong staining was observed in the basal cell layer of bronchi and in type II pneumocytes. Twenty-three of the 35 tumor specimens (43%) showed either undetectable or low expression (0 or 1+) as compared with basal bronchial cells that served as internal controls in essentially all specimens (Fig. 2). Reduced expression was more frequent in adenocarcinoma and poorly differentiated lung cancer specimens than in squamous cell cancer specimens (subtype distribution is shown in Table 1). Specificity of staining was confirmed by the use of a blocking peptide. A subset analysis of the 25 adenocarcinoma specimens, where accurate staging information was available, showed that reduced expression was significantly more common in specimens obtained from patients with stage III/IV (6 of 9) as opposed to stage I disease (6 of 16, P = 0.02 by χ² analysis). Thus, our results show that C/EBPα expression is down-regulated in nearly half of primary lung cancers and that loss of expression appears to be associated with histological subtype, as well as tumor stage.

Generation of Cell Lines Inducibly Expressing C/EBPα. To analyze the effects of C/EBPα expression, we selected two C/EBPα nonexpressing lung cancer cell lines (as determined by Northern blotting), Calu-1 (squamous cell) and H358 (adenocarcinoma-bronchoalveolar type), for additional studies. We stably transfected these cell lines with a mammalian expression vector construct (ppc22) harboring the rat C/EBPα gene under the control of the zinc-inducible metallothionein promoter, as well as the control vector (ppc18; Ref. 15). Clones were selected on the basis of G418 resistance. Inducibility of C/EBPα expression was demonstrated by Western blots on whole cell lysates collected from cells cultured for 24 h with and without the addition of 100 μM ZnSO4 (Fig. 3a). The most highly inducible clones showed ~3-fold (Calu1) to 10-fold (H358) inducibility over baseline. Interestingly, whereas a number of Calu1 clones showed leakiness of the promoter, i.e., baseline C/EBPα expression without induction similar to our experience with myeloid cells (10), none of the isolated H358 transfectants showed significant baseline expression. This suggests that H358 cells may have a selective growth disadvantage even at low levels of baseline C/EBPα expression.

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Fig. 5. Induction of C/EBPα leads to proliferation arrest. a, growth curve of ppc22 or ppc18-transfected H358 cells, uninduced (Zn−) and zinc induced (Zn+). b, BrdUrd proliferation assay. Representative flow cytometry histograms are shown (day 5 of induction): ppc22-transfected H358 cells; A, noninduced; B, induced (inset E). Mock-transfected H358 cells; C, noninduced; D, induced (inset F).
mocytes; they contain phospholipid in a lipid bilayer form and get extruded from the cell through exocytosis to form part of surfactant (28). The presence of fibrolamellar bodies signifies a more mature and terminally differentiated type II pneumocyte (29). Of note is that the H358 cell line was derived from a patient with bronchoalveolar cell carcinoma, a form of lung adenocarcinoma thought to arise from type II pneumocytes. These changes strongly suggest that C/EBP expression on tumorigenicity of these cell lines. Even in mock-transfected (28). The presence of fibrolamellar bodies signifies a more mature and terminally differentiated type II pneumocyte (29). Of note is that the H358 cell line was derived from a patient with bronchoalveolar cell carcinoma, a form of lung adenocarcinoma thought to arise from type II pneumocytes. These changes strongly suggest that C/EBP expression on tumorigenicity of these cell lines. Even in mock-transfected cells, zinc itself caused an 80% reduction in colony-forming ability, ppc22-transfected H358 cells; c, non-induced; D, mock-transfected H358 cells; day 6 of zinc treatment (inset F).

Expression of C/EBP \( \alpha \) Causes Growth Arrest and Apoptosis. In both transfected cell lines, a very significant growth reduction was also observed after zinc induction (H358 cells; Fig. 5a). Zinc treatment itself caused a slight (25%) growth reduction in mock-transfected cells, but this did not reach the level observed in the inducible cell lines. We have performed colony forming assays in methylcellulose to determine the effect of the induction of C/EBP \( \alpha \) expression on tumorigenicity of these cell lines. Even in mock-transfected cells, zinc itself caused an \( \approx 80\% \) reduction in colony-forming ability, and whereas a tendency was seen for further reduction in ppc22-transfected cell lines (\( \approx 90\% \)), these results are hard to interpret because of the effect of zinc on even mock-transfected cells (data not shown). Therefore, we performed studies to further delineate the effect of C/EBP \( \alpha \) on H358 cells.

BrdUrd proliferation assays showed a dramatic reduction in the rate of actively proliferating, BrdUrd-positive H358 cells on induction. A decrease in the number of proliferating cells was already apparent by day 2 (data not shown), and an essentially complete block in BrdUrd incorporation was observed by day 6 (Fig. 5b). No significant effect on BrdUrd incorporation was seen in mock-transfected clones. Annexin/propidium iodide staining of H358 cells demonstrated a significant increase in the numbers of early apoptotic, as well as necrotic/late apoptotic, cells (Fig. 6). This increase first became apparent on day 4, suggesting that apoptotic changes were not primary events. A slight increase in apoptosis was observed in mock-transfected cells grown in the presence of zinc.

Withdrawal of Zinc Induction Leads to Slow Recovery of Proliferative Potential. To determine whether the proliferation arrest caused by C/EBP \( \alpha \) was reversible, ppc22-transfected H358 cells were grown in the presence or absence of 100 \( \mu M \) ZnSO4. In zinc-treated cells, the medium was changed to zinc free on days 1, 2, 3 and 4, respectively, or zinc treatment was continued for the duration of the experiment. Cells were collected on days 4, 7, 10, 15, and 20 (Fig. 7). All cells exposed to zinc ceased to proliferate for several days. Cells in which zinc was withdrawn on day 1 made their first division around day 7 and slowly resumed their normal rate of proliferation by about day 11. Cells in which zinc treatment was suspended on days 2 and 3 resumed proliferation more and more slowly. Most cells in which zinc treatment was withdrawn on day 4 died, but rare colonies started to emerge around days 20–25. Interestingly, all individual cells of these colonies maintained morphological features: increased granularity and vacuolation reminiscent of the induced cells for several cell divisions (\( \approx 6–10 \) days), suggesting that certain C/EBP \( \alpha \)-induced changes can be transmitted from one cell generation to the other even in the absence of continued expression of C/EBP \( \alpha \). Essentially, no viable cells remained in culture after day 15 of continuous zinc treatment.

To determine the time frame that is required for the cells to recover proliferative potential after loss of C/EBP \( \alpha \) expression, we determined C/EBP \( \alpha \) expression 24 and 48 h after the withdrawal of zinc induction. C/EBP \( \alpha \) protein level was reduced drastically 24 h after withdrawal of zinc induction.
Fig. 8. C/EBPα expression is lost rapidly after zinc withdrawal and can be reinduced. Western blot analysis of C/EBPα expression in ppc22-transfected H358 cells. Lane 1, 24 h of zinc induction; Lane 2, cells grown in zinc-free medium for 24 h after 24 h of zinc induction; Lane 3, 72 h of zinc induction; Lane 4, cells grown in zinc-free medium for 48 h after 24 h of zinc induction; Lane 5, retreating cells after 1 day of zinc treatment, day 20; Lane 6, same as Lane 5 retreated with zinc for 1 day.

drawal of zinc induction and was undetectable by 48 h (Fig. 8). This suggests that proliferation resumes ~4–5 days after loss of C/EBPα expression. C/EBPα expression can be reinduced in cells grown out after the withdrawal of zinc treatment (Fig. 8). This proves that their outgrowth is not caused by the emergence of clones that lost the inducible construct plasmid or by the outgrowth of already present cells that did not contain the inducible C/EBPα gene to begin with.

DISCUSSION

Our studies demonstrate the reduction or loss of C/EBPα expression in a large proportion of primary lung cancer samples, as well as in established lung cancer cell lines. The fact that its expression is lost more frequently in established lung cancer cell lines as opposed to primary lung cancer specimens is in line with our prior hypothesis that the expression of C/EBPα hinders the establishment of cell lines because of its antimitotic activity. Our finding that nearly half of all primary lung cancer specimens has lost or reduced expression of C/EBPα is particularly intriguing given the recent finding that 19q13.1, the chromosomal locus where its gene is located, is lost in 50% of non-small cell lung cancer specimens examined (4).

A subset analysis of 25 adenocarcinoma specimens also demonstrated that its expression is lost more frequently in stage III/IV versus stage I adenocarcinoma. This could imply that loss of C/EBPα expression might play a role in tumor progression as observed for certain other tumor suppressor genes, such as E-cadherin (30). Given the small sample size of our immunohistochemical study, this finding will need to be validated in a larger cohort. Similarly, the prognostic significance of the loss of expression will have to be the subject of further investigation.

We have also demonstrated that reestablishment of the expression of C/EBPα in non-small cell lung cancer cell lines leads to reduced growth, proliferation arrest, and increased apoptosis accompanied by morphological changes characteristic of the differentiation of airway epithelial cells. These results suggest that C/EBPα expression converts the cells from a fully malignant to a less proliferative and more differentiated, thus less malignant, phenotype. Therefore, we hypothesize that C/EBPα acts as a bona fide tumor suppressor in lung cancer. The predictable and dramatic proliferation arrest in our transfected cell lines should also provide an excellent model for the additional study of C/EBPα’s antimitotic role.

The mechanism of how the expression of C/EBPα is lost in lung cancers is not known. The chromosomal locus of the C/EBPα gene, 19q13.1, was recently found to be deleted in ~50% of non-small cell lung cancers, suggesting that this region could harbor a tumor suppressor gene (4). We believe that C/EBPα is a strong candidate for this role and have already initiated studies to perform a genetic analysis of the C/EBPα gene in lung cancer, in particular, non-small cell lung cancers. At present, we are in the process of obtaining DNA from laser microdissected tumor tissue, and we have no preliminary data to report as of yet. No studies to date have been published looking for mutations in the coding region of the C/EBPα gene in lung cancers. The promoter region of C/EBPα is rich in CpG islands and could be a target for epigenetic silencing by promoter hypermethylation as well (31).

In our immunohistochemical study, approximately half of the tumors had normal or close to normal C/EBPα expression. Another unanswered question is how these tumors that have maintained C/EBPα expression can possess a fully neoplastic phenotype. Several possibilities exist: (a) these tumors might harbor mutations that cause resistance to the effects of C/EBPα; and (b) it is possible that the expressed C/EBPα is nonfunctional, less functional, or dominant negative because of genomic mutations or post-translational modifications, rendering it less active.

The target genes of C/EBPα and its role in the respiratory epithelium are largely unknown. Both Clara cell secretory protein and surfactant B seem to be regulated by C/EBPα (32). We performed transcriptional profiling studies of the inducible H358 cell lines to identify critical target genes of C/EBPα in lung tissue. Among a number of other highly induced/repressed genes, we found that hepatocyte nuclear factor 3β is highly induced by 6 h after C/EBPα induction. HNF 3β is a member of the forkhead transcription factor family, and along with thyroid transcription factor-1, it is one of the transcriptional master regulators of the differentiated airway epithelial phenotype (20). The importance of this finding is further underlined by the fact that TTF-1 expression is transcriptionally controlled by HNF3β. If such regulation does indeed exist under physiological circumstances, C/EBPα would seem to play an absolute critical role in airway differentiation. We are pursuing additional studies to identify the genes critical for the antiproliferative function of C/EBPα. We believe that our transfected cell lines provide an ideal system for the study of C/EBPα target genes in the lung. The identification of these genes could provide us with a better understanding of airway epithelial differentiation and novel drug targets for the treatment of lung cancer and also help identify novel markers of airway epithelial lineages.

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