Initiators and Promoters of Lung Cancer

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As we expand our knowledge of the initiators and promoters of lung cancer, early detection and intervention strategies show great potential in individuals at high risk, especially smokers and exsmokers. Documented mutations of dominant oncogenes and tumor suppressor genes in human lung cancer cells may represent important steps in the pathogenesis of invasive cancer. The precise molecular events and their sequence that lead to tumor promotion in lung cancer, however, are less well understood. Chemointervention with agents like the retinoids may halt proliferation of cancer cells prior to the development of metastatic competence. Use of anti-growth-factor therapy and peptide hormone antagonists may also have a role in intervention approaches. This paper reviews present understanding of the initiation and promotion of lung cancer, as well as preventive strategies currently proposed for patients at risk.

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The causal link between cigarette smoking and lung cancer can no longer be debated. Tobacco is the dominant etiologic agent for at least 80 percent of lung cancer cases in the United States. As suggested recently by retired Surgeon General Dr. Jesse Steinfeld, all health care professionals must stay involved in concerted primary prevention efforts. However, for the over 80 million current or former smokers in this country who already have an increased risk of developing lung cancer, further efforts are warranted.

The recent publication of the M.D. Anderson group's success in using 13-cis retinoic acid in a population of definitively treated head and neck cancer patients to reduce the development of new primary cancers points to a new avenue for effective lung cancer control. Both head and neck cancer and lung cancer are caused by exposure to cigarette smoke. If their first cancer is controlled, head and neck cancer patients have a very high risk of developing a second primary cancer of upper aerodigestive tract origin.

In the M.D. Anderson randomized trial, none of the patients who received retinoic acid developed lung cancer, whereas four of the patients receiving placebo did develop lung cancers. Despite the focus on head and neck cancer in this trial, the outcome is very significant for lung cancer management.

In this paper, we will review our knowledge of the early stages of cancer growth in the direction suggested by the pioneering work with 13-cis retinoic acid and consider opportunities for the translation of basic scientific insights into possible tools for rational early lung cancer detection and intervention.

Molecular Mechanisms of Tumor Initiation and Promotion in Lung Cancer

Possible Tumor Initiation Events

Multiple genetic abnormalities have been documented in human lung cancer cells. Cytogenetic analysis of lung cancer cells has shown multiple nonrandom breaks involving chromosomes 1, 3, 7, 15, and 17, while studies of the expression and sequence of dominant and recessive proto-oncogenes have revealed amplification, mutation, and deletion of these genes. Such genetic changes occurring in genes that are involved in signal transduction and the regulation of gene expression and cellular proliferation may represent molecular examples of mechanisms central to tumor initiation events.

Dominant Oncogenes: Early molecular biologic experiments demonstrated that certain cellular genes, when mutated, can induce cellular transformation. Such mutated genes, now called dominant oncogenes, are activated forms of cellular genes, called proto-oncogenes, which normally control cellular proliferation. As recently discussed, these proto-oncogenes encode proteins that function as growth factors and growth-factor receptors, signal-transducing proteins, and nuclear proteins involved in gene expression. Mutation or amplification of these genes has been documented in many human cancer cells, including lung cancer cells, suggesting that activation of dominant oncogenes is one important step in the complex pathway that ultimately leads to invasive cancer.

One such family of oncogenes mutated in human lung cancer cells is the ras family. This family includes the N-ras, K-ras, and H-ras proto-oncogenes, which encode the membrane-associated guanosine triphosphate (GTP)-binding proteins believed to be involved in signal transduction. Many human cancers have been found to have "activating" point mutations within these ras proto-oncogenes, typically involving codons 12, 13, or 61. These mutations induce structural changes within the ras protein to produce an activated protein that constitutively binds GTP. Analysis of the deoxyribonucleic acid (DNA) sequence of these ras proto-oncogenes in human lung cancer cells has identified activating mutations, typically involving K-ras, in 15 to 30 percent of human non-small-cell lung cancer (NSCLC) cells. These ras mutations are seen most commonly in adenocarcinomas and rarely in NSCLC of other histologies. Recent clinical studies indicate that such mutations may have prognostic significance. Slebos et al. found a highly significant decrease in both disease-free survival and overall survival in patients with early stage adenocarcinoma whose...
tumors had K-ras mutations. In addition, Mitsudomi et al. found that patients with both early and late stage NSCLC whose tumors had K-ras, N-ras, or H-ras mutations had decreased survival compared to patients whose tumors had no ras mutations.

The NSCLC cells have aberrant expression of another family of dominant proto-oncopenes, the erb B family, whose members include erb B1 and erb B2 (also called Her-2/neu). Both of these proto-oncogenes encode membrane-associated tyrosine kinases that likely function as growth-factor receptors. The erb B1 protein has been identified as the receptor for epidermal growth factor (EGF), while the erb B2 protein is believed to be a receptor for an as-of-yet unidentified growth factor. These proto-oncogenes are frequently abnormally expressed in NSCLC cells. The erb B1, or EGF receptor proto-oncogene is amplified in up to 20 percent of squamous cell tumors, while the erb B1 protein is overexpressed in all histologies of NSCLC (90 percent of squamous cell tumors, 20 to 75 percent of adenocarcinomas, and rarely in large cell or undifferentiated tumors). Moreover, erb B2 proto-oncogene amplification and protein overexpression are also seen in lung cancer cells, predominantly in adenocarcinoma; however, this occurs much less frequently than does overexpression of erb B1.

Another family of dominant oncogenes, the myc family, has been found to be abnormally expressed in lung cancer cells. This family includes the c-myc, L-myc, and N-myc proto-oncogenes, which encode closely related nuclear proteins believed to be involved in regulating gene expression and cellular growth. Of note, L-myc was initially identified as an amplified proto-oncogene in a human small-cell lung cancer (SCLC) cell line. Unlike ras and erb B, myc proto-oncogenes are frequently aberrantly expressed in SCLC cells and only rarely in NSCLC cells. The myc proto-oncogenes are activated by overexpression of cellular proto-oncogenes like erb B, either by amplification or deregulated transcription, which leads to overexpression of the myc proteins. Amplification of myc has been found to involve c-myc, N-myc, and L-myc; however, only one myc family member is amplified in any given tumor. In clinical studies, myc amplification correlates with more aggressive tumors and decreased patient survival, which is consistent with the in vitro finding that SCLC cells demonstrating deregulated myc expression grow more rapidly than do SCLC cells with no deregulation of myc expression.

Other dominant proto-oncogenes expressed in lung tumors include members of the ras family (c-ras-1) and jun family (c-jun), and of the src-related tyrosine kinase oncogenes, src and lck. However, these proto-oncogenes are also expressed in normal lung tissue, and therefore their role in the pathogenesis of lung cancer is unclear.

Recessive Oncogenes: In addition to finding abnormalities in the sequence and expression of dominant oncogenes, molecular biologic studies have demonstrated that the loss or inactivation of certain genes may also be important in the pathogenesis of human cancers. Such genes have been called tumor suppressor genes, antioncogenes, or recessive oncogenes, as they may function to suppress cellular proliferation and as both alleles are mutated or deleted in transformed cells. The first such tumor suppressor gene described was the retinoblastoma (Rb) gene, whose existence was originally predicted by Knudson after genetic studies demonstrated that inactivation of both alleles of a gene led to the development of childhood retinoblastoma. The Rb gene was subsequently cloned, mapped to the long arm of human chromosome 13, and shown to be homozygously lost or mutated in most Rb cells. The Rb gene product is a 110-kd phosphoprotein that undergoes cell-cycle-dependent phosphorylation and is thought to be involved in cell-cycle regulation. The Rb gene has been found to be mutated or deleted in many other human tumors, including osteosarcoma, bladder cancer, and breast carcinoma, and SCLC. Study of Rb expression in lung cancer cells showed that 13 percent of SCLC primary tumors and 18 percent of SCLC cell lines have Rb gene mutations or deletions, while greater than 95 percent of SCLC and 20 percent of NSCLC cells have abnormalities leading to defective Rb protein expression.

Another tumor suppressor gene, p53, is often mutated in human lung cancer cells. In cellular transformation models, the wild-type p53 gene suppresses transformation, while a mutated p53 gene fails to prevent transformation. The p53 protein is known to bind to viral DNA-binding oncoproteins (such as SV40 large T, adenovirus E1B, and papillomavirus E6), and recent studies now suggest that the p53 protein may function by regulating DNA transcription. Loss or mutation of the p53 protein may lead to abnormal gene expression and ultimately deregulated cell growth. The recent discoveries of p53 mutations in colon, breast, brain, and lung cancer cells support this hypothesis and suggest that the wild-type p53 protein may function in many different cells to suppress transformation.

The discovery that the short arm of chromosome 17 (where p53 is located) is frequently deleted in lung cancer cells stimulated the study of p53 gene expression in both NSCLC and SCLC cells. Chiba et al. found p53 point mutations in 42 percent of NSCLC tumors, while D’Amico et al. identified p53 point mutations in 100 percent of SCLC cell lines. Most mutant p53 proteins have a prolonged half-life and can be detected histochemically, while wild-type p53 is undetectable using this technique. These findings support the use of histochemical staining as a screening test for p53 mutations. In one such histopathologic study, 55 percent of resected lung cancers were found to have detectable p53. Direct sequencing of the p53 gene from three of the tumors with detectable p53 confirmed the presence of a p53 point mutation, while sequencing of DNA from a tumor that failed to stain revealed wild-type p53. These results demonstrate that p53 mutations are common in both SCLC and NSCLC.

Another common cytogenetic abnormality in lung cancer cells is the loss or deletion of the short arm of chromosome 3 (3p). Restriction fragment-length polymorphism analysis has revealed that one of the alleles of chromosome 3p is lost in 90 to 100 percent of SCLC cells and 25 to 50 percent of NSCLC cells. Such a deletion, coupled with a mutation within a putative tumor suppressor gene located on chromosome 3p, could be another important step in the development of lung cancer. Possible candidate tumor suppressor genes located on chromosome 3p include the retinoic acid receptor-γ gene, which encodes a DNA-binding protein...
involved in the regulation of gene transcription, and the phosphotyrosine phosphatase gene, which encodes a protein that may function to balance the action of tyrosine kinases, some of which are known dominant oncoproteins, to control cell growth.

As the above studies indicate, deletion or mutation of recessive oncoproteins occurs commonly in both SCLC and NSCLC. The RB gene abnormalities, p53 mutations, and loss of chromosome 3p may each be important steps in the transformation of bronchial epithelial cells into carcinoma. Like mutations of dominant oncoproteins, these irreversible genetic changes in tumor suppressor genes may represent initiation events in this transformation pathway. The substances and events that lead to tumor promotion in lung cancer, however, are less well characterized.

Tumor Promoters

Early cancer cells are potentially responsive to the regulatory effects of retinoids, which induce cancer cells to stop proliferating. In the M.D. Anderson trial, in contrast to the rate of new primary cancers, the rate of head and neck primary cancer relapses was not affected by the administration of retinoic acid, which is consistent with the notion that advanced cancer cells lose responsiveness to retinoids. The concept of chemointervention is to use agents that can control early cancer cells prior to the development of metastatic competence. The period after the initiation of a cancer but prior to the development of invasive cancer is dominated by the processes of tumor promotion. The duration of the tumor promotion phase in the development of a cancer may be many years.

Substances like phorbol esters, steroids, asbestos, and chemicals within tobacco smoke may function as tumor promoters of lung cancer by inducing chronic proliferation of bronchial epithelial cells. The molecular pathways through which such substances promote the transformation of bronchial epithelial cells are poorly understood; however, recent studies suggest that these substances may stimulate production of growth factors, producing continued cellular proliferation and ultimately leading to transformation. Important growth factors in the proliferation of lung cancer cells include epidermal growth factor, gastrin-releasing peptide, and insulin-like growth factors. We and others have proposed that promotion factors comprise a new class of attractive targets for lung cancer intervention.

Success in the development of effective new interventions such as anti-growth-factor agents will depend on the evolution of clinical trial strategies to enable rapid and efficient evaluation of new agents. Clinical intervention approaches will involve the participation of individuals whose respiratory epithelium is potentially undergoing tumor promotion. As previously discussed, tumor promotion occurs during a preclinical phase of cancer, and at present, no diagnostic tests exist to definitely establish which individuals may have early lung cancer. Chest x-ray, the best current diagnostic tool for lung cancer, is first found to be positive only after metastatic dissemination has occurred in at least two thirds of all lung cancer patients.

Initially, clinical prevention trials will require the careful characterization of high-risk populations to permit clinical evaluations of new intervention agents. The tamoxifen intervention trial for breast cancer, which employs a statistical model to identify women with increased risk, exemplifies this type of strategy. The M.D. Anderson trial employing 13-cis retinoic acid as an intervention agent in definitively treated head and neck cancer patients is another example, since these patients were at high risk for new upper aerodigestive tract cancers. An ongoing evolution in clinical trial methodology at many levels will be required to permit rapid evaluation of the large number of new possible intervention approaches.

Lung Cancer Autocrine Growth Factors as a Target for an Intervention Approach

Over the last decade, considerable work has been published on the role of growth factors in lung cancer, which we have recently summarized. Our group at the National Cancer Institute focused on the role of gastrin-releasing peptide (GRP), which can stimulate the growth of both normal and malignant lung cells. Increases in GRP expression coincide with key periods of fetal lung growth and differentiation. High levels of GRP-like activity have been reported in the bronchial lavages of smokers compared with nonsmokers. Taking all the information about the effects of GRP collectively, we postulate that it may function as a tumor promoter for early lung cancer. As we have reported, use of a monoclonal antibody to neutralize GRPs growth stimulatory effect in patients with advanced small cell lung cancer has been well tolerated.

The possibility of blocking GRP as an intervention approach has theoretic appeal. To facilitate such an effort, we have developed a mathematic model to calculate the required dose of anti-growth-factor therapy to neutralize GRPs effects in a specific patient. The concentration of tumor-produced GRP and GRP-receptor density in tumor cells, as well as other relevant kinetic parameters, can be established for a particular patient. Using the model, one can calculate the concentration of anti-GRP monoclonal antibody or other types of GRP antagonists required to block the GRP effect on tumor cell proliferation. Intervention agents such as GRP antagonists will be evaluated in high-risk subjects who are not necessarily patients with overt cancer; this circumstance imposes the need to minimize exposure to potentially toxic intervention agents. Minimal effective doses also can be calculated in a parallel fashion to customize administration of antagonists that block other known growth-factor/receptor interactions.

The discussion of GRP as a target for intervention application is an exercise in developing new strategies in lung cancer control. Instead of relying on the empiricism that has dominated cancer therapy research to date, perhaps our knowledge of cancer biology can provide clues for developing rational new approaches to lung cancer management. Neutralizing GRP-mediated tumor promotion is one such approach to early cancer intervention, but other biologically rational strategies exist as well.

Processing Enzymes as Promotion Factor Targets for Intervention Strategies

Central to the biology of many cancer cells is the endogenous production of peptide hormones, to which the cells are simultaneously capable of responding. Autocrine
growth stimulation in neoplastic cells was first proposed by Sporn and Todaro, and several peptide factors that participate in autocrine loops have been identified. As with GRP, many of these factors have important roles in normal cell growth, signaling, development, and tissue renewal. Attempts have been made to control the growth of cancer cells by preventing interaction between a growth factor and its specific receptor, using antibodies to specific growth factors or receptors, and using peptide hormone antagonists. These approaches are aimed at the peptide/receptor interaction (Fig 1A). The study described above using an anti-GRP monoclonal antibody exemplifies this type of approach. However, since lung cancer cells can produce multiple autocrine growth factors, the utility of this approach as a general anti-promotion tool may be limited. We are exploring additional strategies for neutralizing autocrine growth-factor effects in cancer through the study of common mechanisms of peptide hormone synthesis. These biosynthetic pathways may provide potential sites for simultaneous interruption of multiple autocrine pathways (Fig 1B).

**Biology of Posttranslational Prohormone Processing**

This approach requires identification of common steps in the maturation of a variety of biologically active proteins and peptides. The pro-opiomelanocortin/adrenocorticotrophic prohormone typifies the generation of numerous bioactive molecules from a differentially processed precursor via controlled endoproteolytic mechanisms. Other posttranslational modifications that are common to the production of multiple bioactive peptide hormones include acetylation, sulfation, and α-amidation. For instance, GRP has an absolute requirement for a C-terminal α-amidated methionine for bioactivity. Amidation is one of the best-studied posttranslational modifications of peptide hormones. It is often the final step in the series of processing reactions leading to expression of full bioactivity.

The steps leading to formation of any C-terminal amino acid, such as the methionine amide of GRP, devolve from a sequence found in the prohormone that is representative of the motif for peptide amidation. In the GRP prohormone this sequence is as follows: —MetGlyLysLys—. The only invariant amino acid in the α-amidation process is the glycine. A review of amidated peptides and their precursors can be found in Cutitta et al. Posttranslational synthesis of α-amidated peptides generally requires three enzymatic activities that are present in neurosecretory granules: endoproteolysis C-terminal to basic residues, exo- (carboxy) peptidolysis to remove the resultant basic amino acids, and glycine-directed peptidyld α-amidating activity. The amide group of the peptide hormone is formed by oxidative cleavage of the glycine-extended prohormone by an overall reaction (peptidyl α-amidating monoxygenase [PAM]) catalyzed by two sequential enzymes: peptidyl-glycine α-hydroxylating monoxygenase (PHM) and peptidyl-α-hydroxyglycine α-amidating lyase (PGL). As α-amidation is usually essential for the bioactivity of amidated peptides, all these enzymes must be present and functional in the same compartment as the prohormone for efficient synthesis of bioactive amidated peptide hormones from their prohormones.

We have identified and partially characterized PAM enzymes in extracts of human lung cancer cell lines. Amidation by lung cancer cell PAM enzymes is a two-step process, and both enzymes are identifiable in cell extracts. Both enzymatic activities display similar cofactor and substrate specificities to the analogous rat and bovine enzymes. The PAM activities are present in extracts from cell lines such as small-cell and carcinoid lines and in a subset of NSCLC lines. The activities are highest in cell lines displaying other biochemical features of neuroendocrine differentiation. Serum-free medium conditioned by these cell lines also contains active amidating enzymes. Biochemical and molecular biologic studies are under way to confirm the presence of endoprotease and carboxypeptidase processing enzymes in these cell lines.

**Intervention Strategies Involving Posttranslational Processing Enzymes**

It is evident that the production of an amidated peptide, a seemingly simple chemical peptide modification, is dependent on the correct subcellular routing and activity of both the prohormone and the processing enzymes. The essential involvement of these enzymes in the production of growth-stimulatory peptides, which may be involved in cancer promotion, has important implications for relevant intervention strategies. Inhibition of these processing enzymes would be expected to result in the secretion of inactive, nonprocessed precursor prohormones instead of active peptide hormones, thus interrupting autocrine or paracrine growth stimulation. As far as targeting these processing enzymes in lung cancer is concerned, inhibition could affect a patient's normal neuroendocrine processes if treatment is given systemically. As we have previously suggested, treatment therefore might be better tolerated if applied clinically in an anti-promotion mode while the proliferating cells are still confined to the bronchial epithelium, instead of being applied in a setting where tumor has metastasized through the bronchial wall and systemic therapy is required.

The biochemical requirements of posttranslational processing enzymes suggest several avenues for inhibition of these enzymes. The PAM enzymes, as well as the endoprotease and carboxypeptidase enzymes, are dependent on
divalent cations. The PHM, for instance, is a copper (Cu++)-specific monooxygenase. Treatment of cells both in vitro and in vivo with specific copper chelators (disulfiram and diethylthiocarbamate) has been shown to reduce the amidation of an amidated peptide (murine joining peptide). The authors linked this effect to inhibition of the PHM step in glycine-directed peptidyl α-amidation. Specific substrate analogues also could be used to inhibit posttranslational processing enzymes. Peptide analogues of the substrates of the processing enzymes inhibit by simple competitive mechanisms, but covalent irreversible inhibitors may be of more value in a clinical setting. We have recently discussed the potential clinical utility of potent inhibitors of each posttranslational processing enzyme involved with amidation. Detailed studies with pure enzymes may facilitate development of new inhibitors for each of the posttranslational processing enzymes.

Recent reports on the endoprotease enzymes suggest the existence of a family of enzymes that cleave prohormones in the regulated secretory pathway. If expressed in a cell-specific manner, these enzymes may be able to specifically inhibit prohormone processing while sparing cleavage of constitutive precursors, or alternatively inhibit processing in tumor cells only. This "fine-tuning" of such an approach would only be possible if selective inhibitors could be designed for different members of the families of processing enzymes but might be especially useful in developing candidate intervention agents.

**FUTURE DIRECTIONS**

Our growing understanding of the biology of the early stages of cancer growth has identified a number of potential tools that may prove useful in the detection and intervention of early lung cancer. The potential benefits in detecting early lung cancer and intervening with novel biochemical measures provide an exciting challenge for prevention research. Current and former smokers comprise the most clearly defined group at risk for lung cancer and are an appropriate group for study of early detection and intervention strategies.

Refinement of clinical trial intervention strategies will be required to address specific study conditions. Subjects with elevated baseline risk, for example, may be appropriate candidates for screening with invasive procedures such as bronchoscopy to obtain specimens. If the intervention used following detection of a positive screen is associated with significant morbidity (eg, thoracotomy), then validation of the screening test will need to be more rigorous. However, if less invasive interventions are warranted (eg, administering a moderate-dose retinoid for one year), then predictive requirements for the screening test can be relaxed. The interdependence of early detection research with intervention research is evident, and many context-specific issues will arise as a prevention-oriented lung cancer management model evolves.

Identification of epithelial cells with amplifications, mutations, or deletions of genes involved in signal transduction, regulation of gene expression, or cellular proliferation may permit clinically useful detection of early cancer. Manipulation of the early stage of carcinogenesis to decrease the number of initiated cells or to prevent promoters from acting on these cells may be feasible as fundamental mechanisms of cancer cell biology are elucidated.

Interfering with tumor promotion, the preclinical phase of carcinogenesis prior to establishment of metastatic disease, appears especially promising. In lung cancer, tumor promotion consists of proliferation of the transformed cell on the bronchial epithelial surface. Growth stimulation, coupled with sustained exposure to carcinogen, may be required for the accumulation of sufficient genetic changes to enable neoplastic progression to invasive disease. Multiple agents, both exogenous (eg, phorbol esters, asbestos, and chemicals in tobacco smoke) and endogenous (eg, growth factors), induce cellular proliferation. The attractive feature of growth-factor inhibition lies in the potential to selectively neutralize particular growth-factor effects in cells undergoing transformation.

Intervention approaches may also exploit rational delivery schemes to enhance therapeutic effect. If endobronchially administered anti-GRP agents can block growth stimulation by GRP in early lung cancer cells, this could arrest tumor cell proliferation on the bronchial mucosa without interrupting other normal physiologic processes requiring CRP.

The identification of markers for evaluation as indicators of early lung cancer and for targeting with intervention strategies is a critical part of prevention research. Determining the optimum number and sequence of markers to use in such studies will further add to the understanding of carcinogenesis.

Other applications of markers of early cancer, such as for use as intermediate end points in clinical trials, may develop as an important use of biomarkers. Use of an intermediate end point assumes that the relationship between the marker and the eventual development of cancer is known. Whether the proposed markers of initiation or promotion detected on bronchial epithelial cells can serve as intermediate end point markers or as targets for lung cancer intervention studies requires prospective evaluation of a large number of patients. These markers will be validated with pathologically documented lung cancer as the gold standard. However, if the resultant intervention does not produce significant toxicity or morbidity, then the marker may not require the same precision for validation. A goal for intervention research is to develop clinical strategies to arrest cancer promotion in relevant high-risk populations using compounds with very low toxicity profiles.

A paradigm shift is critical to the application of novel biomarkers and intervention strategies in the clinical setting. If prevention-oriented cancer management is to have success in reducing lung-cancer-related mortality within the next two decades, progress must be made in devising both useful early detection and intervention approaches. To proceed expeditiously, prudence must be exercised to strictly minimize unnecessary morbidity and potential mortality of procedures performed to evaluate false-positive marker studies. Prospects are excellent that continuing research into the basic science of markers of carcinogenesis will provide the foundation for the development of prevention-oriented approaches to lung cancer management. The next decade holds enormous promise for significant progress in this very young field.
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