Correlative Assessment of Morphologic, Immuno-phenotypic, and Genetic Changes in Bronchial Epithelium of Tobacco Smokers*

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Detection of pulmonary malignancy often occurs late in the course of the disease. As a result, prognosis for lung cancer is poor. One reason for late detection is the inaccessibility of the bronchi for biopsy of tissue to study morphology, biologic, and genetic properties of bronchial epithelium. A widely accepted paradigm for neoplastic development in the lungs holds that pulmonary carcinogenesis is the result of stepwise accretion of mutational events in bronchial epithelial progenitor cells and that widespread early genetic changes may occur in the tracheobronchial tree (“field effect”) before the onset of overt invasive carcinoma. If the important early mutational events in pulmonary carcinogenesis could be found, it should be possible to better identify patients at increased risk for lung cancer by genetic analysis of bronchial epithelium at a time when intervention may reduce the risk and lessen the mortality of this tumor.

We have attempted to identify early bronchial lesions by sputum cytology screening of patients with a smoking history of more than 40 pack years and COPD. Patients with moderate or worse dysplasia then undergo laser-induced fluorescence emission (LIFE) bronchoscopy (Xillix, Inc.) and biopsy. Biopsies are studied by conventional histologic techniques and by immunohistochemistry for the expression of Ki-67 and p53. Dysplastic cells are microdissected from biopsied sections and analyzed by polymerase chain reaction (PCR)-based techniques for loss of heterozygosity in chromosome 3p using a large number of primer sets for microsatellite repeats and for mutation of the p53 gene, exons 5-9, by single-strand conformation polymorphism using nested primers. We have studied 25 patients with bronchial epithelial dysplasia of varying severity by these methods. We have observed frequent increases in the vascularity of metaplastic and dysplastic submucosa, sometimes with papillary ampullary-vacuole epithelial projections into the bronchial lumina. Increased expression of Ki-67 was observed in all dysplasias; p53 expression was found in patients with severe dysplasia. Loss of heterozygosity (LOH) at 3p was documented in a narrow region at 3p13-14 in 4 of 6 cases in which adequate material was available for microdissection. p53 mutation was not observed in any of the 6 microdissected biopsies. We conclude that bronchial squamous dysplasia is a frequent finding in patients with a long smoking history, that this histologic change is accompanied by LOH at 3p and increased cell turnover (as reflected by increased Ki-67 expression), and that these latter changes frequently occur before over-expression or mutation of p53 is detectable.

Chrysotile Asbestos Induces PDGF-A Chain-Dependent Proliferation in Human and Rat Lung Fibroblasts In Vitro*

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Inhalation of asbestos in a rat model results in the predominant deposition of fibers onto alveolar duct bifurcations, where a fibroblastic lesion develops. The lesion that remains 1 month following a single exposure is primarily composed of increased numbers and volume of mesenchymal cells and macrophages. Mesenchymal cell number and volume is increased by 25% and 40%, respectively. We now know that lesions persist for at least 6 months following a series of 3-h exposures on 3 consecutive days. The mechanisms driving lung mesenchymal cell proliferation in response to inhaled asbestos fibers is under investigation in our laboratory.

Although investigators have proposed that polypeptide mediators secreted by stimulated alveolar macrophages, such as platelet-derived growth factor (PDGF), are responsible for the mesenchymal cell proliferation following asbestos inhalation, this hypothesis has not yet been proved. It also is unclear why nonfibrogenic particles, which stimulate macrophage growth factor release in vitro, do not result in fibrosis following in vivo exposure. We have observed that fibrogenic asbestos fibers are translocated into the intersti-
tial space, whereas nonfibrogenic carbonyl iron spheres are not. Because asbestos fibers come into direct contact with interstitial lung fibroblasts in vitro, we have exposed primary isoles of both rat and human lung fibroblasts to chrysotile asbestos in vitro to determine whether asbestos fibers can induce a mitogenic effect on lung fibroblasts in the absence of other cell types and exogenous factors.

Our results, using a cell proliferation assay and bromodeoxyuridine incorporation, show that there is a dose-dependent mitogenic effect of sterilized asbestos fibers on quiescent rat lung fibroblasts (RLFs) in vitro. Of note, most of this asbestos-induced RLF growth can be specifically blocked with an anti-PDGF antibody. Furthermore, we have found that PDGF alpha receptor messenger RNA (mRNA) and concomitant surface receptor expression as well as secretion of PDGF-A chain peptide all are upregulated following asbestos exposure in vitro. We also found expression of PDGF-A chain, but not B chain, mRNA on Northern blot analysis of total RNA from RLFs. Thus, chrysotile asbestos upregulates PDGF-A chain peptide expression and the PDGF-alpha receptor in RLFs in vitro. All of these proliferative events are apparently mediated by an autocrine mechanism, where the asbestos fibers induce production of PDGF-A chain along with increases in the alpha receptors that bind the A chain ligand.

It is not known whether PDGF-A chain-dependent autocrine growth is active in vivo in asbestos-induced lung fibrosis. Also, it is not known whether human lung fibroblasts (HLFs) proliferate as do RLFs in response to direct perturbation with asbestos fibers. This latter question must be addressed in view of some controversy in the literature, ie, there are reports suggesting that HLF cell lines do not make PDGF mRNA, whereas other investigators have found that primary HLF isolates do indeed make PDGF-A chain mRNA. Apparently, the mitogenic effect of thrombin on HLFs is mediated through a PDGF-A chain-dependent mechanism. The difference in findings may be explained by the use of cell lines in the first study and primary lung cell isolates in the latter.

Materials and Methods

In order to address whether HLFs proliferate in response to asbestos fibers in vitro, we isolated primary human lung fibroblasts from normal lung tissue. Briefly, primary human lung fibroblasts were obtained through mechanical and enzymatic dissociation of normal human lung tissue resected during lobectomy or pneumonectomy for lung cancer, using a modification of the method we use in obtaining RLF isolates. A portion of the resected lobe distant from a cancerous lung nodule was used. Then a bronchus was cannulated and lavaged repeatedly with ice-cold phosphate-buffered saline solution to remove bronchiol and airspace inflammatory cells. The matching pulmonary artery was cannulated and perfused with phosphate-buffered saline solution/0.02% ethylenediaminetetraacetic acid until the pulmonary vein effluent ran clear. Parenchyma from the blanched peripheral lung tissue (within 1 cm of the pleura to avoid large vessels and bronchi) was resected and diced into 1-mm pieces, placed in Dulbecco's modified eagle's media (DMEM) with 0.05% trypsin, 0.008% collagenase, and 0.005% DNAase and then stirred for 30 min at 37°C before filtering through 100-μm nylon mesh. Filtered cells were washed once in DMEM with 10% fetal bovine serum (FBS), amphotericin B (Fungizone), penicillin (100 U/mL), and streptomycin (25 mg/mL). Cells were seeded at 10⁶ cells per 175-cm² flask and washed twice to remove nonadherent cells after 24 h of incubation at 37°C in 95% air, 5% CO₂. When cultures became confluent, they were passed for immunostaining, and subcultured or cryopreserved in DMEM with 10% dimethyl sulfoxide for future use.

Results

Cell characterization was conducted on HLF isolations from two donors to document that we isolated only fibroblasts before any experiments were performed with the freshly isolated cells. Cells were passed into chamber slides and cultured in DMEM with 10% FBS for 48 h. Then cells were fixed for 15 min with 4% formalin and endogenous peroxidase was quenched with hydrogen peroxide. The slides were treated with the following primary antibodies: monoclonal antihuman alpha-smooth muscle actin, monoclonal antihuman desmin, monoclonal antifactor VIII, monoclonal antivimentin, and monoclonal antihuman neurofilament protein (all Dakopatt). The secondary antibody was biotinylated anti-mouse (Dakopatt). Slides were developed using a streptavidin reagent and diaminobenzidine. Sections from human uterus were used as a positive control for the vimentin, desmin, and smooth muscle actin antibodies. The positive control for antifactor VIII was human placenta and the positive control for antineurofilament protein was human neural tissue. Our human lung isolates stained positively for vimentin, but did not stain for desmin, factor VIII, or neurofilament protein. Approximately 10% of the cells stained weakly for alpha-smooth muscle actin, so we found that our isolates were a mixture of 90% HLFs and 10% myofibroblasts on their second passage following cryopreservation.

Northern blot analysis for PDGF-A chain was conducted on total RNA from confluent second-passage HLFs, grown in DMEM with 0.4% FBS. Total RNA was extracted, separated, and hybridized as previously described. A human PDGF-A chain complementary DNA (from Dr. C. Betscholtz) was used as a template for random priming. The PDGF-A chain probe hybridized to the HLF RNA at the three expected sizes of 2.8, 2.4, and 1.7 kb (not shown). Our Northern blot analysis results confirm that primary HLFs make PDGF-A chain mRNA in vitro.

Moreover, using a colorimetric cell proliferation assay (Promega), we were able to determine that HLFs proliferate in response to human recombinant PDGF-AA (UBI). Since PDGF-AA binds only to the PDGF-alpha receptor, we can conclude that the HLFs express the PDGF-alpha receptor. Therefore, we have found that both components, ligand and receptor, of PDGF-A chain-dependent autocrine proliferation are expressed by HLFs in vitro.

To determine whether HLFs proliferate in response to asbestos in vitro, RLFs and HLFs from two donors, passage 2 through 6, were plated at a density of 3,000 cells per well into 96-well plates overnight in DMEM with 10% FBS. Cells were washed twice in DMEM with 0.4% FBS and rendered quiescent for 3 days in DMEM with 0.4% FBS. Then cells were exposed to varying concentrations (0.05 to 5 µg/cm²) of sheared and sonicated sterile chrysotile asbestos fibers in DMEM with 0.4% FBS for 3 days before quantification using a colorimetric proliferation kit (Promega). Cell-free asbestos fibers in DMEM did not affect the development of the cell proliferation assay. Up to six duplicate samples with
five replicates per sample did not vary by more than 10%. The assay was linear in the range of 3,000 to 6,000 cells per well. Absorbance was converted into percent of control by plotting 3,000 and 6,000 cells per well into control wells containing DMEM with 0.4% FBS in each plate. The difference in the absorbance values between the 3,000 and 6,000 cell wells was considered to be 100%. Figure 1 shows that RLFs exhibit an increase in cell number at similar asbestos doses, and of similar magnitude, to those that we previously published using an automated cell counter. Quiescent HLFs also proliferated in response to chrysotile asbestos. Asbestos was cytotoxic to HLFs at doses above 10 μg/cm² (not shown).

**DISCUSSION**

To further understand this mechanism at the molecular level, we now have shown, in a preliminary set of experiments, that using chimeric PDGF-A chain antisense oligomers, we were able to block the asbestos-induced HLF proliferative response in vitro. The sense-strand oligomer controls did not inhibit cell growth. In addition, the mitogenic response of HLFs to interleukin-1 alpha, but not PDGF-BB, was blocked by the PDGF-A chain antisense oligomers. This is consistent with the literature since interleukin 1 is reported to act indirectly as a mitogen on human dermal fibroblasts via upregulation of PDGF-A chain and its matching alpha receptor, whereas PDGF-BB is a mitogen that does not require involvement of PDGF-A chain.

In summary, our data indicate that asbestos is mitogenic for both RLFs and HLFs in vitro through a PDGF-A-chain-dependent mechanism. Moreover, primary cell isolates from human lungs produce PDGF-A chain and are capable of autocrine growth following exposure to fibrogenic dusts. We speculate that translocation of particles into the interstitial compartment, where they perturb quiescent fibroblasts, may be involved in mediating the proliferative component of lung fibrosis through a PDGF-A chain autocrine mechanism. Using in situ hybridization and immunohistochemistry, we are currently investigating whether the mRNAs that code for the PDGF-A chain and the PDGF-alpha receptor and their corresponding peptides are upregulated in vivo as components of the proliferative events that are operating during fibrogenesis in a murine model of asbestos-induced fibrosis.

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