by enzyme-linked immunosorbent assay (ELISA). Immunohistochemistry of pleural biopsy specimens showed that IL-8 was localized to malignant mesothelioma cells, but not to other types of malignant metastatic deposits.

Normal human mesothelial cells were obtained from patients with pleural effusions secondary to congestive heart failure. Primary cultures were established in serum-free media. Mesothelioma cell lines were obtained from American Type Culture Collection. In vitro mesothelioma cell lines were demonstrated to constitutively express IL-8 messenger RNA while normal resting human mesothelial cells did not constitutively express IL-8. Supernatants of mesothelioma cell lines contained significantly greater IL-8 (5.4±0.6 ng/mL) than supernatants of unstimulated normal human mesothelial cells (0.4±0.1 ng/mL). We conclude that mesothelioma cells constitutively express IL-8, an angiogenic factor that induces neovascularization and local tumor propagation.

The Molecular Epidemiology of Oncoproteins*

Serum p53 Protein in Patients With Asbestosis

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Paul W. Brandt-Rauf, MD, ScD, DrPH

One of the most common somatic genetic alterations in human cancers involves the loss of inhibitory function of the p53 tumor suppressor gene product.1 In many cases, a common mechanism is the loss of one normal p53 allele and/or point mutation of the other p53 allele.1 The resultant effect is a paradoxic increase in intracellular levels of the p53 protein, since many mutant forms of p53 have a much longer half-life than wild-type p53 and mutant forms of p53 can bind to the wild-type stabilizing its normally rapid degradation and extending its half-life.1 This accumulation of p53 protein has been observed in many human tumors, including lung cancers.1,6

The intracellular accumulation of mutant p53 protein in cells in culture results in a corresponding increase in p53 protein in the extracellular supernatant.7 Similarly, individuals with tumors that have increased levels of mutant p53 protein can have correspondingly high levels of p53 in extracellular fluids, such as serum, which can be quantified using antibodies against p53 in enzyme-linked immunosorbent assays (ELISA). For example, we have previously reported the detection of elevated levels of mutant p53 protein in the serum of asbestos-exposed patients with lung cancers with known intracellular alterations in p53 as well as in the serum of asbestos-exposed patients who subsequently developed lung cancers.8,9 The current study is an extension of this work in which we have used two different ELISAs for p53 (a panpotic assay and a mutant-specific assay) to examine the serum levels of p53 protein in the banked serum samples of asbestos patients with and without lung cancers or mesotheliomas and nonasbestosis noncancer control subjects.

Materials and Methods

A previously described cohort of 111 patients with compensable asbestosis was followed at the Institute of Occupational Health in Helsinki from 1978 to 1987 with regular examinations every 1 to 3 years and, between 1981 and 1987, with the periodic collection of serum samples that were stored frozen at –70°C.10,13 Cancer incidence for this cohort was ascertained through the fall of 1994 from the Finnish Cancer Registry. At that time, 38 of 111 patients had developed malignant tumors, including 27 lung cancers (6 adenocarcinomas, 5 squamous cell carcinomas, 4 small cell carcinomas, and 12 lung cancers that were not further specified), 3 malignant pleural mesotheliomas, and 8 other assorted cancers. The cancer cases were primarily middle-aged to elderly, white, male current or ex-smokers with an average of 20 years of asbestos exposure. The noncancer asbestosis patients were virtually identical to the cancer cases in terms of age, sex, race, smoking status, and asbestos exposure. In addition, single serum samples collected in 1983 (and stored frozen at –70°C until the time of analysis) were available from nine normal control subjects seen at the Clinic of the Institute of Occupational Health in Helsinki for noncancer-, nonasbestosis-related problems. These control subjects were slightly younger than the asbestosis cohort (mean age, 53 years compared with 65 years for cancer cases and 68 years for asbestos control subjects) but were otherwise similar in terms of sex and race (all white men).

The serum samples from these groups were assayed for the levels of p53 protein using a panpotic and a mutant-specific p53 ELISA. The panpotic p53 ELISA is a sandwich assay that utilizes a mouse monoclonal capture antibody (PAB 1801) and a rabbit polyclonal reporter antibody that will detect wild-type and mutant human p53 proteins and was performed according to the protocol of the manufacturer (Onogene Science, Uniondale, NY).

For the assay, the equivalent of 100 μL of sample containing 1.5% normal mouse serum (added to minimize the potentially interfering effect of heterophilic antibodies) is added to microtiter wells that are prewashed with the mouse monoclonal capture antibody and incubated at 37°C for 3 h. After washing with phosphate-buffered saline solution, 100 μL of the rabbit polyclonal reporter antibody diluted 1:20 is added to each well and incubated at room temperature for 2 h. After washing, 100 μL of horseradish peroxidase-conjugated goat antirabbit IgG is added to each well and incubated at room temperature for 1 h. After washing again, the wells are incubated with 100 μL of o-phenylenediamine substrate solution in the dark at room temperature for 30 min. Then 100 μL of the stop solution (4N sulfuric acid) is added to each well, and the color is measured at 490 and 630 nm using a microplate reader.

A standard curve is generated from the average absorbances at 490 and 630 nm of solutions of known concentrations of purified, recombinant human p53 (0, 100, 300, 600, 1,000, and 1,500 pg/mL, in duplicate). This assay will detect 100 pg/mL of p53 on a signal level that is approximately twice background and generates a linear standard curve up to 1,500 pg/mL, and the assay does not cross-react with other cellular components of p53-negative cell lines. With serum samples spiked with known quantities of purified, recombinant...
human p53, this assay gives an average recovery ranging from 77 to 128%, depending on dilution. Intra-assay variability ranges from 3.0 to 5.9%, and interassay variability ranges from 10.0 to 15.8%. The concentration of p53 in samples (assayed in duplicate) was determined by interpolation of the average sample absorbance at 490 and 630 nm from the standard curve. The concentration of mutant p53 in samples was determined using the mutant-specific sandwich ELISA based on a mouse monoclonal and rabbit polyclonal antibody, as described in detail previously. 8

A total of 337 serum samples from the asbestosis patients (including the 27 lung cancers, 3 mesotheliomas, and 73 noncancer cases) and 8 serum samples from the normal control subjects were assayed with the pantropic ELISA. A total of 118 serum samples from the asbestosis patients (including the 27 lung cancers, 3 mesotheliomas, and 36 noncancer cases) and 9 serum samples from the normal control subjects were assayed with the mutant-specific ELISA. A positive elevation of the serum level of p53 by either assay was arbitrarily defined as any value greater than 200 pg/mL.

**RESULTS**

The results are presented in Tables 1 and 2. As shown in Table 1, for the pantropic p53 assay, 2 of the 27 lung cancers (7%; 1 adenocarcinoma and 1 squamous cell carcinoma) were serum-positive compared with 0 of 3 mesotheliomas (0%), 1 of 73 noncancer asbestosis control subjects (1%), and 0 of 8 normal control subjects (0%). Similarly, for the mutant p53 assay, 5 of the 27 lung cancers (19%; the same adenocarcinoma and squamous cell carcinoma plus three of the unspecified lung cancers) were serum-positive compared with 0 of 3 mesotheliomas (0%), 2 of 36 noncancer asbestosis control subjects (6%), and 1 of 9 normal control subjects (11%). Although for both assays the percentage of positives was higher for the lung cancer cases compared with either control group, these differences were not statistically significant (Fisher's exact test, p>0.05) owing to the small numbers involved.

As shown in Table 2, for the multiple individual samples from the positive asbestosis cases, the absolute values for the mutant-specific p53 assay tended to be consistently higher than those for the pantropic p53 assay. Since the same cut-off value for positivity was used for both assays, this partially explains the differences in the numbers of serum-positive cases defined by the two assays. Overall, however, the cor-

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**Table 1—Serum p53 Levels in Asbestosis Patients and Control Subjects**

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Pantropic p53</th>
<th>Mutant p53</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±SD, pg/mL*</td>
<td>Positives (%)†</td>
</tr>
<tr>
<td>Lung cancers</td>
<td>246±39</td>
<td>2/27 (7)</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>273</td>
<td>1/6 (17)</td>
</tr>
<tr>
<td>Squamous cell</td>
<td>216</td>
<td>1/5 (20)</td>
</tr>
<tr>
<td>Small cell</td>
<td>—</td>
<td>0/4 (0)</td>
</tr>
<tr>
<td>Unspecified</td>
<td>—</td>
<td>0/12 (0)</td>
</tr>
<tr>
<td>Mesotheliomas</td>
<td>—</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>Asbestosis control subjects</td>
<td>298</td>
<td>1/73 (1)</td>
</tr>
<tr>
<td>Normal control subjects</td>
<td>—</td>
<td>0/8 (0)</td>
</tr>
</tbody>
</table>

*Most samples were nondetectable for p53; these figures represent the means and SDs for the positive samples only.
†Positivity defined as any value greater than 200 pg/mL.

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**Table 2—Timing of Individual Serum Samples from p53-Positive Asbestosis Cases in Relation to Date of Cancer Diagnosis or Follow-up**

<table>
<thead>
<tr>
<th>Case*</th>
<th>Assay</th>
<th>Date of Sample Collection</th>
<th>Date of Diagnosis/ Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung, A</td>
<td>Mutant</td>
<td>410 (+)</td>
<td>460 (+)</td>
</tr>
<tr>
<td></td>
<td>Pantrp</td>
<td>296 (+)</td>
<td>314 (+)</td>
</tr>
<tr>
<td>Lung, S</td>
<td>Mutant</td>
<td>290 (+)</td>
<td>110 (-)</td>
</tr>
<tr>
<td></td>
<td>Pantrp</td>
<td>550 (+)</td>
<td>370 (+)</td>
</tr>
<tr>
<td>Lung, U</td>
<td>Mutant</td>
<td>41 (-)</td>
<td>60 (-)</td>
</tr>
<tr>
<td></td>
<td>Pantrp</td>
<td>300 (+)</td>
<td>50 (-)</td>
</tr>
<tr>
<td>Control 1</td>
<td>Mutant</td>
<td>1100 (+)</td>
<td>1720 (+)</td>
</tr>
<tr>
<td></td>
<td>Pantrp</td>
<td>338 (+)</td>
<td>439 (+)</td>
</tr>
<tr>
<td>Control 2</td>
<td>Mutant</td>
<td>310 (+)</td>
<td>450 (+)</td>
</tr>
</tbody>
</table>

*P=adenocarcinoma; S=squamous cell carcinoma; and U=unspecified carcinoma.
relation between the two assays for the 24 sample pairs in Table 2 was quite reasonable (R=0.52, p<0.01). If all of the sample pairs for both assays are included (ie, including the nondetectables; total N=68), the correlation is even better (R=0.67, p<0.001), as shown in Figure 1.

In addition, in Table 2, it can be seen that for some cases, serum samples were positive for p53 (by one or both assays) years prior to the time of diagnosis. For example, the case of adenocarcinoma was consistently positive by both assays (except for a single borderline pantropic assay value in 1987) up to 7 years prior to the time of cancer diagnosis.

**DISCUSSION**

These results are consistent with those of other studies of p53 protein expression in lung cancer. Increased amounts of tissue p53 protein have been identified in a significant proportion (33 to 57%) of human lung cancers. In addition, we have previously reported using the mutant-specific ELISA to detect elevated serum p53 levels in 3 of 23 lung cancer patients (13%) who demonstrated increased levels of p53 protein in the tumor tissue by immunohistochemistry and/or the presence of mutations in the p53 gene; the presence of p53 was also confirmed in the serum by immunoblotting, and laser densitometric quantitation of the 53-kd bands showed reasonable agreement with the values obtained by the ELISA. Furthermore, other larger studies with these assays have demonstrated strong, statistically significant correlations between the serum levels of p53 protein as determined by the pantropic and the mutant-specific ELISAs and the amount of p53 protein in the corresponding lung tumor tissue as determined by immunohistochemistry. To date in the current study, the tumor tissue from only one case has been available for immunohistochemical analysis (the adenocarcinoma of the lung in Table 2). Using a modification of the immunohistochemical technique of Cattoretti et al with microwave processing of the tissue for epitope unmasking, staining with primary anti-p53 monoclonal antibody D0-1, and computerized densitometric analysis of the optical density of the staining, the tumor tissue from this patient showed a significant (p<0.01) elevation of intranuclear p53 in comparison to negative control cells and adjacent normal lung tissue (Fig 2). This is of note since multiple serum samples in this patient were consistently positive for p53, as described above.

Furthermore, tissue studies also suggest that changes in p53 may occur early in the carcinogenic process in some cases of lung cancer. For example, in one study, p53 gene mutation with concomitant accumulation of the mutant p53 protein was detected in a squamous cell carcinoma of the lung and in the adjacent dysplastic epithelium. In a larger study, p53 immunostaining was identified in 2 of 17 instances of dysplastic epithelium and 8 of 12 instances of carcinoma in situ associated with p53-positive lung cancers. This type of field carcinization effect would be consistent with the results of the current study of increased amounts of serum p53 identifiable years prior to the development of clinically detectable cancer. This is also consistent with studies of other cancers, for example, in which we have identified elevated plasma p53 levels in patients with colon cancer and in patients with premalignant colonic polyps.

Further study will be necessary to demonstrate that the detection of elevated serum p53 levels predicts the subsequent development of cancer. For example, it is possible that the two asbestosis control patients with elevated serum p53 levels will develop cancer in the future. Continued follow-up of this and similar cohorts can help to resolve this issue. Additional discrepancies also need to be examined. For example, the percentages of reported p53 tissue-positive lung cancers appear to be higher than those identified in serum. There are several possible reasons for this. For example, not all tumors with intracellular accumulations of p53 may release significantly detectable amounts of p53 into the circulation. In addition, circulating antibodies to mutant p53 protein have been detected in some cancer patients, includ-
ing those with lung cancer.\textsuperscript{18,19} It is possible that the presence of such antibodies could accelerate the clearance of p53 from blood or interfere with the ability of the assay to identify p53 by masking relevant epitopes. The development of such antibodies may also explain the variability in p53 serum levels over time seen in some of the patients in the current study (such as control 1 and the first unspecified lung cancer in Table 2). Thus, it may be desirable to assay both for serum p53 protein and anti-p53 antibodies in future studies. Indeed, it might be most useful to combine multiple assays for such studies. For example, this cohort of patients with asbestosis has been examined for serum expression of several other oncoproteins, and the individual with adenocarcinoma of the lung who was serum-positive for p53 in this study has been previously identified as serum-positive for transforming growth factor $\alpha$ and the p21 protein of the $\text{ras}$ oncogene.\textsuperscript{9,10} Therefore, ultimately it may be possible to use a battery of serum tests such as these to identify those individuals with the highest risk of disease in such exposed cohorts.

\section*{References}


\section*{Figure 2. Laser densitometric analysis of the sum of the optical densities of intranuclear immunohistochemical p53 staining of normal lung tissue (group 1) and lung cancer tissue (group 2) from a formalin-fixed, paraffin-embedded tissue section from a p53-serum-positive case of adenocarcinoma (Table 2). Approximately seven cells in ten randomly selected fields were analyzed for each group. The difference in the optical density distributions is statistically significant ($p<0.01$).}
Correlative Assessment of Morphologic, Immunophenotypic, and Genetic Changes in Bronchial Epithelium of Tobacco Smokers

Wilbur A. Franklin, MD; Sean Todd, PhD; Robert M. Genmill, PhD; Harry A. Drabkin, MD; Robert Cook, MD; Julie Sorenson, BS; Joy Folkvord, MS; Jerry Haney, MS; Robert Low, MD; Tony Parks, MD; Susan Proudfoot, MSHA; Tim Kennedy, MD, FCCP; and York E. Miller, MD

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 morphologic, 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 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 vasculopithelial projections into the bronchial lumen. 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 overexpression or mutation of p53 is detectable.

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

Joseph A. Lasky, MD, FCCP; James C. Bonner, PhD; Boihong Tonthat, BS; and Arnold R. Brody, PhD

Inhalation of asbestos in a rat model results in the predominant deposition of fibers onto alveolar duct bifurcations, where a fibrotic 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 translated into the interstiti...

*From the Pulmonary and Critical Care Medicine Section, Tulane University School of Medicine, New Orleans (Dry, Lasky and Brody and Ms. Tonthat), and the Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health Sciences, Research Triangle Park, NC (Dr. Bonner).
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