Correlation of Sulfur Mustard Exposure and Tobacco Use with Expression (Immunoreactivity) of p53 Protein in Bronchial Epithelium of Iranian "Mustard Lung" Patients

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A unique chronic obstructive pulmonary disease (COPD), provisionally called "mustard lung," which occurs as a late complication of sulfur mustard (SM) exposure among SM-exposed Iranians, is presently poorly characterized. This investigation evaluates p53 immunoreactivity in bronchial epithelium of individuals with histories of tobacco use and/or SM exposure, as a tool to help define mustard lung. In this study, 68 COPD patients were segregated into two groups, 35 mustard-exposed patients (including 8 smokers) and 33 unexposed patients (including 16 smokers). Disease severity was assessed with pulmonary function tests. p53 protein in bronchial tissue obtained as biopsies was quantitated by immunostaining. Among nonsmokers, 41.2% of unexposed subjects and 14.8% of exposed subjects expressed p53. Among smokers, 25% of the unexposed group and 50% of the exposed group expressed the protein. Initial data trends suggest an additive contribution of SM exposure and smoking to p53 immunoreactivity. These results illustrate the use of p53 immunoreactivity in the characterization of COPD, including mustard lung.

Introduction

During the Iraq-Iran war of the 1980s, sulfur mustard (SM) [bis-(2-chloroethyl)sulfide] was used extensively against Iranian military and civilian personnel.[1] Long-term survivors of these attacks suffer from debilitating medical complications that are related to but distinct from the effects of acute exposure, predominantly eye, skin, and lung lesions,[2–5] and high incidences of respiratory disease.[2,6,7] Respiratory illness among these individuals appears to follow a characteristic pattern that begins, within a few hours to days after contact with mustard vapor or liquid, as acute bronchitis, with significant airway hyperreactivity. Within weeks, severity may decrease but the symptoms continue and become stabilized as a form of chronic obstructive pulmonary disease (COPD), which in most cases persists for the lifetime of the individual. The severity of the disorder appears to correlate with the presence and extent of mustard-induced lung lesions.[2] The process has the hallmarks of a syndrome unique to individuals with histories of mustard intoxication and has become known generically as "mustard lung" in Iranian treatment facilities. The condition is progressive. Years after appearance of the initial symptoms, chronic, often obliterative, bronchiolitis typically develops, resulting in increasingly severe dyspnea and impairment of pulmonary function. Strategies for management of the health needs of SM-exposed persons require reliable approaches to defining the cellular and molecular mechanisms leading from mustard exposure to specific diseases. A major challenge in this broad effort is to identify easily measured biomarkers affected by the chemical reactivity of mustard agents that correlate with the occurrence of disease. For this purpose, p53, a critical tumor-suppressor protein, is an ideal focus of research, because it is expressed by cells in response to DNA damage and other potentially carcinogenic cytotoxic influences. Normally, production of the protein initiates a cascade of events that results in cell cycle arrest and apoptosis, thereby preventing the survival and proliferation of genetically damaged cells.[8] This is a ubiquitous cellular stress response that, in addition to its antitumor effects, may also allow tissue p53 immunoreactivity patterns to be used as predictors of neoplastic disease in persons exposed to genotoxic carcinogens. In the present investigation, it was hypothesized that immunoreactivity of p53 would be elevated in respiratory tract tissue of COPD patients with previous exposure to SM or tobacco use. Underlying this hypothesis is the ability of both carcinogens to cause stable changes in DNA that may remain quiescent for long periods before causing disease.[8–11] For example, guanine to thymidine transversions, the base pair changes likely to result from DNA lesions caused by polyaromatic hydrocarbons in cigarette smoke, are often found in tumor tissue from smokers.[12] Characteristic alterations in DNA are also associated with mustard exposure, including guanine to adenine transitions within the p53 element, which are the base pair changes associated with alkylation of DNA by SM.[9] The aforementioned mutations are associated both with increased production of p53 protein by cells of the respiratory tract and tumor development.[8–12] It is therefore possible that individuals with confirmed exposure to agents known to induce these DNA alterations may exhibit elevated p53 protein levels in tissues that received the heaviest toxicant dosage. In the present study, p53 immunoreactivity in bronchial epithelium of COPD patients is evaluated for correlation with previous exposure to SM and tobacco carcinogens. The approach used in this study may eventually be applied to diagnostic protocols for identification of persons with mustard lung and other respiratory illness, who may be at elevated risk for development of cancer.

Methods

Patient Population

Sixty-eight outpatients diagnosed with COPD presenting with hemoptysis and persistent cough attributable to chronic bron-

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This manuscript was received for review in June 2005. The revised manuscript was accepted for publication in July 2006.

chitis participated in this study, all on a voluntary basis with informed consent. Test and control cohorts included 35 individuals with histories of single, high-dose exposures to SM in the time period of 1985–1987, 8 of whom were current or former smokers and 27 nonsmokers. A control group of 33 individuals with no history of mustard exposure, including 16 current or previous smokers and 17 nonsmokers, were also evaluated. The mean time between mustard exposure and biopsy in the case group was 14 ± 2 years.

Mustard Exposure and Inclusion and Exclusion Criteria

Mustard exposure in this study is defined as any contact with SM in liquid or vapor form, resulting in transient or permanent disability. This definition is based on standards developed in a comprehensive national survey, performed during the time period of 1997–2000, that established a uniform convention for designation of Iranian citizens with war-related chemical injuries. For the purpose of the present investigation, inclusion in the mustard-exposed group was based on four basic criteria, i.e., (1) documented exposure to SM, based on official certification from the Iranian Veterans Affairs organization (Janbazan); (2) records of medical treatment, showing the type and extent of mustard-associated injury and/or disability; (3) a productive cough on most days for 3 months in 2 consecutive years; and (4) hemoptysis in the form of bloody streaks or expectoration of clots. Patients with mass lesions in the chest, as determined by radiological examination, were excluded from the study. A detailed smoking history was obtained from each patient, including the number of years smoking, number of cigarettes smoked per day, and abstinence periods of >1 month. There were no pipe or cigar smokers among the patients studied. Persons designated as former smokers were those who had refrained from smoking for ≥6 months before the study.

Pulmonary Function Test

Airway obstruction was evaluated with spirometry, using American Thoracic Society criteria.[13] Briefly, seated patients, with a nose clip in place, performed a minimum of three forced expiratory maneuvers, with verbal encouragement to blow maximally into a standard spirometer until no air remained to be expelled. Forced vital capacity and forced expiratory volume were recorded, and measurements were repeated until three technically satisfactory curves with reproducible contours were obtained. All of the indices used for analysis were derived from the maneuver with the largest forced vital capacity. Results are reported as the relative level of airway obstruction, graded as none, mild, moderate, or severe (Table I).

Bronchoscopy

A flexible fiberoptic bronchoscope was inserted transnasally, with patients receiving supplemental oxygen through a facemask during biopsy and sample collection. All bronchial biopsies were taken from right upper lobe carina in which there were no visible tumors or suspected infiltration. Mucosal thickening was apparent for each patient.

Histological Analyses

Sample Processing

Tissue samples were fixed in ethanol, embedded in paraffin wax, and stored at 20°C. For tissue staining, slides were placed in a xylene bath for 5 minutes. Excess liquid was tapped off, and the slides were placed in absolute ethanol for 3 minutes, followed by 95% ethanol for 3 minutes. The slides were then placed in distilled water for 3 seconds before application of the staining protocol.

p53 Immunohistochemical Analysis

Immunohistochemical analysis for p53 expression was performed on 5-μm-thick sections that were cut from the specimens and placed on slides coated with poly-L-lysine solution (Sigma-Aldrich, St. Louis, Missouri). Specimens were then dewaxed in xylene and rehydrated in graded alcohol. Endogenous peroxidases were blocked by immersion in 0.1% H_2O_2 in absolute methanol for 20 minutes. Nonspecific antibody binding was blocked by incubation of slides in 20% fetal calf serum in phosphate-buffered saline (PBS) for 20 minutes. Sections were incubated overnight at 4°C with a primary polyclonal rabbit anti-human p53 antibody (CM-1), at a dilution of 1/1000.[14] This step was followed by incubation with biotinylated anti-rabbit secondary antibody diluted 1/400 (Dakopatts, Copenhagen, Denmark) and an avidin-peroxidase conjugate (Dakopatts). Rinses were performed with several changes of PBS between the stages of the procedure. The peroxidase-conjugated secondary antibody was developed with the diaminobenzidine colorimetric reaction, and the sections were lightly counterstained with hematoxylin and mounted with Eukitt (Kindler, Freiburg, Germany).

Negative control assays for immunostaining were carried out by substituting PBS for the primary antibody. Lung biopsy sections known to be strongly immunoreactive for p53 protein were used as positive control samples, using criteria previously described.[14] Results were evaluated and divided into four categories on the basis of intensity of p53 protein staining. Staining intensity scores were designated as follows: 0 = no p53 protein immunoreactivity; 1 = 1 to 5% of cells positive; 2 = 6 to 10% of cells positive; and 3 = >10% of cells positive for p53 protein (Table II). In determining the percentage of positive cells, the entire biopsy section was taken into account. The result of staining was considered positive if there was agreement between two pathologists.

Statistical Analyses

Bronchial biopsy specimens were designated as p53 positive based on a minimal cutoff value of 1% of cells exhibiting immunoreactivity for the protein. Student's t test (two-tailed) was used to evaluate the significance of differences between groups in p53 protein expression, as shown in Table III. The strength of association between mustard exposure and smoking was estimated as the ratio of probability of p53 immunoreactivity measured in samples from unexposed versus mustard-exposed subjects, calculated for both smokers and nonsmokers (Table IV). For all comparative measurements, a $p < 0.05$ was considered statistically significant. Assessment of the influence of mustard exposure on p53 expression in bronchial tissue of nonsmokers and smokers was determined by calculating the odds ratio (OR) = $p(1 - q)/(1 - p)q$; p is defined as the ratio of p53-positive samples to the total number of samples in the nonsmoking unexposed group or the smoking, SM-exposed group, and q is defined as the ratio of p53-positive samples to the total sample number in the group of nonsmoking exposed persons or smok-

ing exposed persons. An OR of <1 indicates a lack of association between mustard exposure and p53 expression, and an OR of >1 indicates a positive relationship between the two variables.

Missing Data

Some participants who contributed biopsies for p53 assessment were not evaluated for airway obstruction with spirometry, although all patients who were tested for airway obstruction contributed corresponding p53 data. These data are displayed separately from other results. The number of patients in each category is shown in Table I.

Results

The distribution of participants according to age, gender, smoking history, and pulmonary function test (PFT) results is shown in Table I. Fifty-two subjects (of the project sample of 68) were given PFTs. Of these individuals, at least mild airway obstruction was observed for 45.5% of 11 unexposed nonsmoking participants, 14.3% of 7 unexposed smoking participants, 65.4% of 26 mustard-exposed nonsmoking participants, and 75% of 8 mustard-exposed smoking participants. PFT results were not analyzed for significance of differences between groups.

Comparisons of immunohistochemical staining scores in biopsies of bronchial tissue from samples drawn from each exposure group are shown in Table II. p53 immunoreactivity of ≥1% of the cells per biopsy was observed for 41.1% of the 17 unexposed nonsmoking participants, 25.0% of the 16 unexposed smoking participants, 14.8% of the 27 mustard-exposed nonsmoking participants, and 50% of the 8 mustard-exposed smoking participants.

The significance of comparisons between groups for biopsy p53 immunoreactivity is shown in Table III. Although none of the distributions differed at the $p < 0.05$ level, some comparisons exhibited trends that may reach statistical significance with larger n values (in future studies). These included (1) mustard-exposed nonsmoking participants (14.8% p53 positive) and unexposed nonsmoking participants (41.2% p53 positive; $p = 0.075$) and (2) mustard-exposed smoking participants (50% p53 positive) and mustard-exposed nonsmoking participants (14.8% p53 positive; $p = 0.060$).

TABLE II

p53 PROTEIN EXPRESSION IN CASE AND CONTROL GROUPS

p53 Staining Intensity Score	Unexposed Nonsmoking Participants ($n = 17$)	Unexposed Smoking Participants ($n = 16$)	SM-Exposed Nonsmoking Participants ($n = 27$)	SM-Exposed Smoking Participants ($n = 8$)
0	10 (58.8)	12 (75.0)	23 (85.2)	4 (50.0)
1	3 (17.6)	4 (25.0)	4 (14.8)	4 (50.0)
2	1 (5.9)	0 (0)	0 (0)	0 (0)
3	3 (17.6)	0 (0)	0 (0)	0 (0)

Five-micron, ethanol-fixed, paraffinized, bronchial biopsy sections were stained with polycolonal rabbit anti-human p53 antibody (CM-1) and biotinylated anti-rabbit and avidin-peroxidase conjugate, developed with diaminobenzadine, and counterstained with hematoxylin. p53 expression was estimated by using a relative scale based on the percentage of cells staining positively in the entire biopsy section, as follows: 0 = no cells stained; 1 = 1 to 5% of cells positive; 2 = 6 to 10% of cells positive; 3 = >10% of cells positive.

ORs were calculated to evaluate the likelihood of p53 expression being affected by past exposure to mustard agent in smokers and nonsmokers (Table IV). SM exposure was determined not to be associated with p53 immunoreactivity in bronchial tissue of nonsmokers, as revealed by an OR of 0.25 for this comparison. Conversely, the OR of 3.00 calculated for the strength of association between SM exposure and production of p53 protein observed in biopsies from smokers suggests the existence of a positive interaction between the two toxicants with respect to cellular changes leading to the p53 expression response.

Discussion

In the present study, detectable p53 protein in bronchial epithelium is considered to be a predictor of cellular damage with carcinogenic potential. Justification for this interpretation is based on the fact that, in healthy cells under normal physiological conditions, p53 protein is usually nondetectable.[15–17] However, when DNA damage or mutation, oncogene activation, and other stressors occur, the protein is stabilized and accumulates in the nucleus, a process that promotes elimination of a cell that may become cancerous.[15–17] In cells containing muta-

TABLE I

CASE AND CONTROL GROUP CHARACTERISTICS

	Unexposed Nonsmoking Participants	Unexposed Smoking Participants	SM-Exposed Nonsmoking Participants	SM-Exposed Smoking Participants
Age in (years) (mean ± SD)	$n = 17$	$n = 16$	$n = 27$	$n = 8$
	45.7 ± 15.1	56.0 ± 19.2	41.2 ± 7.0	39.8 ± 4.9
Gender	$n = 17$	$n = 16$	$n = 27$	$n = 8$
Male	13	15	27	8
Female	4	1	0	0
PFT results obstruction level[a]	$n = 11$	$n = 7$	$n = 26$	$n = 8$
None	6	6	9	2
Mild	1	1	9	3
Moderate	3	0	6	2
Severe	1	0	2	1

[a] The PFT protocol followed in this investigation was conducted according to the American Thoracic Society standard for spirometry established in 1987.[13]

TABLE III

p53 PROTEIN EXPRESSION IN CASE AND CONTROL GROUPS

	p			
	Unexposed Nonsmoking Participants (7 p53 positive, 41.2%; *n* = 17)	Unexposed Smoking Participants (4 p53 positive, 25%; *n* = 16)	SM-Exposed Nonsmoking Participants (4 p53 positive, 14.8%; *n* = 27)	SM-Exposed Smoking Participants (4 p53 positive, 50%; *n* = 8)
Unexposed nonsmoking participants (7 p53 positive, 41.2%)	NA	0.325	0.075	1.000
Unexposed smoking participants (p53 positive, 25%)	0.325	NA	0.443	0.363
SM-exposed nonsmoking participants (4 p53 positive, 14.8%)	0.075	0.443	NA	0.060
SM-exposed smoking participants (4 p53 positive, 50%)	1.000	0.363	0.060	NA

Five-micrometer, ethanol-fixed, paraffinized, bronchial biopsy sections were stained with polycolonal rabbit anti-human p53 antibody (CM-1) and biotinylated anti-rabbit and avidin-peroxidase conjugate, developed with diaminobenzadine, and counterstained with hematoxylin. Sample designation was based on a cutoff value of 1%. All samples in which >1% of cells stained positively for p53 in an entire biopsy section were considered to exhibit elevated expression of the protein.
NA, not assessed.

TABLE IV

p53 PROTEIN EXPRESSION IN CONTROL AND CASE GROUPS AS A FUNCTION OF TOBACCO USAGE

	Proportion (%)		
	Unexposed Participants (*n* = 33)	SM-Exposed Participants (*n* = 35)	OR (95%) Confidence Interval
Nonsmokers	41.2	14.8	0.25 (0.06–1.04)
Smokers	25.0	50.0	3.00 (0.50–17.95)

Percentages of p53-positive phenotype in each group were determined on the basis of immunohistochemical analysis of bronchial biopsies. OR were calculated to determine the strength of association between mustard exposure and p53 expression for non-smokers and smokers.

tions in the gene for p53 or functionally related genetic elements, the capacity of p53 protein to cause apoptosis may be impaired, thereby allowing a tumor to form.[15-17] Therefore, p53 mutations are early events in a wide range of neoplastic diseases, allowing the presence of the protein to be used an indicator of eventual cancer risk.[10,18] Measurement of this parameter in normal tissue, where most of the cells are unaffected, remains technically challenging. However, prospective studies have largely supported the value of positive p53 expression in indicating lung cancer risk, showing that lung cells that lack a normal p53 protein are genetically unstable and thus more prone to carcinogenesis.[19,20] In this study, p53 expression was analyzed in the context of previous toxicant exposure (SM and tobacco), taking into account disease severity, which was assessed as PFT results. PFT outcomes were not compared among groups of participants, to allow determination of statistically significant differences in grouped values. However, the qualitative comparisons shown in Table I reveal broad trends in this variable. Here, the percentage of patients experiencing airway obstruction was higher in the mustard-exposed group than in the group with no SM exposure, an observation consistent with the observed pathogenesis of mustard lung. The correlations between smoking and PFT outcomes shown in Table I are less clear. Elevated pulmonary obstruction among mustard-exposed versus unexposed smokers is consistent with a possible additive effect of the two influences. However, the lower level of pulmonary obstruction observed among smokers versus nonsmokers in the unexposed group is anomalous and suggests that demonstration of clear PFT response trends with respect to smoking status requires a larger sample size.

At the date of this writing, no comprehensive study of mustard lung has been conducted. The present investigation was undertaken to establish whether p53 immunoreactivity constitutes a valid useful parameter for the broad effort to characterize consistent features of the disorder. Results of immunohistochemical staining of bronchial tissue (Tables II and III) failed to reveal statistically significant correlations between production of p53 protein and history of SM exposure or tobacco use. However, two trends emerged that may become significant in ongoing investigations with larger numbers of participants. The first is the observation that, among nonsmoking participants, those with SM exposure exhibited lower p53 immunoreactivity than did those without previous SM contact (Table III). If future studies confirm that this result was not an anomaly of the present study attributable to the small sample size, then the existence of a (counterintuitive) p53-suppressive effect of SM may be identified. A second trend that may emerge as a significant feature of respiratory illness in mustard-exposed persons is the apparent correlation between p53 immunoreactivity and smoking combined with SM exposure approached statistical significance (Table III). Moreover, mustard exposure was determined to be associated with increased levels of the p53 protein in participants with smoking histories, as shown by the OR of 3.00 for this comparison (Table IV).

The scope of this investigation is too limited to make definitive recommendations regarding the usefulness of p53 immunoreactivity in characterizing the pathogenesis of mustard lung or predicting the risk of transition to neoplastic disease for particular patients. The major limitations to broad use of the data presented here include small sample size and broad immuno-

staining intensity cutoff scores for designation of a particular sample as "p53-positive." However, this research was undertaken only to provide a preliminary guide to a larger ongoing study that will incorporate p53 expression patterns as one of several major parameters for characterization of mustard lung. The trends described here allow a more-focused approach to developing case definitions of this syndrome that can be used as the basis for improved treatment of mustard victims.

Acknowledgment

We acknowledge Stephanie C. Fox, JD, of QueenBeeEdit (Bloomfield, Connecticut), for her hard work in editing and formatting this article.

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