Immunohistochemical Localization of Glutathione S-Transferases in Human Lung

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ABSTRACT

Glutathione S-transferases (GST) detoxify a number of carcinogenic electrophiles including diol-epoxide metabolites of polycyclic aromatic hydrocarbons. The distribution of GSTs A1/A2, M1, M2, M3, and P1 has been studied in lung tissue from 32 subjects by immunohistochemistry. The staining intensity for GSTA1/A2 and GSTM1 was found to be the most abundant GSTs in human lung, being present in the bronchial and bronchiolar epithelium of all individuals studied. The staining intensity for GSTA1/A2 varied more than that for GSTP1 between individuals. GSTA1/A2, a polymorphic µ-class enzyme, was ambiguously detected in lung tissue and, if expressed, is present at very low levels. GSTM2, a striated muscle-specific isozyme, occurred minimally in the epithelium of the terminal airways, and GSTM3, an enzyme of broad extrahepatic occurrence, was observable in the ciliated airway epithelium and smooth muscle of the lung. The staining for GSTM3 varied from minimal to very intense between individuals; in the bronchial epithelium, it was more abundant in current smokers than in ex-smokers. The immunostaining for GSTs in general was most intense in the bronchial epithelium decreasing in the distal airways, in contrast to the previously described peripheral localization of the polycyclic aromatic hydrocarbons activating the P450IA1 enzyme. The localization of GSTs in the bronchial wall suggests that GST polymorphisms may contribute to susceptibility, especially to bronchial tumors of tobacco smokers.

INTRODUCTION

The binding of electrophilic metabolites of carcinogens to macromolecules, especially DNA, is a critical event in chemical carcinogenesis. GSTs detoxify a number of carcinogenic electrophiles, including diol-epoxide metabolites of tobacco-derived PAH by catalysis of the conjugation with reduced glutathione. Four multigene families of GSTs have been characterized in humans, namely, α, µ, θ, and δ (1–3). Information available to date shows that PAH diol-epoxides are detoxified by GSTs P1 (3, 4), M1 (3), M2,4 and M3,5 Individual variation due to a genetic polymorphism occurs at the GSTM1 locus where a null allele is so frequent that ~50% of the population are homozygous GSTM1 null and have a null phenotype (5, 6). The expressed GSTM1 enzyme is considered to offer some protection against smoking-related lung cancer, although the evidence in the literature is conflicting. In a few studies, the GSTM1-null phenotype has been shown to be more common in lung cancer patients than in the general population (7–9), whereas no association has been observed in others (10). The lack of the GSTM1 gene has been associated especially with the squamous cell lung cancer of cigarette smokers (11–13).

The localization of PAH-activating and -detoxifying enzymes in lung tissue, as well as individual differences in their activity, is of essential importance in the understanding of the effect of individual and environmental factors in pulmonary carcinogenesis. We have reported previously that the presence in lung tissue of a cytochrome P450IA1, which takes part in the conversion of PAH compounds into carcinogenic diol-epoxides, is more closely associated with peripheral than with bronchial lung cancer. Moreover, we have shown that the localization of this enzyme explains this connection with peripheral cancer (14, 15). We describe in this paper the localization of GSTs A1/A2, M1, M2, M3, and P1 in lung tissue and individual variation in the expression of the µ-class GSTs M1 and M3.

MATERIALS AND METHODS

Patients. The patients were selected from a series of consecutive patients who underwent surgery, i.e., a pulmonectomy or lobectomy, for a tumors lung lesion between August 1988 and June 1992. All of the patients were interviewed personally about their smoking and occupational history during their stay at the hospital. The distribution of GSTs A1/A2, M1, M2, M3, and P1 in the lung was studied from 12 patients (5 women and 7 men), ages 19 to 75 years (median, 66 years). There were four nonsmokers, five exsmokers, and three current smokers; eight had a malignant pulmonary tumor and four had a benign or low-grade malignant pulmonary tumor. These patients were selected to represent the male and female gender, as well as different smoking habits and diagnoses, to exclude a confounding effect of any factor on the immunohistochemical detection of enzymes.

Individual expression of GSTM1 and M3 and the GSTM1 genotype were studied also in 20 additional cases, mainly male lung cancer patients with different smoking histories. The current smoking status of the 32 patients is given in Table 2. In the histological examination their diagnoses were as follows: 5 benign or low-grade malignant tumors; 3 inflammatory lesions (including 2 tuberculomas); 24 carcinomas; 7 adenocarcinomas; 14 squamous cell carcinomas; 1 adenosquamous carcinoma; and 3 small cell carcinomas.

Preparation of Lung Samples. Fresh tissue specimen, a lung or a lobe of lung, was prepared within 1 h after resection. Pieces of parenchymal lung tissue were mounted in OCT compound (Ames Division, Miles, Elkhart, IN), frozen immediately in liquid nitrogen, and stored at −70°C. After preparation of fresh samples, the specimen was sewn up, filled with 10% formalin through the bronchi, and fixed overnight. At least two samples per lobe, taken from the central and peripheral lung tissue, were available for this study. The samples were from the histologically normal lung tissue; samples from the tumor, inflammatory lesion, or tissue behind a bronchial obstruction were not used.

Preparation of Antibodies. The antibodies against the human µ-class GSTM1 M2 and M3 subunits were raised in female New Zealand White rabbits by giving them injections of a primary 100 µg of protein in complete Freund’s adjuvant, followed at weeks 6 and 8 by two booster injections in incomplete Freund’s adjuvant. The rabbits were bled out at week 10. The anti-M1 IgG showed some cross-reactivity with M2 but very little with M3 and anti-M2 cross-reacted with M1, whereas anti-M3 did not cross-react with any of the other GSTs. The cross-reactivities of the antibodies, as determined by Western blot analyses, have been reported in detail elsewhere (16). The antisera against human α-class and θ-class GST (Bioprep, Dublin, Ireland) were specific for enzymes within their own class and displayed no cross-reactivity for any of the µ-class GST subunits.

The monoclonal antibody 1-7-1 (against a 3-methylcholanthrene-inducible rat cytochrome P-450) recognizes both P450IA1 and P450IA2 (17, 18). Antibody 1-7-1 was a generous gift from Drs. Sang S. Park and Harry V. Gelboin (National Cancer Institute, NIH, MD).

Immunohistochemistry. GSTs were studied in paraffin-embedded tissue. In addition, both GSTs and P450IA were studied in the serial frozen sections from 21 patients with inductible P450IA for comparison of the localization of carcinogen-activating and -detoxifying enzymes.
Paraffin-embedded lung tissue 4 µm thick was cut, placed on slides coated with 0.05% poly-L-lysine hydrobromide, air dried, and incubated overnight at 38°C. The sections were dewaxed with xylene, hydrated through a series of alcohol solutions, and then incubated in 0.3% (v/v) hydrogen peroxide in absolute methanol for 30 min to quench endogenous peroxidase activity. The sections were subsequently incubated in goat normal serum (1:300) followed by incubation overnight at 4°C, and then incubated for 1 h at 20°C with rabbit polyclonal antisera, anti-GSTAJ/A2 (1:500), M1 (1:1000), M2 (1:1000), M3 (1:2000), or P1 (1:300) diluted in Tris buffer containing 0.05% poly-L-lysine hydrobromide, air dried, and incubated overnight at 38°C. The sections were dewaxed with xylene, hydrated through a series of absolute methanol for 30 min to quench endogenous peroxidase activity. The sections were subsequently incubated in goat normal serum (1:30) for 15 min, followed by an avidin-biotin complex reagent for 30 min. After each step, the sections were washed repeatedly with 0.05 M Tris buffer (pH 7.6). The sections were then exposed to a reaction solution containing the chromogen diaminobenzidine.

For paraffin sections, two different proteinase pretreatments were tried. The sections were predigested either with 0.1% trypsin in Tris buffer for 8 min at 20°C or with 1% pepsin in distilled water and sulfuric acid at pH 1.5 for 10 min at 38°C. The pretreatments had no effect on the signal with anti-GSTM1 and anti-GSTM2 antibodies;they weakened the staining for GSTA1/A2 and GSTP1 and enhanced that for GSTM3. Because better results were obtained with pepsin predigestion, this procedure was used for GSTM3.

Frozen sections 6–8 µm thick were cut on a cryostat, dried overnight at 20°C, and heat-fixed at 37°C for 1 h before undertaking the immunohistochemical procedure. The avidin-biotin complex method which was used for the paraffin-embedded tissue was applied with the following modifications: the blocking of endogenous peroxidase activity was not performed; the sections were incubated for 30 min at 20°C with the primary antisera against GSTs and monoclonal antibody 1-7-1 (1:10000); phosphate-buffered saline was used instead of Tris buffer; and 3-amino-9-ethylcarbazole was used as a chromogen.

A positive case from a previous series was used as a positive control, and replacement of the antibody by the buffer was used as a negative control. In frozen sections, HyHel-9 monoclonal antibody against egg white lysozyme was also applied. All negative, ambiguous, and weakly positive staining results were confirmed from another tissue sample. The slides were read by an experienced pulmonary pathologist (S.A.) without knowledge of the real identification of the samples and without information on smoking or the diagnosis of the patient.

**Determination of GSTM1 Genotype.** DNA was isolated from peripheral blood samples which were collected into EDTA or heparin tubes the day before surgery and stored at −20°C until use. GSTM1 polymorphism was studied using a PCR-based method, described in more detail elsewhere (19). On the basis of the sequences of GSTM1 and related genes of the multigene family, three oligo primers for the exon 4 and exon 5 regions of the genes were used. Primers 1 and 2 could also anneal to another class µ gene, while primer 3 was specific for the GSTM1 gene. When the three primers were used together in a PCR assay, a 158-base pair fragment was consistently found, whereas the polymorphic 231-base pair fragment could be seen only in the GSTM1-positive genome. The constant 158-base pair fragment was amplified as an internal control, excluding the possibility of a false interpretation due to failure in the PCR reaction.

**Table 1 Localization of GST classes and cytochrome P450A in human lung**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>P1</th>
<th>A1/A2</th>
<th>P450A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bronchial wall</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epithelium</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Smooth muscle</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bronchial glands</td>
<td>(+)</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>(+)</td>
</tr>
<tr>
<td>Endothelium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Peripheral lung</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bronchiolar epithelium</td>
<td>(+)</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Terminal</td>
<td>(+)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Alveolar epithelium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Type 1</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Type 2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Endothelium</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Alveolar macrophages</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(+)</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* The presence of GSTs and P450A in different cell types: -, absent; (+), minimal occurrence; +, present; ++, abundant.

**RESULTS**

The distribution of the GSTs in different compartments of the airways was similar in all individuals studied (Table 1), with the exceptions that will be mentioned later. However, the intensity of staining varied with the antibody and, in the case of GSTs M3 and A1/A2, with the individual. All the GSTs studied, except GSTM2, were localized mainly in the ciliated bronchial and bronchiolar epithelium, showing less staining in the distal than in the proximal Airways. This pattern of staining is contrary to that seen for cytochrome P450A, which occurred in the alveolar and bronchiolar epithelium but was almost absent in the epithelium of bronchi larger than 1 mm in diameter (Fig. 1; Table 1).

**GSTM1.** The immunostaining for GSTM1 varied from being absent to being a weak immunoreaction in the bronchial and bronchiolar ciliated epithelial cells and in bronchial smooth muscle (Table 1). The poor quality of the immunohistochemical signal and the fact that it does not correlate with the PCR genotyping assay (Table 2) suggests that the staining observed with anti-GSTM1 is due to a cross-reaction with other µ-class GST proteins, which indeed had a staining pattern similar to that of M1 in frozen sections of the peripheral lung.

**GSTM2.** The immunostaining for GSTM2 showed patchy distribution of immunopositive areas within mainly negative peripheral lung tissue. Immunostaining was seen in the apical part of the Clara cells, in the brush border of the cuboidal cells of the terminal bronchioli, and in the type 1 epithelial cells of respiratory bronchioli and alveolar ducts. The immunostaining of alveolar epithelium was not seen in...
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Fig. 1. Comparison of immunostaining for GSTP1 and P450IA in bronchiolar and bronchial epithelium. In serial frozen sections from a bronchiolus (L, lumen), both GSTP1 (a) and P450IA (b) are present in ciliated epithelium. × 150. In a small bronchus 1 mm in diameter, epithelial staining for GSTP1 is intense (c), whereas very weak staining for P450IA (arrows) can be observed (d). Immunoperoxidase, no counterstain, × 260.

frozen sections. Weak staining was observed in the brush border of ciliated epithelial cells and the smooth muscle of the bronchi (Table 1).

GSTM3. The immunostaining for GSTM3 varied from minimal to intense according to the individual. It was detected in the bronchial and bronchiolar epithelium, in bronchial glands, and in bronchial and vascular smooth muscle (Table 1). In the bronchial epithelium, a cytoplasmic immunoreaction was observed in most of the ciliated cells, whereas mucous-secreting goblet cells remained negative (Fig. 2a). The immunoreaction of the walls of the terminal and respiratory bronchioli was inconspicuous. Alveolar macrophages showed weak staining occasionally.

The phenotypic variation in the expression of the μ-class GSTs M1 and M3 was studied because of the known polymorphism of the GSTM1 gene and the variation observed here in the expression of GSTM3. The staining results for GSTM1 and M3 in the bronchial epithelium compared with the GSTM1 genotype and smoking are given in Table 2.

The groups of current smokers and exsmokers in Table 2 were similar with regard to age, gender, and diagnosis; they were mainly
male lung cancer patients with a mean age of 63 ± 7 (SD) years. The immunostaining for GSTM3 in the bronchial epithelium was significantly more intense in current smokers than in exsmokers who had stopped smoking less than 5 months ago (two-tailed test at α = 0.002; the Mann-Whitney U = 3.5), whereas the difference was not significant between current smokers and exsmokers for longer than 1 year (Table 2). The staining did not differ between patients with squamous cell or adenocarcinoma. It was not possible to compare statistically nonsmokers with exsmokers and current smokers or to study the effect of age and gender on immunostaining, because the nonsmokers were younger, they more often were women, and they had a nonmalignant disease more frequently than the exsmokers or current smokers.

**GSTA1/A2.** GSTA1/A2 (subunits A1 and/or A2) genes were present in every case studied, but the intensity of immunostaining differed...
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between individuals. The localization of GSTA1/A2 was solely bronchial and bronchiolar in some individuals but also occurred in the alveolar epithelium of other individuals. The distribution of the α-class enzymes was widest of all the GSTs studied, extending from the epithelium of the central bronchi (Fig. 2b) to the type 1 and type 2 alveolar epithelium. GSTA1/A2 was also seen in bronchial and vascular smooth muscle and in alveolar macrophages (Table 1).

**GSTP1.** The intensity of the immunostaining for GSTP1 was rather constant between individuals. The slight variation may reflect intraparenchymal as well as interindividual differences. An intense staining reaction was observed in the bronchial and bronchiolar epithelium (Fig. 1, a and c; Fig. 2c). Bronchial smooth muscle, epithelium of the bronchial glands, alveolar type 1 and type 2 epithelium, and alveolar macrophages showed weak staining occasionally (Table 1).

**DISCUSSION**

The pulmonary occurrence and cell type localization of GSTs were investigated in order to acquire information concerning their localization in lung tissue with respect to the detoxication of electrophiles arising from the metabolism of carcinogenic components of tobacco smoke. At present, the only known substrates for GSTs in cigarette smoke are those derived from PAH, namely, PAH diol-epoxides. The most studied PAH diol-epoxide, benzo(a)pyrene-7,8-diol-9,10-epoxide, is a poor substrate for GSTA1/A2, a relatively good substrate for GSTM1 (3), -M2, and -M3, and better still with GSTP1 (3, 4).

The present immunohistochemical study revealed that GSTs in general are most abundant in the ciliated epithelium of the bronchi, decreasing in the distal airways, in contrast to the peripheral localization of the PAH-activating cytochrome P450IA1. In the immunostaining of bronchial epithelium, the mucus-secreting goblet cells remained negative for all of the GSTs studied. Typically, goblet cells are numerous in the bronchial epithelium of tobacco smokers, since in the course of chronic bronchitis, ciliated epithelial cells have been replaced gradually by mucus-secreting cells. This phenomenon probably decreases the detoxication capacity of the epithelium by GSTs and may add to the harmful effects of smoking with respect to the development of lung cancer.

According to the present study, GSTA1/A2 and GSTP1 are the principal GSTs in most individuals. Both were always present in bronchial and bronchiolar epithelium, the staining for GSTP1 being more consistent than that for GSTA1/A2. In an earlier study (20), analysis of lung tissue by high performance liquid chromatography demonstrated that GST subunits A1, A2, M1, M3, and P1 were present in amounts varying with the individual but with GSTP1 being the most abundant.

The distribution of GSTs within the airways was almost similar in all subjects, although the intensity of immunostaining, especially for GSTA1/A2 and GSTM3, varied interindividually. We cannot fully exclude the possibility that the disease may have had an effect on the intensity of staining. We tried to avoid this by using only histologically normal tissue. The distribution of GSTs was the main target of this study, and we decided to use surgical tissue material from patients with different kinds of tumorous lung lesions because personal interview data were available and all tissue samples were prepared freshly in a similar manner.

Among the μ-class enzymes, apparent GSTM1 was observed to stain weakly in the bronchial and bronchiolar epithelium. The staining was not consistent with the GSTM1 genotype determined from lymphocyte DNA, suggesting that cross-reaction with other μ-class enzymes obscured the distinction between GSTM1-positive and null phenotypes. Consequently, the level of GSTM1 expression in all pulmonary cell types was too low to be detectable by immunohistochemistry. In a recent study (9), GSTM1 enzyme protein has been detected by enzyme-linked immunosorbent assay in the lung tissue of individuals with the GSTM1-positive genotype.

The homozygous deletion of the GSTM1 gene has been associated with an increased risk of squamous cell lung cancer (11-13). The localization of GSTs mainly in the bronchial wall might be important in determining this association. We have observed earlier that the cytochrome P450IA1, which activates PAH compounds, is located in the peripheral lung in the cell types which usually give rise to peripheral adenocarcinomas (14); furthermore, we have not found a case of peripheral pulmonary adenocarcinoma without inducible P450IA1 (15). Accordingly, the lack of a carcinogen-toxicifying enzyme in the bronchial wall could similarly favor the development of squamous cell carcinomas, which usually originate from the bronchial epithelium. However, in view of the relatively high content of GSTP1 and GSTM3 in the lung and the low occurrence of GSTM1, any effect of GSTM1 expression on susceptibility to lung cancer probably originates in an extrapulmonary compartment such as in the liver.

A significant proportion of PAHs from tobacco smoke passes into systemic blood to be activated or detoxified in extrapulmonary tissues (21). The liver is the major organ of detoxication and, among the four GST genes taking part in the detoxication of tobacco-derived carcinogens, only the GSTM1 locus is active in the liver. It is probable that more carcinogenic electrophiles enter the circulation from the liver in GSTM1-null individuals than from those which are GSTM1 positive. This mechanism might explain the association of the GSTM1-null phenotype with increased risk of cancer of not only the lung but several other organs such as the stomach, colon, urinary bladder, and larynx (22, 23).

The immunostaining for GSTM3, an enzyme with a wide distribution in extrahepatic tissues (24), was particularly interesting, showing the greatest interindividual differences of all the GSTs in lung tissue, varying from minimal staining to staining intensities similar of those of GSTP1 and GSTA1/A2. There was a significant decrease of GSTM3 in the bronchial epithelium of the patients who had stopped smoking less than 5 months ago as compared with current smokers. It is uncertain whether the difference observed in the bronchial epithelium represents a local rather than an overall metabolic situation in the lung. The findings on GSTM3 concerning the interindividual variation in the expression and induction by smoking suggest that GSTM3 warrants further studies as regards susceptibility to lung cancer.

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