Aryl Hydrocarbon Hydroxylase Activity in Pulmonary Macrophages and Lymphocytes from Lung Cancer and Noncancer Patients


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SUMMARY

Aryl hydrocarbon hydroxylase activity was measured in pulmonary alveolar macrophages and peripheral blood lymphocytes from 47 patients with primary lung cancer and 56 patients bronchoscopied for other indications. In macrophages from nonsmoking patients without cancer, the aryl hydrocarbon hydroxylase level was 16 ± 2 (S.E.) milliunits/10⁶ cells, whereas nonsmokers with primary lung cancer had values of 24 ± 3 milliunits/10⁶ cells (p < 0.05). Cigarette smokers consistently had higher macrophage enzyme values than nonsmokers (p < 0.01), although smokers without cancer (enzyme activity, 77 ± 11 milliunits/10⁶ macrophages) and smokers with lung cancer (enzyme activity, 95 ± 14 milliunits/10⁶ macrophages) had similar enzyme levels (p > 0.2). Induction of aryl hydrocarbon hydroxylase in cultured lymphocytes from nonsmokers (expressed as fold induction) was significantly lower for lung cancer patients (1.17 ± 0.29) than for patients without lung cancer (3.10 ± 0.29; p < 0.001). Enzyme induction was similar in lymphocytes from smokers whether or not they had lung cancer. For individual patients without lung cancer, enzyme activity in pulmonary macrophages and enzyme induction in cultured lymphocytes were correlated (correlation coefficients, 0.821 for nonsmokers and 0.902 for smokers; p < 0.001 for each group). Enzyme values for cells from smokers with primary lung cancer did not correlate, whereas an inverse relationship was present with cells from nonsmoking lung cancer patients (correlation coefficient, −0.935; p < 0.001). The mechanisms of the dissociation between aryl hydrocarbon hydroxylase activity in pulmonary macrophages and the induction of the enzyme in peripheral blood lymphocytes in patients with lung cancer are presently unknown. For whatever reasons this phenomenon exists, it may be of diagnostic value in the early detection of lung cancer.

INTRODUCTION

Cigarette smoking is epidemiologically related to lung cancer (17, 33-35), but the mechanisms involved in the production of these carcinomas have not been delineated. One hypothesis is that malignant transformation may be initiated by exposure of the bronchial epithelium to aromatic polycyclic hydrocarbons such as BP⁴ and BA (which are present in cigarette smoke and are capable of inducing malignant tumors in laboratory animals) (2, 3, 20, 23).

The enzyme system that metabolizes polycyclic aromatic hydrocarbons in tissues is AHH, an inducible monoxygenase complex localized in the endoplasmic reticulum of many tissues in the body (6, 24, 25, 27). By hydroxylation, AHH transforms polycyclic aromatic hydrocarbons into more hydrophilic products, which are usually less carcinogenic. During this process, epoxides, which may be more potent carcinogens than are their parent hydrocarbons, are transiently produced as initial intermediate products (9, 13—16, 27, 29). The diol-epoxide, an even more reactive intermediate that may be the proximal carcinogenic hydrocarbon, is also formed through the remetabolism of initial hydroxylated dihydrodiols (32, 39). In addition, many of the primary AHH metabolites may be remetabolized by this same monoxygenase complex to compounds with enhanced carcinogenicity (39). By generating increased quantities of epoxides and/or diol-epoxides and by remetabolizing initial metabolites, high levels of AHH may be detrimental.

Individual differences in the rate of hydroxylation of polycyclic hydrocarbons could be a factor in determining susceptibility to carcinogenesis by these agents. There is evidence that AHH inducibility may be genetically determined in laboratory animals and man (12, 21, 26, 36). Higher levels

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1 The abbreviations used are: BP, benzo(a)pyrene; BA, benzanthracene; AHH, aryl hydrocarbon hydroxylase; PAM, pulmonary alveolar macrophages; JMM, Joklick's modified Spinner's minimum essential medium supplemented with 10% fetal calf serum, heparin (10 units/ml), phytohemagglutinin (10 ml/liter), and pokeweed mitogen (10 ml/liter).
of AHH induction have been reported by Kellerman et al. (22) for cultured lymphocytes from lung cancer patients than were reported for cultured lymphocytes from normal individuals, which suggests that AHH inducibility and the propensity to develop lung cancer may be associated.

Previous studies in our laboratory have demonstrated a positive correlation between inducibility of AHH in cultured lymphocytes and AHH activity in PAM from normal individuals (7). In the present investigations, we have studied AHH in both PAM and lymphocytes obtained from patients who were bronchoscooped for a variety of medical problems. With cells from patients without lung cancer, we confirmed the previously reported positive correlation between macrophage and lymphocyte AHH activity. However, with cells from patients with primary lung cancer, PAM and lymphocyte values were not positively correlated.

MATERIALS AND METHODS

Study Subjects. Lavage with 0.9% NaCl solution, which was performed through an Olympus Model 5-B2 fiberoptic bronchoscope (7) at the time of diagnostic bronchoscopy, was used to obtain PAM from 103 patients (Table 1). The purpose of the study was explained to all subjects, written informed consent was obtained on a form approved by the Baylor Institutional Review Board for Human Research. The data were grouped separately for noncancer patients (whose diagnoses included pulmonary tuberculosis, pneumonia, bronchitis, hemoptysis, benign tumor, sarcoidosis, and lung abscess) and primary lung cancer patients (whose diagnoses were confirmed histologically). None of the individuals with carcinoma had undergone prior X-ray or chemotherapy treatments. Records of all patients were carefully screened to make certain they were not receiving medication that might be expected to alter the AHH activity of their cells. All patients were hospitalized at the time of the study.

Both clinical groups were further subdivided into non-smokers and cigarette smokers. For purposes of this study, a patient was arbitrarily classified as a nonsmoker if he had not used cigarettes or tobacco products for 6 months or more. This interval was selected because previous studies in our laboratory had indicated that the high AHH activity in PAM from smokers reverts to lower nonsmoker levels within 3 months following cessation of smoking (8). In most cases the interval since termination of smoking for former smokers was much longer (means of 24 years for cancer patients and 6 years for noncancer patients). Nonsmokers who had lung cancer and had previously smoked cigarettes had a mean cumulative smoking history of 65 pack-years, whereas patients without lung cancer had a mean smoking history of 30 pack-years. Twelve of the noncancer patients and 1 of the lung cancer patients who were nonsmokers (by our criteria) had never smoked at any time in the past. The histological classifications of the tumors from smokers and nonsmokers with lung cancer appear in Table 2.

Preparation of Lymphocyte Cultures. Blood for this study was obtained on the same day from the majority of the patients whose PAM were examined. Mononuclear cells were separated from 10 ml of venous blood by Ficoll-Hypaque sedimentation (5). Cells were washed, resuspended in 10 ml 0.9% NaCl solution, and counted with a Model ZBI Coulter counter (Coulter Electronics, Hialeah, Fla.). Microscope slides for differential counts were stained with Wright-Giemsa stains (85 to 96% of the cells obtained were lymphocytes). From 1 to 3 million cells per ml of whole blood were obtained from each individual. Approximately 1 million cells were then added to each culture vial containing 5 ml JMM (F-13; Grand Island Biological Co., Grand Island, N. Y.) The mitogens were reconstituted from lyophilized preparations. All culture reagents were purchased from Grand Island Biological. The JMM and other reagents used in these experiments were prepared with Harleco Ultra Pure water. Two sets of cultures were incubated in quadruplicate. One set contained cultures to which 10 $\mu$M BA (Sigma Chemical Co., St. Louis, Mo.) had been added at 0 time to induce AHH activity; the other set contained samples without BA. Lymphocytes were cultured for 96 hr at 37° in 5% CO$_2$-air, after which 3 ml of the medium were carefully removed from each vial without disturbing the cells. The cells were resuspended in the remaining 2 ml of medium, and 40 $\mu$l were removed from each vial to count the cells and check viability. The pH of the cell suspension was adjusted to 8.0 with 0.05 n NaOH, and 25 $\mu$g BP (Sigma) in 5

### Table 1

<table>
<thead>
<tr>
<th>Subjects whose PAM were studied for AHH activity</th>
<th>Noncancer</th>
<th>Lung cancer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>56</td>
<td>47</td>
</tr>
<tr>
<td>Male</td>
<td>51</td>
<td>44</td>
</tr>
<tr>
<td>Female</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Cigarette smokers</td>
<td>36</td>
<td>36</td>
</tr>
<tr>
<td>Nonsmokers*</td>
<td>20</td>
<td>11</td>
</tr>
<tr>
<td>Age</td>
<td>20–83 yr</td>
<td>35–83 yr</td>
</tr>
<tr>
<td>Mean</td>
<td>51</td>
<td>60</td>
</tr>
</tbody>
</table>

* Individuals who had never smoked or who had not smoked for at least 6 months prior to being studied.
μl acetone were added to each vial. All cultures were further incubated for 1 hr at 37° and then assayed fluorometrically for AHH activity.

**Preparation of PAM.** PAM were obtained by lavage as described above. Aliquots of the lavage sample were pooled and centrifuged in the cold at 180 x g for 20 min. Cells were washed twice in 0.9% NaCl solution, suspended in JMM, and counted in a hemocytometer. Differential counts were made on slides stained with Wright-Giemsa (80 to 96% of the cells obtained were PAM), and cell viability was determined by trypan blue exclusion. One to 2 million cells were placed in culture vials containing 2 ml JMM (pH 8.0), and 25 μg BP in 5 μl acetone were then added to each vial. The control sample reaction was stopped immediately by adding 8 ml of 10% acetone in hexane. After incubation for 1 hr at 37°, BP metabolites were extracted from the cultures with acetone-hexane as described below.

**AHH Assay.** AHH was measured fluorometrically by determining the amount of hydroxylated BP produced by cultured lymphocytes or PAM. The reaction was stopped after 1 hr by the addition of 8 ml of 10% acetone in hexane to each cell culture. Cells were vortex mixed to suspend them and then centrifuged for 5 min at 500 x g to separate aqueous and organic phases. A portion (4 ml) of the upper (organic) phase was transferred to 1 ml of 1 N NaOH in a 13-x 100-mm test tube and mixed vigorously. The phases were separated by centrifugation for 5 min at 500 x g, and the lower (aqueous) phase was measured in the Amino SPF-125 spectrophotofluorometer (American Instrument Co., Silver Spring, Md.) equipped with a mercury lamp with excitation at 396 nm and emission at 522 nm. Quinine sulfate (1 μg/ml in 0.1 M H2SO4) was used as a working standard (excitation at 350 nm and emission at 450 nm) after comparison with authentic 3-hydroxybenzo(a)pyrene. One unit of enzyme activity is defined as the fluorescence equivalent to production of 1 pmole of 3-hydroxybenzo(a)pyrene per min. Values were corrected for an extraction efficiency of 80%. Values for AHH in cultured lymphocytes were expressed as noninduced, as BA induced, or as fold induction [(induced lymphocyte AHH activity/noninduced lymphocyte AHH activity) – 1]. The coefficients of variation for the lymphocyte and the macrophage AHH assays used in this study were 17% (N = 17) and 13% (N = 6), respectively.

**RESULTS**

To determine whether patients with lung cancer had altered levels of AHH in PAM, we compared enzyme levels in PAM from individuals with and without lung cancer (Chart 1). Enzyme values in PAM from smokers (whether or not they had lung cancer) were 5-fold higher than those in PAM from nonsmokers (p < 0.001 for noncancer and p < 0.01 for lung cancer patients; t test for nonpaired data). Among nonsmokers, patients with carcinoma had significantly higher AHH levels in PAM than did patients without lung cancer (p < 0.05).

Cultured lymphocytes from both clinical groups were examined for noninduced (basal) and BA-induced AHH activity (Charts 2 and 3). Among patients without cancer, noninduced AHH values were significantly higher in lymphocytes from smokers than in those from nonsmokers (p < 0.01). For lung cancer patients, no significant differences were observed for smoker and nonsmoker basal AHH levels in lymphocytes (p > 0.3). Among nonsmokers, an increase in basal AHH activity was observed in lymphocytes from patients with carcinoma (p < 0.001). However, similar basal levels were observed in lymphocytes from smokers with or without lung cancer (p > 0.1).

The absolute levels of BA-induced AHH activity were also
cyte AHH values. These problems have been encountered by workers who use the lymphocyte AHH assay and are apparently associated with variations in mitogen-induced metabolic activation and with individual variations in responsiveness to factors in culture media (unpublished results from our laboratory and personal communications with T. Yamauchi, Department of Biology, M. D. Anderson Hospital, Houston, Texas, and H. Guirgis, Department of Preventative Medicine, Creighton University School of Medicine, Omaha, Neb.). Initial studies in our laboratory with normal volunteers demonstrated a positive correlation between in vitro lymphocyte AHH fold induction and fresh PAM AHH activity (7). To investigate this association further, we compared lymphocyte and PAM AHH activity in the present group of patients.

Very weak correlations were observed when absolute values for AHH in BA-induced lymphocytes and fresh macrophage AHH levels were compared for smokers from either the noncancer or the lung cancer group (correlation coefficients, 0.200 for noncancer and 0.122 for lung cancer patients; p > 0.1 for both groups). However, when AHH activity in PAM and fold induction of AHH in lymphocytes from noncancer patients were compared (Charts 5 and 7), a positive correlation [similar to that noted previously with cells from normal volunteers (7)] was demonstrated with cells from both smokers and nonsmokers (correlation coefficients, 0.902 and 0.821, respectively; p < 0.001 for each group). When values for smokers without lung cancer were graphed, the majority (69%) fell within the boundaries defined by 100 milliunits/10^6 PAM and 2-fold induction of AHH in lymphocytes (Chart 5).

Because the noninduced and BA-induced lymphocyte AHH activities have been used to determine an individual's AHH inducibility, these 2 values were compared for the same individuals. Good correlations were observed when these 2 variables were examined for patients without lung cancer [correlation coefficients, 0.909 (p < 0.001) for smokers and 0.822 (p < 0.001) for nonsmokers]. Basal and induced-AHH levels in lymphocytes from cancer patients were also correlated [correlation coefficients, 0.606 (p < 0.01) for smokers and 0.537 (p > 0.05 < 0.1) for nonsmokers]. When values for basal and induced AHH activity in lymphocytes from individual patients were examined, the linear correlation observed suggested that inducibility (expressed as fold induction) would be a valid method for expressing the induction capacity of lymphocytes.

Among noncancer patients, significantly higher (p < 0.001) fold induction of AHH was observed with lymphocytes from nonsmokers than with those from smokers (Chart 4). Similar induction of AHH was noted in lymphocytes from patients with lung cancer whether or not they were smokers (p > 0.05). Among nonsmoking patients, those with carcinoma of the lung had significantly lower lymphocyte AHH fold induction than did those without lung cancer (p < 0.001), whereas differences in fold induction between cancer and noncancer smokers were not significant (p > 0.1).

Because AHH is widely distributed in tissues (25), a comparison of AHH activity in 2 separate tissues might aid in discriminating differences among individuals in the capacity of the AHH system to respond. In addition, study of AHH induction in tissue other than lymphocytes is desirable due to variability and lack of reproducibility of individual lympho-
When we examined the relationship between AHH activity in PAM and lymphocytes from smokers and nonsmokers with lung cancer, a different association between AHH levels in the 2 tissues was encountered (Charts 6 and 7). With cells from lung cancer patients who were smokers, no consistent relationship was found between AHH activity in PAM and fold induction in cultured lymphocytes. When these values were graphed, only 25% of the smokers with lung cancer had low values that fell within the boundaries defined by 2-fold induction in lymphocytes and 100 milliunits/10⁶ PAM (Chart 6). An inverse relationship was noted between AHH in PAM and lymphocytes from nonsmokers with lung cancer (correlation coefficient, −0.935; p < 0.001).

Patients with lung cancer generally had either high lymphocyte inducibility and low macrophage AHH activity or vice versa.

No association was found between AHH activity in the cells tested and dietary intake or age of either the noncancer or the lung cancer subjects. Also, no relationship was observed between the number of cigarettes consumed by a smoker and the amount of AHH activity present in his cells, regardless of whether or not the individual had carcinoma of the lung. In addition, no relationship was apparent between specific types of lung cancer and the quantity of AHH activity present among patients with cancer.

DISCUSSION

Levels of AHH activity were consistently higher in PAM from smokers than in those from nonsmokers, whether or not the subject had lung cancer. These findings confirm previous reports in which high AHH levels were induced in pulmonary tissues of animals exposed to cigarette smoke (1, 38) and in PAM from normal subjects who were cigarette smokers (7, 8). It is apparent that cigarette smoke can induce increased amounts of AHH in the cells of the respiratory tract, the portal entry for cigarette smoke.

AHH can be detected in a number of tissues in man (10, 28, 30), and cigarette smoking can induce high AHH levels in tissues physically distant from the lung, as demonstrated by elevated AHH levels in the placentas from women who smoked during pregnancy (37). Similarly, when BP is instilled into the lungs of mice, it is rapidly disseminated throughout the tissues of the animal (18). Such treatment with BP typically results in AHH induction in tissues remote from the lung (25). In the present study, we may have noted a similar induction of AHH in another tissue when we found that cultured lymphocytes from smokers have higher basal enzyme levels than cells from nonsmokers. In addition, lymphocytes from smokers are more responsive to AHH induction by BA. When basal and induced levels of AHH in cultured lymphocytes are expressed as fold induction, values for cells from smokers without cancer are significantly less than those for cells from nonsmokers without cancer. This is a reflection of increased basal AHH values in lymphocytes from smokers, which reduces the ratio of induced to noninduced AHH. These data indicate that cigarette smoking affects not only the free cells in the lungs of smokers but also cells that circulate in the peripheral blood.

Nonsmokers with lung cancer had lymphocyte basal AHH levels and fold induction values that resembled those of smokers. As with cigarette smokers, lower values for fold induction of AHH were related to a significant elevation in basal AHH activity in lymphocytes from nonsmokers with cancer.

The elevated basal AHH values in lymphocytes could be related to the past smoking history of these individuals. All but 1 of the patients with lung cancer who were classified as nonsmokers had formerly smoked, although an average of over 20 years had passed since smoking had been discontinued. Such an interval is consistent with the initiation-promotion theory of carcinogenesis (4, 31), which hypothesizes that a latent period (possibly representing a number.
of years) may be required after exposure to carcinogens, such as those present in cigarette smoke, before the actual onset or promotion of cancer occurs. This interval may be related to the length of time required for the development of lung cancer. Garland et al. (11) have shown that an average of 11 years is needed for the progression of squamous cell carcinoma from a single cell to a detectable tumor, and an even longer period (26 years) is required for the development of adenocarcinoma into a roentgenographically detectable lesion. If this is true, a former smoker may remain at increased risk of manifesting lung cancer long after active use of cigarettes has ceased. This relationship with smoking is further supported by the finding in our study that smokers and nonsmokers with lung cancer had squamous cell carcinoma of the lung as the predominant cancer type. Because nonsmokers rarely develop this type of lung cancer (33–35), it is conceivable that this finding may be related to the individual’s past smoking history.

When AHH values for freshly lavaged PAM and AHH fold induction in cultured lymphocytes were simultaneously compared for the same individual, noncancer and lung cancer patients exhibited different patterns of AHH activity. In patients without lung cancer, a positive correlation was found between enzyme values in both cell types. These findings are consistent with previous reports that show correlation between PAM enzyme values and fold induction of AHH in peripheral lymphocytes from normal, healthy individuals (7).

With PAM and lymphocytes from lung cancer patients, comparable levels of enzyme activity were usually not expressed in both types of cells. In cancer patients 1 cell type characteristically possessed moderate or high levels of AHH activity, whereas enzyme activity in the other cell type remained low. The present series was almost equally distributed into patients with moderate to high AHH activity in lymphocytes, but whose PAM activity was low, or those with low lymphocyte and high PAM AHH activity.

The present patients have been studied after the onset of clinically detectable cancer, but before any radiation or chemotherapy was administered. Prior to the development of cancer, these patients may have had different tissue levels of monooxygenase activity only to experience an alteration in the enzyme activity in either PAM or lymphocytes or both through secondary consequences of cancer.

It is possible that cancer in the lung may promote the development of abnormal cellular function. For example, changes in the blastogenic responsiveness of cultured lymphocytes to mitogenic agents may be found with advanced lung cancer (19). Such altered blastogenic responsiveness would be expected to affect AHH inducibility because the assay requires that actively dividing cells be present in cultures.

Alternatively, cells from lung cancer patients could be producing a different ratio to metabolites, which would not be detected by the normal fluorometric assay for AHH. A larger proportion of dihydrodiols, quinones, tetrahydroxy metabolites, or unknown metabolites might be produced by the metabolism of BP by the AHH system in cells from these patients. However, proportionally few quantities of the phenol derivatives may be formed, and the conventional AHH assay (which is based on the assumption that the phenol derivatives are the major BP metabolites produced by the AHH system) would give the investigator a false low AHH activity reading. Cells from lung cancer patients might actually be producing high levels of AHH, but the AHH assay used in this study would be unable to detect these levels.

Chronic exposure of the bronchial epithelium and PAM to inhaled carcinogens (such as BP and BA) in cigarette smoke may lead to conversion of these substances into epoxides (13, 15, 16) and/or diol-epoxides (39) with enhanced carcinogenicity. The binding of these transiently formed intermediate products to DNA or protein within the cell might be related to cell transformation (9, 15, 29). Further investigations will be necessary to delineate the mechanisms responsible for the dissociation between AHH values in PAM and those in lymphocytes from lung cancer patients. Whether aberrations in AHH activity in cells from cancer patients are related directly to the etiology of lung cancer or are secondary effects resulting from the onset of cancer has not been resolved.

Regardless of the problems involved in understanding AHH responsiveness in lung cancer patients and the absence of direct evidence to show a relationship between AHH induction and pulmonary carcinogenesis, lymphocyte AHH fold induction and AHH activity in PAM might be useful in the early detection of carcinoma of the lung. A long-term study of basal and induced lymphocyte AHH activity in former smokers who have not yet developed lung cancer could prove important in determining whether former smokers who have elevated basal lymphocyte AHH values have an increased propensity to develop lung cancer.

If AHH abnormalities in the cells of cancer patients are found to be present before lung cancer is clinically obvious, a comparison of lymphocyte and PAM enzyme values might be of value in the early detection of carcinoma of the lung. For patients with high risk for developing lung cancer, lymphocytes and PAM (obtained at the time of diagnostic bronchoscopy) could be tested for AHH induction. Such studies might complement cytology and radiography in the early detection of lung cancer, especially in those patients in whom bronchoscopy studies fail to establish the diagnosis.

REFERENCES


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