Dissociation between Aryl Hydrocarbon Hydroxylase Activity in Cultured Pulmonary Macrophages and Blood Lymphocytes from Lung Cancer Patients

Theodore L. McLemore, R. Russell Martin, Nelda P. Wray, Elroy T. Cantrell, and David L. Busbee

ABSTRACT

The in vitro induction of aryl hydrocarbon hydroxylase was measured in pulmonary alveolar macrophages and peripheral blood lymphocytes from 14 cigarette smokers with primary lung cancer and 15 smokers with a variety of nonneoplastic pulmonary diseases. Enzyme levels were measured in cells cultured with or without the inducer benzanthracene. For both induced and noninduced cultures, there were no differences in mean levels of cultured macrophage or lymphocyte enzyme activity between noncancer and lung cancer patients. Absolute levels and fold induction of aryl hydrocarbon hydroxylase in cultured macrophages and lymphocytes from individual noncancer patients were positively correlated [noninduced (r = 0.640, p < 0.04), induced (r = 0.801, p < 0.001), and fold induction (r = 0.942, p < 0.001)]. However, comparison of these values in cultured macrophages and lymphocytes from individual lung cancer patients demonstrated no positive correlation [noninduced (r = 0.083, p > 0.3), induced (r = 0.306, p > 0.3), and fold induction (r = −0.625, p < 0.02)]. Comparison of enzyme activity in macrophages freshly lavaged from the lung and benzanthracene-induced activity in cultured macrophages revealed a positive correlation for both noncancer (r = 0.865, p < 0.005) and lung cancer (r = 0.971; p < 0.001) patients. Similarly, comparison of enzyme activity in fresh macrophages and fold induction values in cultured macrophages was also well correlated for both groups of patients (r = 0.876, p < 0.001 for non-cancer patients; r = 0.908, p < 0.001 for lung cancer patients). Multivariant analysis of enzyme characteristics in several tissues may be useful in lung cancer diagnosis and in assessment of lung cancer risk. However, the use of a single tissue such as lymphocytes for evaluation of the relationship between aryl hydrocarbon hydroxylase activity and cancer susceptibility is questionable.

INTRODUCTION

Chronic inhalation of cigarette smoke (20, 43, 45, 46) and other exogenous tar derivatives (19) has been epidemiologically associated with the development of lung cancer. The observation that environmental carcinogens such as those present in cigarette tar may be enzymatically activated to proximal carcinogens only after entry into the host (31) has stimulated interest in biochemical mechanisms which might contribute to the metabolic activation of these compounds after their entry into the body. AHH4 is a membrane-bound monooxygenase system located in most tissues of the body (5, 8, 30, 35, 39, 50, 52). It is capable of converting hydrophobic polycyclic aromatic hydrocarbons (which are components of cigarette smoke condensate) into more hydrophilic products which are more easily excreted and which usually are less carcinogenic. In the process of metabolizing and remetabolizing these hydrocarbons, the AHH system produces transient intermediate products with enhanced mutagenic (11, 14, 40, 44, 51) and carcinogenic (13, 15, 42) activities. Production of increased quantities of intermediates, particularly the diol-epoxides (44, 49, 51), during hydrocarbon metabolism by AHH might be detrimental to individuals chronically exposed to polycyclic aromatic hydrocarbons.

The AHH enzyme system in PAM is inducible by cigarette smoke in normal individuals (7, 8) and in patients with or without primary lung cancer (33). It is also inducible in cultured mitogen-activated lymphocytes by polycyclic aromatic hydrocarbons (2, 5–7, 10, 16–18, 23, 25–27, 29, 33, 36, 37, 41). Individual variation has been observed in the degree of AHH inducibility in cultured lymphocytes (2, 7, 10, 18, 25, 33, 36) and fresh or cultured PAM (7, 8, 32–34). For individuals without lung cancer, the degree of AHH inducibility in cultured lymphocytes correlates well with absolute levels of AHH measured in fresh PAM obtained by pulmonary lavage (7, 33). For lung cancer patients, the degree of in vitro inducibility of lymphocytes and the absolute levels of AHH in freshly obtained PAM (in which the enzyme is induced in vivo by smoking) are regularly disso-

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4 The abbreviations used are: AHH, aryl hydrocarbon hydroxylase; PAM, pulmonary alveolar macrophages; BA, benzanthracene; JMM, Joklik’s modified SPF Minimum Essential Medium (F-12; Grand Island Biological Co., Grand Island, N. Y.), supplemented with 10% fetal calf serum (Lot A282021; Grand Island Biological Co.), heparin (10 units/ml, Lot 30251; Curtin-Matheson, Houston, Texas), phytahemagglutinin (10 ml/liter, Lot C663217; Grand Island Biological Co.), and pokeweed mitogen (10 ml/liter, Lot A856886; Grand Island Biological Co.), and prepared with Harleco ultra-pure water (Lot 62186); BP, benzo(a)pyrene.
The mean cumulative cigarette use was 55 pack years for cancer and 50 years for noncancer patients. All these induction of AHH induction were obtained from 15 noncancer patients with lung cancer, as well as patients with other pulmonary disorders, including pulmonary tuberculosis, sarcoidosis, pneumonia, pulmonary abscess, hemoptysis of undetermined origin, and interstitial fibrosis. Twelve patients with primary lung cancer were histologically diagnosed as having squamous cell carcinoma, while one had adenocarcinoma and another had large cell carcinoma. Prior to study, the investigative nature of the project was explained to all subjects and written informed consent was obtained on a form approved by the Institutional Review Board for Human Research at Baylor College of Medicine. All patients were studied on institutional diets at the time of this study, and charts of the patients were carefully screened to ensure that no therapy was being administered which might be expected to affect AHH levels.

**Preparation of PAM.** PAM were obtained at the time of diagnostic bronchoscopy with the use of an Olympus 5-B2 fiberoptic bronchoscope, as previously described in (8), by use of saline lavage with 50 to 100 ml of sterile 0.15 N NaCl. PAM were separated by centrifugation, washed, and resuspended at a known concentration in JMM. One million cells were placed in culture vials containing 5 ml of JMM and cultured for 24 hr with and without 10 μM BA, as previously described in (32).

Assays for fresh AHH activity were performed in triplicate with the use of 1 to 2 million PAM in culture vials containing 2 ml JMM which were assayed as described below without further culture to determine fresh macrophage AHH activity.

**Preparation of Lymphocyte Cultures.** Mononuclear leukocytes were separated from 10 ml venous blood by Ficoll-Hypaque sedimentation (4). Cells were washed with heparinized saline (10 units/ml) and resuspended in JMM. The cells were then quantitated with the use of a Model ZBI Coulter Counter (Coulter Electronics, Inc., Hialeah, Florida), and differential counts were made from slides stained with Wright-Giemsa stain. These preparations were typically 85 to 90% lymphocytes. Leukocyte preparations (0.5 × 10⁷ cells/ml) were then cultured with and without 10 μM BA for 96 hr at 37°C in a mixture of CO₂ and air, as previously described in (32), and AHH activity was measured.

**Fluorometric Analysis of AHH.** AHH was measured in PAM and lymphocytes after incubation for 1 hr with BP as previously described in (33). Data for AHH activity was expressed in milliuunits/10⁶ cells, where 1 unit of enzyme activity is equivalent to the production of 1 pmol per min of 3-hydroxybenzo(a)pyrene. The total number of lymphocytes obtained after 96 hr of culture was used to calculate the enzyme activity in lymphocytes. Values were adjusted for an extraction efficiency of 70%. Values for AHH in cultured PAM and lymphocytes were expressed as noninduced (cultured without BA), induced (cultured in the presence of BA), or fold induction [(induced AHH value/noninduced AHH values) − 1]. The fluorescence values were at least 4 times background where the background had approximately 20% variation from day to day.

**RESULTS**

Intraindividual coefficients of variation for AHH in PAM and lymphocytes from one nonsmoking male subject assayed on several occasions are presented in Table 1. The lymphocytes from this individual had a coefficient of variation similar to that reported in other persons (18, 27). Although there were fewer replicates of PAM, the variation in these cells was less than that in lymphocytes.

Chart 1 shows the levels and induction of AHH in cultured lung macrophages and cultured lymphocytes. The cells were obtained from noncancer patients or from patients with primary lung cancer. The mean levels of AHH were not different between noncancer and cancer patients for either noninduced or BA-induced cells. In both groups of patients the noninduced PAM had the same levels of AHH as noninduced lymphocytes. Similarly, the BA-induced PAM had about the same AHH levels as did BA-induced lymphocytes. A comparison of mean fold induction values of AHH in either cell type also showed no difference between noncancer and cancer patients.

Multivariant analyses of the data from individuals were also performed. The low levels of AHH in noninduced PAM and lymphocytes made some comparisons difficult. Nevertheless, the regression of noninduced PAM AHH on noninduced lymphocyte AHH showed a correlation (r = 0.640; p < 0.04). In cells from lung cancer patients, no such correlation was found (r = 0.083). Other comparisons are presented in Charts 2, 3, and 4.

The comparison in Chart 2 shows that in noncancer patients, AHH in BA-induced lymphocytes was correlated with AHH in BA-induced cultured PAM; (r = 0.801, p < 0.001). In contrast, AHH in BA-induced PAM and lymphocytes were dissociated for lung cancer patients; (r = 0.306, p > 0.3). Nine of the 15 noncancer patients had AHH levels greater than 100 milliuunits/10⁶ cells in both cell types, while no cancer patients demonstrated AHH levels greater than this in both cell types. However, all but 3 lung cancer patients exhibited high enzyme activity in either PAM or lymphocytes.

Computation of induction ratios (17, 26, 36, 37), or fold variation...
Table 1

<table>
<thead>
<tr>
<th>AHH characteristic</th>
<th>PAM</th>
<th>Lymphocytes</th>
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<tbody>
<tr>
<td></td>
<td>(\bar{X})</td>
<td>S.D.</td>
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<tr>
<td>Fresh(^c)</td>
<td>13</td>
<td>1.68</td>
</tr>
<tr>
<td>Noninduced(^d)</td>
<td>20</td>
<td>2.76</td>
</tr>
<tr>
<td>BA-induced(^e)</td>
<td>54</td>
<td>5.54</td>
</tr>
<tr>
<td>Fold induction(^f)</td>
<td>1.75</td>
<td>0.195</td>
</tr>
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</table>

\(^a\) Coefficient of variation.
\(^b\) Number of determinants.
\(^c\) AHH activity (expressed as milliunits/10\(^6\) cells) representing the in situ enzyme activity in macrophages freshly lavaged from the lung.
\(^d\) AHH activity (expressed as milliunits/10\(^6\) cells) for cells cultured for 96 hr without BA in the medium.
\(^e\) AHH activity (expressed as milliunits/10\(^6\) cells) for cells cultured for 96 hr with BA in the medium.
\(^f\) AHH induction values represent (BA-induced AHH/noninduced AHH) - 1.

induction (7, 10, 33) has been used previously to describe inducibility of AHH in cultured cells. A comparison of AHH fold induction in cultured PAM and lymphocytes is made in Chart 3. The pattern of dissociation is again observed in cells from lung cancer patients \((r = -0.625, p < 0.02)\), but noncancer patients had very good correlation for in vitro inducibility in PAM and lymphocytes \((r = 0.942, p < 0.001)\). Only 2 of the 14 lung cancer patients demonstrated inducibility less than 2-fold in PAM and lymphocytes, while 8 of the 15 noncancer patients had inducibility of less than 2-fold in both cell types. All of the noncancer patients had paired inducibilities which fell within the 95% confidence limits of the regression line (shaded area). However, only 2 lung cancer patients exhibited inducibilities within the 95% confidence limits established for noncancer patients.

In an earlier study we examined AHH activity in only fresh PAM and cultured lymphocytes (33). This previous study demonstrated a dissociation between fresh PAM AHH activity and lymphocyte AHH inducibility for individual lung cancer patients and a positive correlation of their values in individual noncancer patients. This study confirmed these previous observations with the use of a smaller group of patients (Chart 4; \(r = 0.948\) and \(p < 0.001\) for noncancer patients and \(r = 0.306\) and \(p > 0.3\) for lung cancer patients).

Chart 1. Induction of AHH in cultured PAM and peripheral blood lymphocytes from cigarette smokers. One unit of enzyme activity equals production of 1 pmol of 3-hydroxybenz(a)pyrene per min. Noninduced values represent cells cultured without an inducer in the medium, whereas BA-induced values represent cells cultured in the presence of the inducer BA. Bars, the mean ± S.E.; O, PAM value; A, lymphocyte value.

Chart 2. Comparison between AHH levels in BA-induced PAM and lymphocytes from individual noncancer or lung cancer patients \((r = 0.801\) and \(p < 0.001\) for noncancer patients and \(r = 0.306\) and \(p > 0.3\) for lung cancer patients).

Chart 3. Comparison of AHH fold induction values in PAM and lymphocytes from noncancer or lung cancer patients \((r = 0.942\) and \(p < 0.001\) for noncancer patients and \(r = -0.625\) and \(p < 0.02\) for lung cancer patients). The regression line and the mean ± 2 S.E. of estimate lines for the noncancer patient values appear on both graphs.
patients and \( r = -0.466 \) and \( p > 0.05 \) for lung cancer patients). All values for noncancer patients fell within the 95% confidence limits of the regression line, whereas the values of only 4 of the 14 lung cancer patients were located within these confidence limits.

We were concerned that the in vivo induction of AHH in fresh PAM by cigarette smoke might not reflect the in vitro induction of the enzyme in PAM by BA. We, therefore, compared the levels of AHH in freshly harvested PAM with BA-induced enzyme levels in cultured PAM from individual cigarette smokers with and without lung cancer (Chart 5). This comparison revealed a high degree of correlation between AHH values for either noncancer \( (r = 0.865, p < 0.005) \) or lung cancer patients \( (r = 0.971, p < 0.001) \). Similarly, the levels of AHH in freshly lavaged PAM from all patients studied correlated with AHH fold induction in cultured PAM (Chart 6; \( r = 0.876 \) and \( p < 0.001 \) for noncancer patients and \( r = 0.908 \) and \( p < 0.001 \) for lung cancer patients). These data indicate that levels of AHH in freshly lavaged PAM from individual smokers with or without lung cancer reflect the in vitro inducibility of these cells.

**DISCUSSION**

In recent years, investigators have attempted to establish a relationship between carcinogen metabolism and lung cancer risk in humans. In animal studies, a clear association has been demonstrated between high AHH inducibility and susceptibility to carcinogenesis by certain polycyclic aromatic hydrocarbons (28). Unfortunately, this attractive relationship has not been equally demonstrable in humans. The prototype tissue for evaluation of AHH activity in human studies has been peripheral blood lymphocytes. Initial studies evaluating the in vitro inducibility of AHH in lymphocytes from lung cancer patients who are cigarette smokers have been unable to conclusively establish whether increased AHH levels are associated with an increased risk of developing lung cancer. The majority of investigations have provided evidence suggesting that higher AHH inducibility is associated with lung cancer (10, 12, 16, 26, 41). However, some reports have been unable to confirm this correlation (23, 36).

Inconsistencies in the results obtained from these laboratories might be related to fluctuations in AHH levels in lymphocytes. There is approximately a 20% coefficient of variation within individuals from day to day (18, 27), and great variability is observed for AHH values among different laboratories (6, 17, 25, 29, 33, 37). The variability of these enzyme values is attributable to a number of factors, including different methods of culturing, identifying, and counting lymphoblasts, as well as differences in AHH assay procedures among various laboratories.

The current data demonstrate that PAM AHH has less intraindividual variation than lymphocyte enzyme levels. We have, therefore, attempted to attenuate the problems associated with lymphocytes by using simultaneous analyses of AHH in multiple tissues (e.g., PAM and lymphocytes). Earlier studies using cells obtained from a given individual on the same day have documented a good agreement between values for AHH in fresh PAM and cultured lympho-
cytes from healthy volunteers (7) or noncancer patients (33). However, when similar measurements were compared in individual lung cancer patients, AHH values in cells from the 2 different tissues were dissociated (33). Until this study was performed, there was uncertainty as to whether enzyme induction in lymphocytes represented the capacity for induction in other tissues. These present data establish that AHH induction in cultured lymphocytes reflects the in vitro inducibility of the enzyme in macrophages from noncancer patients. These findings also suggest that measurement of AHH in more than one tissue obtained on the same day from a given individual may distinguish differences between noncancer and lung cancer patients. This distinction is most apparent when fold induction for both lymphocytes and PAM from individual patients is examined. Since this dissociation between AHH levels is consistently found in lung cancer patients, a comparison between AHH levels in cultured PAM and lymphocytes could be of diagnostic aid when lung cancer is suspected, but bronchoscopy fails to establish a definitive diagnosis.

Because AHH levels in cells from lung cancer patients are apparently abnormal, we were concerned that AHH levels in fresh PAM may not reflect the in vitro enzyme inducibility of these cells. In addition, we could not be certain that the in vivo induction of AHH by cigarette smoke was comparable to the in vitro induction of PAM by BA. Comparison of AHH in fresh and cultured PAM yielded a positive correlation with cells from both noncancer and lung cancer patients, suggesting that the in vivo enzyme induction reflects the induction of the enzyme in cultured PAM, even after the development of lung cancer. Since PAM are maximally induced in vitro by BA with the use of our culture procedure (32), these data suggest that AHH is also maximally induced in PAM in the lungs of smokers by cigarette tars. For individuals without evidence of lung cancer, the inducibility of AHH in lymphocytes appears to be a good indication of the inducibility of the enzyme by cigarette smoke in the lungs.

We have not yet delineated the mechanisms responsible for the observed dissociation between AHH values in cultured PAM and lymphocytes from individual lung cancer patients. The presence of soluble inhibitory or stimulatory factors in lavage supernatant is probably not responsible for deviations in PAM AHH induction. Dissociation between values for PAM and lymphocytes is noted whether fresh or cultured PAM are examined. Fresh and cultured AHH values for macrophages remain correlated regardless of whether the patient has lung cancer. It is unlikely that one cell type underwent suppression of AHH activity in some individuals and enhancement of AHH activity in others, especially since this variability was not present in the comparable group of cigarette smoking subjects without evidence of cancer. It is also unlikely that the effects observed are some type of aberration related to cigarette smoking, since comparison between AHH in PAM and lymphocytes from nonsmokers with and without lung cancer also demonstrates distinct differences between the 2 patient groups (33).

It is probable that no single mechanism is operant and that abnormalities may involve PAM in some patients and lymphocytes in others. Abnormalities involving macrophage function have been described in individuals with lung cancer (1, 9, 24), and dysfunction of lymphocyte AHH in lung cancer patients has also been previously recognized (23, 34, 36, 37). It has not been determined whether this alteration is associated with the AHH enzyme complex itself or whether it is related to changes in lymphocyte responsiveness to mitogens, as previously reported in advanced lung cancer patients (22). Paigen et al. (36) attempted to circumvent this problem by analyzing AHH in lymphocyte cultures from families of lung cancer patients. Their report was unable to confirm an association between AHH inducibility and lung cancer, but the sample size presented was much too small to provide definitive information.

Changes in the functions of a variety of enzyme systems have been noted in the cells from patients with carcinoma (47, 48). It is not unreasonable to assume that inherent alterations in the PAM or lymphocyte AHH system could occur as well. In fact, there is evidence suggesting that polycyclic aromatic hydrocarbon metabolism by the AHH system might be altered in cells from lung cancer patients. Comparisons between interindividual binding of BP metabolites to DNA in cultured human bronchi from patients with and without lung cancer have shown that individuals with lung cancer have BP metabolites which more readily bind to DNA (21). Other binding studies, performed on similar human bronchial tissues, indicate that the major metabolite bound to DNA is the proximal carcinogenic hydrocarbon 7β,8α-dihydrodiol-9α-10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (52). These 2 studies suggest that the increased binding of BP metabolites to DNA from lung cancer patients could be due to an increase in the rate of formation of the proximal carcinogenic diol-epoxide in the bronchial epithelium of these individuals. In addition, unpublished results from our laboratories (T. L. McLemore, D. L. Busbee, and E. T. Cantrell) have demonstrated that the major BP metabolites produced by PAM from noncancer patients are the phenolic derivatives, whereas these do not appear to be the major metabolites produced by PAM from certain lung cancer patients. Furthermore, recent high-pressure liquid chromatography studies of BP metabolism in PAM from certain lung cancer patients suggest that increased quantities of the tetrols, diols, and quinones, with relatively small amounts of the phenolic metabolites, are produced by cells from these individuals (3). Abnormal values for AHH in PAM (or lymphocytes) from lung cancer patients in this study might therefore be the result of alteration in metabolism of polycyclic hydrocarbons by the AHH enzyme system. The fluorometric assay used in these studies is based on the quantitation of the phenolic derivatives of BP (the substrate used for the assay). If cells from certain lung cancer patients were to metabolize BP to larger quantities of other metabolites such as diols, quinones, etc., these products would not be quantitated by the fluorometric method used in this assay, and resulting AHH values would be falsely low. Total BP metabolism could therefore be similar in PAM and lymphocytes from lung cancer patients, but because of alteration in the levels of the phenolic derivatives, fluorometric analyses of AHH would demonstrate a lack of correlation of AHH activity between the 2 types of tissues. Further studies will be necessary to examine this possibility.

The current study does not resolve the question of...
whether high levels of AHH are detrimental to cigarette smokers. However, the majority of patients with lung cancer have high levels of AHH in either PAM or lymphocytes. If these levels were high in both types of cells prior to the onset of cancer, then large quantities of AHH might be harmful through increased production of polycyclic aromatic hydrocarbon metabolites with enhanced mutagenic (11, 14, 40, 44, 51) and carcinogenic (13, 15, 42) activities. However, when AHH is not elevated until after the onset of cancer, this hypothesis for the relationship between AHH activity and carcinogenesis would not apply.

Thus, using the peripheral blood lymphocyte for the determination of AHH in patients with lung cancer is questionable. Although previous studies using lymphocytes have shown high AHH inducibility related to the presence of lung cancer (10, 12, 16, 26, 41), these results might not be valid, since AHH levels in lymphocytes from lung cancer patients do not necessarily reflect levels of the enzyme in PAM or freshly excised lung tissue (34). Considering these limitations, retrospective studies using patients with lung cancer might not be an appropriate method for determining the relationship between AHH inducibility and chemical carcinogenesis in the lung. Rather, this would require prospective population studies using individuals at high risk of developing lung cancer, with classification of these individuals according to AHH inducibility prior to the development of clinically apparent cancer.

As investigators continue to study AHH as a possible etiologic determinant of chemical carcinogenesis in the lungs of cigarette smokers, it is becoming more apparent that use of the comparison of AHH levels in multiple tissues from the same individual might be more reliable than measurement of the enzyme in any single tissue.

REFERENCES


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