Expression of the Tumor Aldehyde Dehydrogenase Phenotype during 2-Acetylaminofluorene-induced Rat Hepatocarcinogenesis

Ronald Lindahl, Susan Evces, and Wen-Lu Sheng

ABSTRACT

In aromatic amine-induced rat hepatomas, the aldehyde dehydrogenase (AIDH) phenotype is qualitatively and quantitatively different from that of normal liver. To identify the mechanism(s) underlying the expression of the tumor-specific AIDHs, we have followed the time course of appearance of the new phenotype during hepatoma formation in Sprague-Dawley rats following brief dietary exposures to 2-acetylaminofluorene (0.02%; 32 days). Tumor promotion by phenobarbital (0.05% in the diet) was also used to compare the effects of a variety of tumor induction protocols on the AIDH phenotype. No change in the AIDH phenotype is detectable by total activity assay, gel electrophoresis, isoelectric focusing, or immunochemical methods during or following exposure to carcinogen or promoter until tumors are grossly observed in liver. Concomitant with tumor appearance, the tumor-specific AIDH phenotype appears. The phenotypic change is limited to the tumor; morphologically and histologically normal liver directly adjacent to the tumor and normal lobes of a tumor-bearing liver do not possess the tumor AIDH phenotype. No correlation exists between tumor size and the degree of deviation of the AIDH phenotype from normal. Nor is there any correlation between the degree of AIDH phenotype deviation and the histology of the various tumors observed. We conclude that the tumor-specific AIDH phenotype is not associated with altered liver metabolism due directly to carcinogen or promoter exposure. Rather, the mechanism of this phenotypic change requires that transformation-associated, stable genetic changes occur in the cells affected by carcinogen that are later expressed as the altered AIDH phenotype.

INTRODUCTION

In normal rat liver, AIDH activity [aldehyde:NAD(P) oxidoreductase (EC 1.2.1.3 and 1.2.1.5)] is distributed among 3 isozymes found in mitochondria and microsomes (12, 22). These isozymes differ in substrate and coenzyme preference, substrate Km, immunochemical properties, and sensitivity to various inhibitors.

Recent work in our laboratories has shown that hepatomas induced in male Sprague-Dawley rats by AAF have a unique AIDH phenotype (5, 10–12, 14). The phenotype is characterized by an increase in total AIDH activity due to the appearance of several cytosolic isozymes not detectable in normal liver. In contrast to the normal liver AIDH isozymes, these tumor isozymes are very active with aromatic aldehyde substrates and NADP as coenzyme (10, 14). They also have distinct electrophoretic mobilities, isoelectric points, and immunochemical properties. The tumor AIDH phenotype is not due to reexpression of fetal AIDH activity nor to the anomolous expression of genes controlling AIDH activity in some other adult tissue (10).

Two additional cytosolic AIDHs can be induced in normal rat liver. One by PB in certain genetically defined lines of rats (1, 9); the other by a variety of xenobiotics including TCDD in both PB-responsive and -nonresponsive lines (2, 3, 15). Although the tumor AIDH isozymes differ in a number of properties from the TCDD-inducible isozyme, the TCDD-inducible isozyme is immunochemically identical to at least one of the tumor isozymes (15). Neither the tumor nor TCDD-inducible isozymes is related to the PB-inducible isozyme (3, 15).

Maximal induction of AIDH activity occurs within 3 days of PB exposure and within 14 days of TCDD exposure (1, 3). Inasmuch as some of the hepatomas possessing the tumor AIDH phenotype were induced by a tumor induction protocol using PB (18) and at least one tumor AIDH isozyme and the TCDD-inducible normal liver isozyme are identical immunochemically (15), it was of interest to determine the time course of appearance of the tumor AIDH phenotype during AAF-induced hepatocarcinogenesis.

The purpose of this study was to determine whether the tumor AIDH phenotype is the result of altered liver metabolism due directly to carcinogen and/or promoter exposure or the result of transformation-associated, stable genetic changes induced in cells affected by carcinogen.

MATERIALS AND METHODS

Male Sprague-Dawley rats (CD/SD) from Charles River Breeding Laboratories (Wilmington, Mass.) were used. AAF (Aldrich Chemical Co., Milwaukee, Wis.) and PB (Sigma Chemical Co., St. Louis, Mo.) were incorporated into the control diet (Wayne Lab Blox) at 0.02% (w/w) and 0.05% (w/w), respectively, by Teklad Test Diets (Madison, Wis.). The appropriate diet and tap water were provided ad libitum throughout the experiment.

The tumor induction protocol of Peraino et al. (18) was used. Total experiment duration was 463 days. Animals, divided into 4 groups, entered the experiment at 20 days of age. One group (AAF-control) received the carcinogen-containing diet for 32 days followed by the control diet for the duration of the experiment. A second group (AFF-PB) received carcinogen for 32 days and were then transferred to the PB-containing diet. A third group (PB) received the PB-containing diet beginning at Day 1. Exposure to PB was until Day 438, after which animals in both the AAF-PB and PB groups were transferred to the basal diet. A fourth group (control) received the basal diet without addition for the entire experiment.

Two animals from the AAF-control and AAF-PB groups were sacrificed every fourth day from Day 3 to 124 and every seventh day from Day 131 to 215, and 3 animals each were sacrificed every 30th day from Day 249 to 463. Two animals from the PB and control groups

1 This work was supported by Grant CA-21103 from the National Cancer Institute. This work was presented at the 72nd Annual Meeting of the American Association for Cancer Research, April 27 to 30, 1981, Washington, D. C. (16).

2 To whom requests for reprints should be addressed.

3 The abbreviations used are: AIDH, aldehyde dehydrogenase; AAF, 2-acetylaminofluorene; PB, phenobarbital; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

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were sacrificed every 14th day from Day 1 to 215, and 3 animals each were sacrificed every 30th day from Day 249 to 463. This schedule was followed unless severe illness of any animal made death prior to the next scheduled sacrifice likely.

Animals were killed by suffocation as a result of sublimation of solid CO₂ in a large container. The liver was removed, washed free of blood, and carefully examined for any gross abnormalities. A piece of the medial lobe and biopsies of any gross lesions were fixed in 10% buffered formalin. The remaining tissue was quick frozen in a solid CO₂:acetone bath and stored at −80° until needed.

After thawing, normal livers were prepared as 33% homogenates in 60 mM sodium phosphate buffer, pH 8.5, containing 1 mM EDTA and 1 mM β-mercaptoethanol. Tumors were prepared as 20% homogenates in the same buffer. Homogenates were made to 1% with Triton X-100 (v/v final concentration) and incubated at 0° for 30 min. The solubilized homogenates were centrifuged at 48,000 × g for 30 min, and the resulting supernatants were drawn off for AIDH determinations.

AIDH activity was assayed at 25° by monitoring the change in A₃₄₀ caused by NADH or NADPH production during the oxidation of aldehyde substrate in a modification of the assay described previously (10). The reaction mixture contained 1.0 ml of 60 mM sodium phosphate buffer, pH 8.5, containing 1 mM EDTA and 1 mM mercaptoethanol, 1.0 ml of 16 mM NAD or NADP, 0.025 ml of 100 mM propionaldehyde or a saturated benzaldehyde solution, unless otherwise stated, 10 or 25 µl of enzyme, and water to 3.0 ml. Enzyme was added last, and the increase in A₃₄₀ was recorded at 1-min intervals for 5 min. All appropriate corrections were made for enzyme-independent changes in A₃₄₀.

On occasion, pyrazole (100 µM) was included in assays to inhibit alcohol dehydrogenase. No change in AIDH activity was seen when pyrazole was present. Activities were expressed as µU/mg protein (1 µU = 1 nmol NAD(P)H produced per min). Protein concentrations were determined by the method of Lowry et al. (17) with bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis was performed at 4° using the Canalco (Ames Co., Elkhart, Ind.) system and reagents, except that both gels and well buffer contained 0.25% (v/v) Triton X-100. Gels were stained for AIDH as described previously (10), using 40 mg nitroblue tetrazolium, 55 mg NAD and NADP, 2.0 mg phenazine methosulfate, and 75 µl of substrate in 50 ml of sodium phosphate buffer, pH 7.5. Control gels were stained without added substrate to test for substrate-independent staining or without coenzyme to test for aldehyde oxidase (6).

Analytical isoelectric focusing in polyacrylamide gel slabs was done with the LKB (LKB Produkter AB, Bromma, Sweden) apparatus and methods. The gels were stained for AIDH as described above.

Ouchterlony double diffusions were performed as described previously (14). For the immune reaction, slides were incubated in a humid environment for 24 hr at 37° and stained for protein with 0.1% Ponceau S in 10% glacial acetic acid or for AIDH activity as described above.

Histopathological examinations were performed on hematoxylin and eosin-stained tissue sections. The classification of lesions observed followed the established criteria for rat hepatomas (7).

RESULTS

No significant differences were observed in liver or body weight gain between groups during the experiment (Chart 1). Significant increases in the liver:body ratio did occur relative

<table>
<thead>
<tr>
<th>Treatment* group</th>
<th>AAF</th>
<th>PB-Control</th>
<th>No. of animals examined</th>
<th>First tumor observed (day)</th>
<th>No. of animals sacrificed post-first tumor</th>
<th>No. of tumors with tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAF-Control</td>
<td>32</td>
<td>431</td>
<td>129</td>
<td>305</td>
<td>19</td>
<td>12/7</td>
</tr>
<tr>
<td>AAF-PB</td>
<td>32</td>
<td>406 PB</td>
<td>25 Control</td>
<td>129</td>
<td>274</td>
<td>24/12</td>
</tr>
<tr>
<td>PB only</td>
<td>0</td>
<td>438 PB</td>
<td>25 Control</td>
<td></td>
<td></td>
<td>51</td>
</tr>
<tr>
<td>Control only</td>
<td>0</td>
<td>463</td>
<td></td>
<td></td>
<td></td>
<td>51</td>
</tr>
</tbody>
</table>

* See "Materials and Methods" for details of treatments and sacrifice schedules.
to controls in animals receiving AAF and/or PB during approximately the first 100 days of treatment (Chart 1). For the duration of the experiment, the liver:body ratio in animals maintained on the PB-containing diet was generally slightly higher than in AAF-control or control animals.

The livers of animals receiving AAF showed numerous acidophilic, basophilic, and clear-cell regions of cellular alteration and considerable ductular proliferation. Among foci, acidophilic regions predominated. Livers of animals which received PB following AAF showed an increase in the number of foci and retained ductular proliferation. There was also considerable hypertrophy of hepatocytes, generally associated with regions of ductular proliferation. As early as Day 100, livers of AAF-PB animals possessed microscopically detectable neoplastic nodules. The livers of animals transferred to the unsupplemented control diet retained numerous foci, but bile duct proliferation ceased and generally remained considerably less than in AAF-PB livers. Animals receiving only PB showed considerable hepatocyte hypertrophy throughout the study. In these animals, the hypertrophy was associated with ductular proliferation, although the hyperlasia was not as severe as in AAF-PB livers. No foci, nodules, or other lesions were observed in sections from PB or control livers.

A total of 36 tumors occurring in 19 animals was examined for the AIDH phenotype in this study (Table 1). Numerous smaller tumors (<1 to 2 mm in diameter) were also observed, especially in AAF-PB livers. However, their small size precluded examining both the AIDH phenotype and histology of these lesions, and they were not included. The tumors included neoplastic nodules and a variety of hepatocellular carcinomas of varying degrees of differentiation.

In grossly normal liver, total AIDH activity did not differ significantly between treatment groups at any time (Chart 2; Table 2). A slight elevation in AIDH activity with propionaldehyde and NAD occurred in both the AAF-control and AAF-PB groups immediately following transfer to either the control or PB-containing diet; however, the activity with benzaldehyde and NADP, the major marker of the tumor AIDH phenotype, was not elevated in any normal liver at any time (Chart 2). Moreover, no change in AIDH phenotype was detectable by the 3 additional methods used, gel electrophoresis, isoelectric focusing, and immunochemical methods, in any grossly normal liver examined during or following AAF or PB exposure.

Concomitant with the appearance of tumors macroscopically, the tumor AIDH phenotype appeared (Chart 3; Table 2). The phenotypic change was limited to a tumor. Morphologically normal liver directly adjacent to a tumor (Fig. 1, AFF/PB 135), tumor-bearing lobes, and normal lobes of a tumor-bearing liver (Figs. 1 and 2; Table 2) did not possess the tumor AIDH phenotype. There was no correlation between tumor size (as an approximate indicator of tumor age) and the degree of deviation of the AIDH phenotype from normal. For the tumors

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>Activityb</th>
<th>Activityc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td>Tumor</td>
<td>Host liver</td>
</tr>
<tr>
<td>Treatmenta</td>
<td>Activity</td>
<td>Activity</td>
</tr>
<tr>
<td>AAF-Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>129</td>
<td>20.3 ± 0.3</td>
<td>39.3 ± 11.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(12.9–149.9)</td>
</tr>
<tr>
<td>AAF-PB</td>
<td>20.5 ± 0.3</td>
<td>84.8 ± 14.6</td>
</tr>
<tr>
<td></td>
<td>(18.3–286.9)</td>
<td>(6.5–1016.2)</td>
</tr>
<tr>
<td>PB only</td>
<td>20.8 ± 0.6</td>
<td>7.6 ± 0.3</td>
</tr>
<tr>
<td>Control only</td>
<td>20.3 ± 0.4</td>
<td>7.2 ± 0.3</td>
</tr>
</tbody>
</table>

a See "Materials and Methods" for details.

b Activity is significantly higher than in host liver (p < 0.01).

c Activity is significantly higher than in AAF-control tumors (p < 0.05).
Chart 3. Distribution of total AIDH activity in hepatic tumors induced by AAF with or without PB promotion. □, host liver; □□□□□□□□, tumor(s) from the same liver examined for AIDH phenotype. Numbers on abscissa, animal identification numbers; increasing number corresponds to successively later sacrifice dates at which animals with tumors were found as indicated in Chart 2; P/NAD, activity with propionaldehyde and NAD; B/NADP, activity with benzaldehyde and NADP.

Fig. 1. Polyacrylamide gel isoelectric focusing of various normal rat liver and tumor AIDHs. Samples were electrofocused for 5 hr at 4° and stained for AIDH with benzaldehyde and NADP. Samples include preparations of at least one normal and/or host (H) liver from each of the 4 treatment groups, a number of tumors (T) induced by AAF or AAF-PB, and the liver of an animal exposed to TCDD, a known inducer of rat liver AIDH when administered acutely. Also included were sections taken 0 to 5 and 5 to 10 mm from a well-defined tumor mass (AAF/PB 135). Numbers, animal identification numbers. The isoelectric points of all activities are approximately pH 7.0.
examined, there was no correlation between tumor histology and the expression of the tumor AIDH phenotype.

Differences in AIDH activity were seen in tumors induced in different animals within the same treatment group (Chart 3), as well as between different tumors within a single liver (Chart 3; Figs. 1 and 2). In all cases (e.g., AAF/PB 131, Chart 3 and Figs. 1 and 2), there was an excellent correlation between total activity, isozyme patterns on gel electrophoresis and isoelectric focusing, and reactivity with anti-hepatoma AIDH serum.

Differences in the expression of the tumor AIDH phenotype also extended to different treatment groups (Chart 3; Table 3). In tumors induced in animals receiving AAF followed by PB, 80% (19 of 24) had significantly higher NADP-dependent AIDH activity than their host livers, and only one tumor did not possess NADP-dependent activity greater than its host liver. In tumors induced by AAF only, 50% (6 of 12) had benzaldehyde-NADP-dependent activity significantly greater than their corresponding host liver, and 5 tumors had NADP-dependent activity considerably less than host liver.

Among tumors induced in animals receiving only AAF, 50% showed no evidence of the tumor AIDH phenotype by any criteria, while only 20% of the tumors from the AAF-PB group lacked any evidence of the tumor AIDHs (Table 3). However, of the 36 tumors examined, only one (Chart 3B, AAF/PB 129, Tumor 1) could not be clearly distinguished from its host liver. All other tumors showed a noticeable phenotypic deviation from normal liver as an obvious increase or decrease in NAD- and/or NADP-dependent AIDH activity, coupled with changes also demonstrable by at least one other method (Chart 3).

Table 3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tumors with elevated B-NADP activity</th>
<th>Tumors with PAGE phenotype of tumor AIDHs</th>
<th>Tumors cross-reacting with anti-hepatoma AIDH serum</th>
<th>Tumors with no evidence of tumor-specific AIDH phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAF-PB</td>
<td>6/12</td>
<td>3/12</td>
<td>4/12</td>
<td>6/12</td>
</tr>
</tbody>
</table>

* See “Materials and Methods” for details.
* Number of tumors per total that have activity with benzaldehyde and NADP at least 2.5 times higher than host liver.
* Number of tumors per total which possess the tumor-specific multiple isozyme pattern according to polyacrylamide gel electrophoresis with very high benzaldehyde-NADP activity characterized as tumor specific.
* Number of tumors per total which cross-react with anti-hepatoma-specific AIDH serum in Ouchterlony double diffusions.
* Number of tumors per total which do not possess the tumor-specific AIDH phenotype by at least one of the 3 principal criteria used.

DISCUSSION

These results indicate that the changes in AIDH activity occurring during AAF-induced hepatocarcinogenesis are tumor specific. The change in phenotype occurs concomitant with the appearance of tumors, several months after carcinogen exposure. The phenotypic change is limited to the tumor; neither normal liver directly adjacent to the tumor nor normal lobes of a tumor-bearing liver possess the tumor AIDH phenotype. Inasmuch as this phenotype is characterizable by a variety of qualitative and quantitative methods of varying degrees of sensitivity, we are confident that, using the protocol employed here, no change occurs in AIDH activity during hepatocarcinogenesis prior to the appearance of the tumor-specific phenotype. This is significant since a number of changes, both gross morphological and histological, occur prior to tumor appearance without detectable change in AIDH activity.

Moreover, additional studies in this laboratory have identified significant changes in ornithine decarboxylase and RNA polymerase and y-glutamyltranspeptidase activity which are correlated with various of these morphological and histological changes very early in hepatocarcinogenesis. In toto, our observations suggest that changes in AIDH activity may be a useful marker for one or more changes occurring late in the progression toward neoplasia.

The expression of the tumor-specific AIDH phenotype is in contrast to the induction of 2 additional novel rat liver AIDH phenotypes by other xenobiotics PB (1) and TCDD (3), both of which cause the appearance of new AIDH isozymes with days of a single exposure. The contrast in induction is especially interesting in the case of the TCDD-inducible isozyme. We have shown previously that at least one of the tumor-specific isozymes and the TCDD-inducible AIDH are very closely related immunochemically (15). However, they differ in their response to the AIDH inhibitor, disulfiram (13), and their inductions clearly follow entirely unrelated time courses.

Since chronic dietary exposure to PB did not induce the NAD-specific, PB-inducible AIDH, we conclude that the

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Fig. 2. Polyacrylamide gel electrophoresis of tumor AIDHs. This liver (Chart 3B, AAF/PB 131) was one of several which had multiple tumors at sacrifice. Three tumors and morphologically normal host liver were compared. Gels were electrophoresed for 3 hr at 2.5 mA/gel at 4° and stained for AIDH activity. Gels 1 and 2, host liver 131; Gels 3 and 4, 131 Tumor 1; Gels 5 and 6, 131 Tumor 2; Gels 7 and 8, 131 Tumor 3. Odd-numbered gels were stained for AIDH with propionaldehyde and NAD. Even-numbered gels were stained with benzaldehyde and NADP.

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Sprague-Dawley rats used in this study were genetically nonresponsive for PB AIDH inducibility (1). This is in contrast to a previous study in which we found that PB induced an NAD-specific AIDH in a significant proportion (14 of 40) of Sprague-Dawley rats exposed previously to AAF but in none of the PB-fed (0 of 12) controls. Although some variability of tumor-specific AIDH phenotype expression was anticipated, one unexpected source was the striking variability in benzaldehyde-NADP activity in different tumors induced in animals receiving only AAF. Some tumors possessed very low activity while others showed the classical tumor AIDH phenotype. However, even those tumors with very low NADP-dependent activity had NAD-dependent AIDH activity at or above control levels, and all tumors were distinguishable from their host liver. In contrast, only one tumor induced by AAF-PB did not possess NADP-dependent activity noticeably greater than its host liver, and although specific activities do vary, the overall uniformity of response was much greater in AAF-PB-induced tumors.

Perin et al. (20) and Sessa et al. (21) have reported that NAD-dependent AIDH activity is significantly reduced in hepatic tumors induced in Sprague-Dawley rats by feeding 4-dimethylaminobenzene for 5 months and in Yoshida ascites AH 130 hepatomas carried in Wistar rats. These workers do not measure NADP-dependent AIDH activity, nor do their tissue preparation or assay procedures appear optimized for determination of total hepatic AIDH activity. Feinstein (4) has reported that tumors induced in Sprague-Dawley rats by 18 days of exposure to 4-dimethylaminobenzene possess the tumor-specific AIDH phenotype and a group of Morris hepatomas of varying degrees of differentiation do not. Whether these differences in AIDH activity are due to differences in methodology or reflect actual differences in the biology of the various primary and transplantable tumors examined will require their direct comparison.

We propose that the mechanism of this phenotypic change requires that transformation-associated, stable genetic changes occur in initiated cells that are later expressed as the tumor AIDH phenotype. Tumors induced by exposure to only AAF can express the tumor phenotype, indicating the essential role of carcinogen in this process. Inasmuch as a significantly greater proportion of the tumors induced by combined carcinogen-promoter exposure possess the tumor-specific AIDH phenotype, a critical role for the promoter in the induction process is implied. The uniform nature of tumor AIDH phenotype expression in AAF-PB-induced tumors suggests that the phenotype may be induced by PB as one of its promotive functions (19). However, the possibility exists that PB is functioning only as an AIDH inducer, in this case an anomalous inducer in cells damaged previously by carcinogen.

We are investigating possible physiological roles for the tumor-specific AIDHs in tumor metabolism. We have demonstrated that the activities of 2 enzyme systems directly involved in hepatic aldehyde metabolism, aldehyde oxidase and the aldehyde reductases, as well as alcohol dehydrogenase (8), are not significantly altered in AAF-induced hepatic tumors compared to normal rat liver. This confirms that the tumor-specific AIDH phenotype is not part of an overall modification of aldehyde metabolism in these tumors. However, the increased AIDH activity shifts overall aldehyde metabolism much more toward oxidation from the reduction predominant in normal liver.

Additional roles for the tumor AIDHs may be related to changes in amino acid and/or lipid metabolism in these tumors, relative to normal liver. Such changes may generate increased levels of aldehydes requiring removal by AIDH. Also, the fact that the tumor-specific AIDHs are NADP-dependent suggests that AIDH may play a role in providing additional reducing equivalents to meet the increased biosynthetic needs of these tumor cells. We are currently examining these possibilities.

REFERENCES

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