Changes in Aldehyde Dehydrogenase Activity during Diethylnitrosamine- or 2-Acetylaminofluorene-initiated Rat Hepatocarcinogenesis

Sheri M. Wischusen, Susan Evces, and Ronald Lindahl

Biochemistry Program and Developmental Biology Section, Department of Biology, The University of Alabama, University, Alabama 35486

ABSTRACT

Hepatomas induced in postweanling male Sprague-Dawley rats by sequential dietary 2-acetylaminofluorene (AAF):phenobarbital (PB) exposure possess an aldehyde dehydrogenase (ALDH) phenotype qualitatively and quantitatively different from that of normal liver. To assess the generality of this phenotype, we have evaluated the ability of another family of carcinogens and an additional tumor induction protocol to induce this change. One-day-old female Sprague-Dawley rats were given injections i.p. of either diethyl nitrosamine (DEN) or AAF (0.15 µmol/g body weight in corn oil). Animals were weaned onto a 30% protein diet containing 0.05% PB. At intervals up to 27 weeks after weaning, animals were sacrificed, and the ALDH phenotype of both normal liver and any lesions was characterized. Elevated NAD(P)-dependent, benzaldehyde-oxidizing activity, the major marker of the tumor-specific ALDH phenotype, was not detected in any normal liver during the experiment. Only DEN:PB-treated animals developed hepatic tumors. Twenty-one tumors were found in 14 animals, the first being observed at 105 days of age. Sixteen of the tumors possessed the tumor-specific ALDH phenotype as determined by changes in total ALDH activity, isozyme patterns, and/or immunochemical methods.

Another significant change in hepatic ALDH, characterized by elevated NAD-dependent activity, was observed in normal liver of some animals receiving PB following either DEN (seven of 34) or AAF (11 of 34). Only one animal in each of the control groups (one of 17 PB controls; one of 16 basal-diet controls) had marginally elevated NAD-dependent ALDH activity. This ALDH activity is distinguishable from the normal liver ALDHs and from the tumor-specific phenotype by a number of properties, but it appears identical to a promotion-associated hepatic ALDH observed previously in some animals initiated with dietary AAF followed by dietary PB promotion.

These results extend induction of the tumor-specific ALDH phenotype to another family of carcinogens, the nitrosamines, and confirm that the phenotypic change is due to an initiator-induced, stable genetic change that is expressed relatively late in hepatocarcinogenesis. The appearance of an additional, independent change in ALDH activity during the promotion phase of hepatocarcinogenesis suggests that changes in ALDH activity may also be useful in understanding the interactions of initiators and promoters during tumorigenesis.

INTRODUCTION

In aromatic amine-induced rat hepatomas, the ALDH4 phenotype [aldehyde:NAD(P) oxidoreductase, EC 1.2.1.3 and 1.2.1.5] is qualitatively and quantitatively different from that of normal liver (8, 11–14). The tumor-specific ALDH phenotype is characterized by increased total ALDH activity due to the appearance of several cytosolic isozymes not detectable in normal liver. The tumor isozymes preferentially oxidize aromatic aldehyde substrates using NADP as coenzyme. They also have electrophoretic mobilities, isoelectric points, and immunochemical properties distinct from normal liver ALDH.

In normal rat liver mitochondria and microsomes, at least 3 ALDH isozymes can be differentiated on the basis of substrate and coenzyme preference, substrate and coenzyme KM, immunological properties, and sensitivity to inhibitors (12, 21). Little, if any, ALDH activity is found in normal liver cytosol.

In addition to the basal normal liver isozymes, several ALDHs can be induced in normal liver cytosol by various xenobiotics (2–6, 10, 17, 19, 20). PB induces an ALDH activity in several genetically defined lines and certain strains of rats (2, 5, 10, 19). The induction response is controlled by a single codominant autosomal locus (R), with up to a 10-fold increase in cytosolic ALDH activity occurring within days of PB exposure in homozygous responsive animals (2, 5). A marked increase (up to 100-fold) in cytosolic ALDH also occurs within days of a single exposure to TCDD in both PB-responsive and nonresponsive lines of rats (3, 4, 17). The TCDD- and PB-inducible normal liver ALDHs differ in a number of physical and functional properties and appear to be the products of 2 separate genes (3, 4).

The tumor-specific ALDHs are physically and functionally distinct from the PB-induced isozyme (4, 15). However, the tumor-specific isozymes are closely related to the TCDD-inducible normal liver isozyme, although the time courses of their induction are clearly distinct (4, 13, 15).

Recently, yet another change in ALDH activity has been observed in normal rat liver during the promotion phase of AAF:PB-induced hepatocarcinogenesis (1). This new phenotype, although also cytosolic, is kinetically, electrophoretically, and immunochemically distinct from the basal normal liver ALDHs, the tumor-specific phenotype, and the TCDD-inducible normal liver ALDH. Interestingly, the new phenotype is very similar to the PB-induced normal liver isozyme but requires both AAF and PB exposure for its induction (1, 3, 4).

To date, the tumor-specific ALDH phenotype has only been induced by dietary exposure of postweanling animals to the aromatic amines AAF or dimethylnitrosamine, with or with-

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3 To whom requests for reprints should be addressed.
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out promotion by PB (7, 13). The purpose of this study was to assess the generality of the tumor-specific ALDH phenotype and the promotion-associated changes in ALDH by evaluating the ability of an additional family of carcinogens, represented by DEN, and another tumor induction protocol to induce these changes.

MATERIALS AND METHODS

Sprague-Dawley rats (CD/SD) obtained from Charles River Breeding Laboratories (Wilmington, Mass.) were used. DEN and AAF were obtained from Sigma Chemical Co. (St. Louis, Mo.) and Aldrich Chemical Co. (Milwaukee, Wis.), respectively. PB (Sigma) was incorporated at 0.05% (w/w) into a semisynthetic 30% protein test diet by Teklad test diets (Teklad, Madison, Wis.). Wayne Lab Blox (Allied Mills, Inc., Chicago, Ill.) served as the control diet.

Virgin females, 3 to 5 months of age, and males of reproductive age were pair-mated overnight. The appearance of a copulation plug the next morning was used to indicate mating. Pregnant rats were housed separately and given water and Wayne Lab Blox ad libitum. Conditions were kept constant at a temperature of 23°C and a 12-hr light, 12-hr dark cycle.

The tumor induction protocol of Peraino et al. (18) was used. One day after birth, the pups were sexed, and the females were randomly assigned to one of 4 groups (Chart 1). Rats in Group A were given a single i.p. injection of DEN (0.15 µmol/g body weight) in 0.05 ml corn oil. Group B animals were given a single i.p. injection of AAF (0.15 µmol/g body weight) in 0.05 ml corn oil. Groups C and D served as controls and were given single i.p. injections of 0.05 ml corn oil. At 21 days, the pups were weaned. Groups A, B, and C were placed on the 30% protein diet containing 0.05% PB. Group D was given Wayne Lab Blox. At intervals after weaning, members of each group were sacrificed (Chart 1).

Long-Evans rats responsive or nonresponsive for induction of hepatic ALDH by PB were originally obtained from Dr. R. A. Deitrich, University of Colorado School of Medicine. Normal adult male responsive and nonresponsive animals were fed the 0.05% PB:30% protein diet ad libitum for 7 days and sacrificed on Day 8.

Animals were sacrificed 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 -70°C until needed.

Preparation of Tissues. After thawing, normal livers were prepared as 33% homogenate supernatants in 60 ITIM sodium phosphate buffer, pH 8.5, containing 1 mM EDTA and 1 ml 2-mercaptoethanol as described previously (13). Tumors were prepared identically but as 10 or 20% homogenates, 33% homogenate supernatants in 60 ITIM sodium phosphate buffer, pH 8.5, containing 1 mM EDTA and 1 ml 2-mercaptoethanol as described (13). Tumors were prepared identically but as 10 or 20% homogenates, 33% homogenate supernatants in 60 ITIM sodium phosphate buffer, pH 8.5, containing 1 mM EDTA and 1 ml 2-mercaptoethanol as described (13).

Histopathological examinations were performed on hematoxylin:eosin-stained sections. The classification of lesions observed followed the established criteria for rat hepatomas (9).

RESULTS

No significant differences in body weight were observed during the experiment (Chart 2). By Day 147, slight increases in liver weight and liver:body ratio were observed in the DEN:PB-treated group. These increases continued for the duration of the experiment.

Livers of animals receiving PB following DEN or AAF showed a few clear-cell foci as well as ductular proliferation. There was also considerable hypertrophy of hepatocytes, generally associated with regions of ductular proliferation. As early as Day 77, livers of DEN:PB- and AAF:PB-treated animals possessed microscopically detectable neoplastic nodules. Animals receiving only PB showed considerable hepatocyte hypertrophy throughout the study. In these animals, the hypertrophy was associated with ductular proliferation, although the changes were not as severe as in the carcinogen-treated livers. No foci, nodules, or other lesions were observed in sections from PB: or basal diet:control livers.

Tumors appeared only in the DEN:PB-treated group. The first tumors appeared only in the DEN:PB-treated group. The first tumors were observed at Day 105. In total, 21 tumors were found in 14 animals. Histologically, the tumors included 6 poorly differentiated hepatocellular carcinomas, 9 fairly well- to well-differentiated adenocarcinomas, and 6 well-differentiated trabecular carcinomas.

Thirteen of the 21 tumors examined had elevated ALDH activity with benzaldehyde and NADP, the primary indicator of the tumor-specific ALDH phenotype (Chart 3; Table 1). The change in ALDH activity was limited to the tumor; morphologically normal host liver had normal levels of activity (Chart 3; Table 1).

Three additional methods were used to confirm the presence of the tumor-specific ALDH phenotype. By gel electrophoresis, the tumor-specific ALDH activity differed qualitatively and quantitatively from the normal liver ALDH activity detectable by this technique (Fig. 1; Table 2). Thirteen of 19 tumors examined possessed the tumor-specific ALDH phenotype on polyacrylamide gel electrophoresis gels. No morphologically normal liver showed this isozyme pattern. There was good correlation between the presence of the tumor-specific ALDH isozymes on polyacrylamide gel electrophoresis gels and the demonstration of several NADP-dependent isozymes with isoelectric points near pH 6.8 to 7.0 by isoelectric focusing. Normal rat liver showed no banding in this range.

Eleven of 20 tumors cross-reacted with a hepatoma-specific
antibody population in rabbit anti-rat tumor-specific ALDH sera (Table 2). There was a direct correlation between cross-reactivity with anti-hepatoma-specific ALDH and tumor-specific ALDH activity, in that all of the tumors that cross-reacted with the anti-hepatoma serum had elevated ALDH activity with benzaldehyde and NADP (above 60 mU/mg protein). In total, 16 of 21 tumors showed at least one characteristic of the tumor-specific ALDH phenotype (Table 2).

In addition to the tumor-specific ALDH phenotype, another change in ALDH activity was observed in some grossly normal livers in both the DEN:PB- and AAF:PB-treated groups. It was characterized by ALDH activity with propionaldehyde and NAD higher than normal basal levels (Table 1; Chart 4). Seven of 34 DEN:PB-treated and 11 of 34 AAF:PB-treated animals possessed higher than normal NAD-dependent activity (>2 S.D. higher than the mean for the basal diet:control group; 50.6 mU/mg protein).

Table 1

<table>
<thead>
<tr>
<th>ALDH activity during DEN- or AAF-induced rat hepatocarcinogenesis</th>
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<tbody>
<tr>
<td><strong>Specific activity</strong></td>
</tr>
<tr>
<td><strong>Normal liver</strong></td>
</tr>
<tr>
<td><strong>Total</strong></td>
</tr>
<tr>
<td>DEN:PB</td>
</tr>
<tr>
<td>AAF:PB</td>
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<tr>
<td>PB control</td>
</tr>
<tr>
<td>Control</td>
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</tbody>
</table>

- See "Materials and Methods" for details.
- Average mU/mg protein for the numbers of determinations shown (n).
- Total ALDH activity for all nontumor tissue.
- ALDH activity in animals from "Total" that had neither promotion-associated ALDH activity nor tumors.
- ALDH activity in animals from "Total" that was elevated with propionaldehyde:NAD (2 S.D. higher than the mean for the basal diet control group, 50.6 mU/mg protein).
- Morphologically normal lobe of a tumor-bearing liver, often, but not always, the same lobe from which the tumor was obtained.
- P:NAD, propionaldehyde (substrate):NAD (coenzyme); B:NADP, benzaldehyde (substrate):NADP (coenzyme); NA, not applicable.
- Activity is significantly higher than the basal level at least the p < 0.01 level of significance by the Mann-Whitney U test.
- Activity is significantly higher than the basal level at the p < 0.05 level of significance by the Mann-Whitney U test.
- Activity is significantly higher than in the host group at the p < 0.05 level of significance by the Mann-Whitney U test.
- Numbers in parentheses, range.
Changes in ALDH during Hepatocarcinogenesis

Elevated NAD-dependent ALDH activity was first detected after 1 week on the PB diet and was found in greatest frequency in both DEN- and AAF-initiated animals at Day 147 (Chart 4). By 210 days, the NAD-dependent ALDH activity had returned to near basal levels.

The slight increase in ALDH activity with benzaldehyde and NADP seen in animals with the promotion-associated ALDH activity as well as in some host livers (Table 1; Charts 3 and 4) is due to the ability of the promotion-associated isozyme to use benzaldehyde and NADP as substrate and coenzyme, respectively, although not as efficiently as propionaldehyde and NAD. No additional evidence for the tumor-specific ALDH phenotype was found in these tissues.

Gel electrophoresis indicated that the increased NAD-dependent ALDH activity was due to a new isozyme appearing in these livers that was distinct from the normal liver and tumor-specific isozymes but identical to the ALDH inducible in normal liver by PB in PB-responsive animals (Fig. 1). All of the livers that possessed elevated NAD-dependent ALDH activity showed this distinct electrophoretic pattern (Table 3). Isoelectric focusing failed to resolve the promotion-associated ALDH.

Although 11 of the 21 tumors examined had elevated NAD-dependent ALDH activity, 10 of these showed a corresponding increase in NADP-dependent ALDH activity, indicative of the classic tumor-specific activity (Chart 3). None of the tumors with elevated NAD-dependent activity showed evidence of the slowly migrating promotion-associated ALDH isozyme.

The promotion-associated ALDH activity was cytosolic and readily oxidized phenylacetaldelyde with NAD as coenzyme, 2

Fig. 1. Polyacrylamide gel electrophoresis of various rat liver ALDHs. Gels were electrophoresed at 2.5 ma/gel for 2 hr at 4° and stained for ALDH as described (11). 1 and 2, normal liver from PB:control animal; 3, cytosolic fraction of AAF:PB-treated animal expressing promotion-associated ALDH activity; 4, cytosolic fraction of responsive Long-Evans rat expressing PB-inducible ALDH; 5, cytosolic fraction of nonresponsive Long-Evans rats; 6 and 7, tumor from DEN:PB-treated Animal 28 (Chart 3); 8 and 9, tumor from DEN:PB-treated Animal 51 (Chart 3). Gels 1, 3 to 6, and 8 were stained with propionaldehyde and NAD. Gels 7 and 9 were stained with benzaldehyde and NADP. Gel 2 was stained like Gel 1, but without added substrate. In addition to no staining in the absence of substrate, no NADP-dependent ALDH activity was observed electrophoretically in normal liver or in liver expressing the promotion-associated ALDH.

Table 2

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Tumors with elevated benzaldehyde:NADP activity</th>
<th>Tumors with polyacrylamide gel electrophoresis phenotype of tumor ALDHs</th>
<th>Tumors cross-reacting with anti-hepatoma ALDH serum</th>
<th>Tumors with no evidence of hepatoma-specific ALDH phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEN:PB</td>
<td>13/21</td>
<td>13/19</td>
<td>11/20</td>
<td>5/21</td>
</tr>
</tbody>
</table>

* See “Materials and Methods” for details.

** Number of tumors per total that have ALDH activity with benzaldehyde:NADP at least 2 S.D. higher than mean for control group.

† Number of tumors per total which possess the tumor-specific ALDH 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 ALDH serum in Ouchterlony double diffusions.

§ Number of tumors per total which do not possess the tumor-specific ALDH phenotype by at least one of the 3 principle criteria used.

Fig. 4. Distribution of ALDH activity in morphologically normal rat liver treated with DEN or AAF. See “Materials and Methods” for details. Numbers in parentheses, number of individuals in each group that had ALDH activity with benzaldehyde (P) and NAD significantly greater than the mean for control group.
characteristics of the normal liver PB-inducible ALDH (Table 4).
As we have observed in the past, the tumor-specific ALDHs were also cytosolic but could not oxidize phenylacetaldehyde to an appreciable extent (Table 4). The promotion-associated ALDH activity did not cross-react with antibodies to the tumor-specific ALDHs.

Histologically, livers exhibiting the promotion-associated phenotype did not differ greatly from carcinogen-treated livers having basal levels of ALDH activity. Foci and areas of hypertrophic hepatocytes, as well as areas of bile duct proliferation, were only slightly greater than in livers that did not exhibit this activity.

**DISCUSSION**

These results extend earlier reports of a tumor-specific ALDH phenotype occurring in rat hepatocarcinogenesis (8, 11–14). That DEN-induced tumors express the phenotype indicates that at least one family of carcinogens in addition to aromatic amines, the nitrosamines, is capable of causing this induction. As with aromatic amine-induced lesions, no change in ALDH activity was detected during or following carcinogen or promoter exposure until tumors are grossly observed in a liver. The tumor-specific ALDH phenotype appears concomitant with the appearance of tumors. The phenotypic change is limited to the tumor; morphologically normal host liver does not possess this ALDH phenotype. There is no correlation between tumor size (as an approximate indicator of tumor age) or tumor histology and the degree of deviation of the ALDH phenotype from normal.

Inasmuch as tumors induced by a single exposure to DEN express this phenotype, multiple or extended exposure to initiator is not required for induction of the tumor-specific ALDH phenotype. Although exposure to an initiator is absolutely required for expression of the tumor-specific ALDH phenotype, the tumor-specific activity is not dependent upon exposure to a promoter for its induction. We have shown previously that tumors induced by 32-day dietary AAF exposure without PB promotion exhibited this phenotype (13). Since a single exposure to AAF did not induce tumors or tumor-specific ALDH activity, it is probable that a tumorigenic dose of initiator is required to bring about this phenotypic change. Finally, the tumor-specific ALDHs have been observed previously only in tumors induced in male rats (7, 13). The present results indicate that the tumor-specific ALDH phenotype can also be induced in females if the appropriate protocol is used.

Expression of the tumor-specific ALDH phenotype in DEN:PB-induced tumors was extremely variable. This response is similar to tumors induced by dietary AAF exposure; some tumors possessed very low activity while others exhibited the classical tumor-specific ALDH activity (13). In contrast, dietary AAF:PB-induced tumors were very uniform in their expression of the tumor-specific ALDH phenotype (13). It is interesting to note that, in general, the tumor-specific ALDH activity was lower in tumors induced by 32-day dietary AAF exposure without PB promotion than in single tumor-bearing livers (Chart 3).

The results reported here also confirm the existence of a change in ALDH activity occurring during the promotion phase of hepatocarcinogenesis. Allen and Lindahl (1) described recently a new ALDH phenotype induced in grossly normal rat liver by combined initiator-promoter treatment under conditions where neither initiator nor promoter alone is capable of such an induction. With the protocol used here, both DEN and AAF can cause this promotion-associated change in ALDH phenotype. This is
especially significant, since AAF cannot induce tumors nor the tumor-specific ALDH phenotype by this protocol.

The promotion-associated ALDH activity is a nonbasal, cytosolic, NAD-dependent isozyme (1). It is distinct from the normal and tumor-specific ALDH phenotypes on the basis of total activity, substrate and coenzyme preference, electrophoretic mobility, sensitivity to disulfiram, and immunochemical characteristics (1, 13). The promotion-associated isozyme is, however, very similar by these criteria to the PB-inducible ALDH activity found in normal livers of several genetically defined lines of rats (1, 2, 4). Most significantly, Allen and Lindahl (1) have demonstrated that the promotion-associated ALDH activity cross-reacts with antibodies to the normal liver PB-induced isozyme.

Combined with our earlier study (1), we have now observed this change in a total of 25 of 74 animals chronically or acutely exposed to AAF followed by PB and in 7 of 34 DEN:PB-treated animals. No animal (0 of 40) receiving only dietary AAF possessed this phenotype. Only one of 37 PB-treated control and one of 41 basal diet control animals have shown marginally elevated NAD-dependent ALDH activity.

All the available data support our hypothesis that the tumor-specific ALDH phenotype is due to an initiator-induced, transformation-associated, stable genetic change expressed late in hepatocarcinogenesis (13). Such specificity makes this phenotype an excellent probe for examining the mechanisms of action of various carcinogens, as well as serving as a marker for one or more latent events occurring in the neoplastic progression.

The promotion-associated ALDH phenotype represents a genetically independent change in ALDH activity. This change in phenotype, requiring both initiator and promoter for its expression, may be useful in studying the interactions between initiators and promoters. It will be especially valuable if tumor promotion protocols in addition to dietary PB treatment can also induce this change.

REFERENCES

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