Regulation of Aldehyde Dehydrogenase Activity in Five Rat Hepatoma Cell Lines¹

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ABSTRACT

Significant changes in aldehyde dehydrogenase (ALDH) activity occur during rat hepatocarcinogenesis in vivo. An NADP-dependent tumor ALDH isozyme has been studied extensively. To better understand the nature, origin, and importance of this dependent tumor ALDH isozyme has been studied extensively. To better understand the nature, origin, and importance of this dependent tumor ALDH isozyme has been studied extensively. To better understand the nature, origin, and importance of this dependent tumor ALDH isozyme has been studied extensively. To better understand the nature, origin, and importance of this dependent tumor ALDH isozyme has been studied extensively.

INTRODUCTION

Work in our laboratory has shown that rat hepatomas induced by a number of chemical carcinogens have a unique ALDH phenotype (1, 9, 10, 13). The tumor 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 differ from the normal liver ALDH isozymes in a number of physical and functional properties (9, 14). In vivo, expression of the tumor ALDH phenotype is highly variable both between tumors and within a single tumor (28).

In normal rat liver, ALDH activity is localized primarily to the mitochondrial and microsomal fractions, with little or no ALDH activity detectable in cytosol. At least 4 normal liver ALDH isozymes can be differentiated on the basis of substrate and coenzyme preference, substrate and coenzyme kinetic properties, and sensitivity to inhibitors (12, 27). Normal liver ALDH is primarily NAD-dependent, and it oxidizes small aliphatic aldehydes.

In addition to these basal isozymes, several ALDHs can be induced in normal liver in vivo by various xenobiotics (5, 6), including 3-MC, BP, and PB. BP and 3-MC induce both NAD- and NADP-dependent ALDH in several strains of rats (26). PB induces a cytosolic NAD-dependent ALDH in certain genetically defined lines and certain strains of rats (4).

To date, our studies of ALDH and changes in the activity of this enzyme during hepatocarcinogenesis have been performed in vivo (1). Under such conditions, design and interpretation of experiments for the study of the regulation of hepatic ALDH activity are complex. For this reason, we proposed to establish an in vitro system, using stable, hepatoma-derived cell lines to study the genesis and regulation of hepatoma ALDH. Three well-established hepatoma cell lines, H4-II-EC3 (20), HTC (25), and MaCa-RH7777 (2), and 2 newly established lines, JM1 and JM2 (18), were selected. These lines have been derived from hepatocellular tumors induced by a variety of tumor-induction protocols and have been maintained in vitro for varying periods of time.

This paper describes the ALDH phenotype of these 5 cell lines and presents an initial analysis of the mechanisms underlying regulation of hepatic ALDH activity. The results reported also support our hypothesis (13) that the tumor ALDH phenotype is the result of transformation-associated mutational events occurring in initiated cells rather than alteration in liver metabolism due to the administration of a carcinogen.

MATERIALS AND METHODS

Chemicals. BP, 3-MC, PB, actinomycin D, cycloheximide, α-amanitin, dimethylsulfoxide, disulfiram, NAD, NADP, nitroblue tetrazolium, and phenazine methosulfate were from Sigma Chemical Company (St. Louis, MO). Aldehydes were obtained from Aldrich Chemical Company (Milwaukee, WI). Heat-inactivated fetal bovine serum was from HyClone Company (Logan, UT; Lot 100415). DME medium was purchased from K. C. Kee, Wl. Heat-inactivated fetal bovine serum was from Hyclone Company (Logan, UT; Lot 100415). DME medium was purchased from K. C. Kee, Wl.

Received May 29, 1984; accepted August 8, 1984.

¹ This work was supported by Grant CA-21103 from the National Cancer Institute. Presented at the 75th Annual Meeting of the American Association for Cancer Research, May 9 to 12, 1984, Toronto, Ontario, Canada (7).
² Submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry in the Graduate School of The University of Alabama.
³ To whom requests for reprints should be addressed.
⁴ The abbreviations used are: ALDH, aldehyde dehydrogenase (aldhyde:NAD<P) oxidoreductase, EC 1.2.1.3 and 1.2.1.5; BP, benzo(a)pyrene; 3-MC, 3-methylcholanthrene; PB, phenobarbital; GST, γ-glutamyl transpeptidase; DME, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide.


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addition of inducer, cells were harvested by trypsin treatment and frozen.

Known in vivo inducers of various ALDHs, including 3-MC, BP, and PB,
cycloheximide were dissolved in distilled water. α-Amanitin was dissolved
in DMSO. ALDH activity was induced by 3-MC and BP as before, except
that actinomycin D, α-amanitin, or cycloheximide was added to a final
concentration of 0.5 μg/ml, 2 μg/ml, or 7 μg/ml, respectively. Inhibitors
were added either simultaneously with inducers or 12 hr later. Cells were
harvested 48 hr after addition of inducers and stored at −80° until
assayed.

RESULTS

The 5 cell lines examined possess a wide spectrum of ALDH activities (Table 1). This range is similar to that seen for primary
tumors in vivo. HTC, JM1, and JM2 have a high constitutive
tumor ALDH activity, as characterized by elevated NADP-de-
dependent activity using benzaldehyde as substrate. H4-II-EC3 has
intermediate tumor ALDH activity. McAr-RH7777 does not pos-
sess detectable levels of the tumor ALDH activity. On a compar-
ative basis, the NAD-dependent ALDH specific activity of McAr-
RH7777 is below the normal liver range.

Analytical gel electrophoresis indicates that the increased

Table 1

<table>
<thead>
<tr>
<th>Substrate/coenzyme</th>
<th>Cell lines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HTC</td>
</tr>
<tr>
<td>Propanaldehyde-NAD</td>
<td>112.5</td>
</tr>
<tr>
<td>Benzaldehyde-NAD</td>
<td>486.6</td>
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Primary rat liver activity:

<table>
<thead>
<tr>
<th>Primary rat liver activity</th>
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</thead>
<tbody>
<tr>
<td>Rat liver activity</td>
</tr>
<tr>
<td>HTC</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>84.8 ± 14.6</td>
</tr>
</tbody>
</table>

a Data for at least 2 determinations for cell lines. Ranges of activities were routinely within 10% of the mean.

b Induced by dietary 2-acetylamidofluorene/PB exposure (13).

c Average for 24 tumors and 51 normal livers, ±S.D.
NADP-dependent activity is due to new cytosolic ALDH isozymes appearing in HTC, JM1, JM2, and H4-II-EC3 (Fig. 1). The electrophoretic mobility of these new isozymes is identical to those of the tumor ALDH activity seen in vivo.

Histochemically, the 3 high-activity lines HTC, JM1 and JM2 stained intensely for ALDH using benzaldehyde and NADP (Fig. 2). The cell culture is heterogeneous in that not all cells stain, and staining is more intense in regions of cell aggregation compared to monolayer regions. The NADP-dependent ALDH activity of the 3 high-activity cell lines is very sensitive to disulfiram inhibition (data not shown). The cell lines (McA-RH7777 and H4-II-EC3) with lower enzyme activities do not stain histochemically for ALDH (Fig. 2).

The relationship of ALDH phenotype to another known tumor biochemical marker, GGT, was examined in the 5 cell lines (Fig. 2). Histochemically, McA-RH7777 is moderately GGT-positive but ALDH-negative. HTC is moderately GGT-positive and strongly ALDH-positive. JM1 and JM2 are slightly GGT-positive but strongly ALDH-positive. H4-II-EC3 is negative for both ALDH and GGT. Consistent with these histochemical observations, Richards et al. (22) have reported that HTC and McA-RH7777 have moderately high GGT activity, while the GGT activity of H4-II-EC3 is very low. These results indicate that there is little correlation between ALDH activity and GGT activity.

The specific activities, electrophoretic profiles, and histochemical localization studies indicate that HTC, JM1, and JM2 are representative of ALDH-positive hepatocellular neoplasms seen in vivo. By the same criteria, McA-RH7777 is representative of an ALDH-negative neoplasm. The marginally elevated specific activities, the electrophoretic profiles, and negative staining by histochemistry indicate that H4-II-EC3 is representative of a marginally ALDH-positive neoplasm.

Known in vivo inducers of ALDH were tested for their ability to increase the ALDH activity in the 5 cell lines (Table 2). Treatment of cells with 3-MC (1.0 mM) increases the NADP-dependent ALDH activity in H4-II-EC3 and McA-RH7777 cell lines up to 34- and 11-fold, respectively. BP at 1 mM also

**Table 2**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Control&lt;sup&gt;a&lt;/sup&gt;</th>
<th>3-MC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>BP&lt;sup&gt;c&lt;/sup&gt;</th>
<th>PB&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4-II-EC3</td>
<td>69.0</td>
<td>2366.3 (34x)</td>
<td>1198.3 (17x)</td>
<td>445.4</td>
</tr>
<tr>
<td>McA-RH7777</td>
<td>17.4</td>
<td>198.1 (11x)</td>
<td>837.7 (46x)</td>
<td>59.1 (3x)</td>
</tr>
<tr>
<td>HTC</td>
<td>1022.1</td>
<td>2155.4 (2x)</td>
<td>2580.2 (2.5x)</td>
<td>2042.0 (2x)</td>
</tr>
<tr>
<td>JM1</td>
<td>826.0</td>
<td>857.8 (1x)</td>
<td>1195.9 (1.4x)</td>
<td>460.1</td>
</tr>
<tr>
<td>JM2</td>
<td>949.8</td>
<td>1741.0 (2x)</td>
<td>1832.5 (2x)</td>
<td>1628.4 (2x)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data for at least 2 determinations. Ranges of activities were routinely within 10% of the mean.

<sup>b</sup> Control cultures received 0.1% DMSO in serum-free medium. Control specific activities are those at the time of maximal induction by appropriate inducer.

<sup>c</sup> Final concentration, 1 mM.

<sup>d</sup> Numbers in parentheses, multiple of induction, compared to appropriate control.

Chart 1. Kinetics and dose response of induction of ALDH in H4-II-EC3 by BP, 3-MC, and PB. Cells were treated as described in "Materials and Methods" and harvested by trypsin treatment at the indicated intervals, and ALDH activity was determined. 1, 1 mM; III, 0.1 mM; 1, 0.01 mM; O, medium control; D, 0.1% DMSO control. Ranges of activities were routinely within 10% of the mean.
increases (up to 17- and 48-fold) the NADP-dependent ALDH activity in both lines. 3-MC and BP at 1 mM increase ALDH activity 2-fold in HTC and JM2 but do not increase NADP-dependent ALDH activity in JM1. Corresponding but only moderate increases in NAD-dependent ALDH activity are seen following 3-MC or BP-treatment of all lines.

Treatment of cells with PB (1.0 mM) causes only marginal increases in NADP-dependent ALDH activity in 4 of 5 cell lines and actually decreases the activity in JM1. Treatment with PB does not increase NAD-dependent ALDH activity in any line. Insulin and dexamethasone do not increase the NAD- or NADP-dependent ALDH activity of any cell line tested (data not shown).

The kinetics and dose-response of the increase of ALDH activity was tested in H4-II-EC3 following 3-MC, BP, and PB treatment (Chart 1). Increased ALDH activity can be detected as early as 6 hr after inducer addition. Maximal increases were observed at about 48 hr (BP, 1 mM), 96 hr (3-MC, 1 mM), or 24 hr (PB, 1 mM) after addition of the appropriate inducer. The increases in ALDH activity were dose dependent over the range of inducer concentrations tested (Chart 1). After maximal levels were reached, ALDH activity gradually declined. In general, similar changes in the time course of ALDH induction were also observed in HTC and McA-RH7777 following treatment with 3-MC and BP (Chart 2). The ALDH activity in the control groups of HTC slightly increased during incubation in serum-free medium.

To distinguish whether the increase in ALDH activity was due to an increase in the total number of cells, to increased ALDH activity in cells already expressing ALDH, or to induction of ALDH activity in ALDH-negative cells, H4-II-EC3 and McA-RH7777 were examined for ALDH histochemically after treatment with 3-MC (Fig. 2). By cell count, there was no increase in the total number of cells. However, the proportion of ALDH-positive cells increased after inducer addition in both lines, indicating the induction of ALDH activity in previously negative cells.

As noted earlier, after maximal induction by 3-MC or BP, ALDH activity gradually declined (Chart 1). To determine whether the decline in ALDH activity observed was due to regulation of the enzyme activity occurring in induced cells or to metabolism or inactivation of the inducer, the ability of preinduced H4-II-EC3 cells to respond to a second exposure to an inducer was tested (Chart 3). Previously induced cells respond to second inducer exposure with a second elevation (approximately 2-fold) of ALDH activity. While addition of fresh medium temporarily increases ALDH activity in uninduced controls, no such response is seen in previously uninduced cells receiving only fresh medium.

To determine whether the increase in ALDH activity is due to an enhanced biosynthesis of the enzyme, inhibitors of transcription and translation were examined for their effect on ALDH inducibility in H4-II-EC3 and HTC (Table 3). When added with inducer, actinomycin D and cycloheximide almost completely blocked induction of NADP-dependent ALDH activity by 3-MC in H4-II-EC3 (Table 3). Under the same conditions, actinomycin D and cycloheximide inhibited BP-induction of ALDH in H4-II-EC3 also. Similarly, α-amanitin significantly inhibited ALDH induction by either 3-MC or BP in H4-II-EC3. In HTC, all 3 inhibitors lowered ALDH to below basal levels when added simultaneously with 3-MC or BP (Table 3). Reductions in actinomycin D, α-amanitin, and cycloheximide inhibition were observed when H4-II-EC3 or HTC were treated with 3-MC or BP for 12 hr prior to inhibitor addition (Table 3). These observations indicate that new tran-
in vivo (11, 13, 28).\(^5\) The broad spectrum of activities in the transformation-associated mutational events occurring in initiator expression seen in chemically induced hepatocellular carcinomas takes at least 30 passages in our laboratory. The tumor ALDH phenotype. Each one has maintained its ALDH transcription and translation are required not only for inducibility, but also to maintain high ALDH basal activity in HTC.

**DISCUSSION**

Four of the 5 rat hepatoma cell lines examined express the tumor ALDH phenotype. Each one has maintained its ALDH phenotype at relatively constant levels during approximately 2 years of culture (at least 30 passages) in our laboratory. The range of ALDH activities observed in these 5 cell lines is also representative of the heterogeneity in tumor-ALDH phenotype expression seen in chemically induced hepatocellular carcinomas in vivo (11, 13, 28).\(^5\) The broad spectrum of activities in the various cell lines and their long-term stabilities in vitro support the hypothesis that the tumor ALDH phenotype is the result of transformation-associated mutational events occurring in initiated cells that are subsequently expressed as the tumor ALDH phenotype (13, 28).

The high basal activity lines HTC, JM1, and JM2 respond minimally to 3-MC and BP induction, while the low activity lines, Mca-RH7777 and H4-II-EC3, each respond to induction with significant increases in NADP-dependent ALDH activity. Interestingly, H4-II-EC3 and Mca-RH7777 respond differently to 3-MC and BP. H4-II-EC3 is mainly responsive to 3-MC, and Mca-RH7777 responds preferentially to BP. Razouk et al. (21) have reported that H4-II-EC3 and Mca-RH7777 differ in their microsomal epoxide hydrolase activities, with Mca-RH7777 possessing approximately 6 times more benzpyrene 4,5-oxide hydrolase activity than does H4-II-EC3. Novicki et al. (18) have reported that JM1 and JM2 are deficient in cytochrome P-450 and NADPH-cytochrome C reductase compared to normal liver. Miyake et al. (16) reported that Mca-RH7777 had detectable but possibly abnormal cytochromes P-450, P-420, and b5. Whether the differences in inducibility of ALDH in the different cell lines are due to altered regulatory mechanisms at the level of the ALDH gene(s) or to cell line differences in ability to metabolize inducers is currently unknown.

Deitrich et al. (5) reported that 3-MC increased hepatic NAD-dependent cytosolic ALDH activity approximately 20-fold in Long-Evans rats. Törmönen et al. (26) demonstrated that both multiple BP and multiple 3-MC exposures increased NADP-dependent hepatic ALDH activity of Wistar rats approximately 30-fold. Nakanishi et al. (17) also examined the effect of 3-MC on ALDH activity in livers of Wistar, Sprague-Dawley, and Long-Evans rats. Their results suggested both inter- and intrastrain variability in ALDH inducibility.

Perin et al. (19) and Sessa et al. (24) have reported low NAD-dependent ALDH activity in Yoshida AH 130 hepatoma as well as the non-liver-derived Sarcoma 180 and Ehrlich carcinoma lines. Canuto et al. (3) also examined Yoshida hepatoma AH-130 and reported decreased NAD(P)-dependent ALDH activity. While these observations are consistent with the variability in ALDH inducibility of the cell lines used in this study, the suggestion of Perin et al. (19) that diminution of ALDH activity may be related to the neoplastic transformation must be reexamined.

That new transcription and translation are required for induction confirms derepression of gene(s) for ALDH not detectable.

---

**Table 3**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Specific activity (mlU/mg of protein)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H4-II-EC3</td>
<td>HTC</td>
</tr>
<tr>
<td>Control</td>
<td>171.2</td>
</tr>
<tr>
<td>3-MC (1 mM)(^d)</td>
<td>2281.7</td>
</tr>
<tr>
<td>BP (1 mM)</td>
<td>980.8</td>
</tr>
<tr>
<td>0 hr(^b)</td>
<td></td>
</tr>
<tr>
<td>3-MC + actinomycin D</td>
<td>435.0</td>
</tr>
<tr>
<td>3-MC + α-amanitin</td>
<td>ND(^d)</td>
</tr>
<tr>
<td>3-MC + cycloheximide</td>
<td>362.2</td>
</tr>
<tr>
<td>BP + actinomycin D</td>
<td>590.4</td>
</tr>
<tr>
<td>BP + α-amanitin</td>
<td>ND</td>
</tr>
<tr>
<td>BP + cycloheximide</td>
<td>365.7</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>85.1</td>
</tr>
<tr>
<td>3-MC</td>
<td>3135.2</td>
</tr>
<tr>
<td>BP</td>
<td>2987.7</td>
</tr>
<tr>
<td>0 hr(^b)</td>
<td></td>
</tr>
<tr>
<td>3-MC + α-amanitin</td>
<td>1340.1</td>
</tr>
<tr>
<td>BP + α-amanitin</td>
<td>1157.2</td>
</tr>
</tbody>
</table>

\(^\text{a}\) Data for at least 2 determinations. Ranges of activities were routinely within 10% of the mean.

\(^\text{d}\) Actinomycin D, α-amanitin, and cycloheximide were added simultaneously with inducers (0 hr) or 12 hr later (12 hr). Cells were harvested 48 hr after addition of inducer.

\(^\text{b}\) Cells were prepared and treated with 1 mM 3-MC or BP as described in Materials and Methods.

\(^\text{c}\) ND, not determined.
in normal rat liver as the mechanism underlying expression of the tumor ALDH phenotype. The minimal response to inducers in the high basal activity lines HTC, JM1, and JM2, coupled with the ability of transcription and translation inhibitors to reduce ALDH activity of HTC to below basal levels, further indicates that, in these lines the gene(s) encoding the NADP-dependent ALDH has been permanently derepressed in a large portion of the cell population. Although it is impossible to determine with certainty, all of the available data are consistent with this derepression occurring during the genesis of primary tumors from which these lines are derived (up to 18 years ago in the case of HTC).

REFERENCES


Fig. 2. ALDH and GGT activity in situ for 5 rat hepatoma cell lines. A, ALDH, benzaldehyde-NADP, HTC; B, ALDH, benzaldehyde-NADP, JM1; C, ALDH, benzaldehyde-NADP, JM2; D, GGT, HTC; E, GGT, JM1; F, GGT, JM2; G, ALDH, benzaldehyde-NADP, H4-II-EC3; H, ALDH, benzaldehyde-NADP, H4-II-EC3 induced by 1 mM 3-MC, 72 hr; I, GGT, H4-II-EC3; J, ALDH, benzaldehyde-NADP, McA-RH7777; K, ALDH, benzaldehyde-NADP, McA-RH7777 induced by 1 mM 3-MC, 72 hr; L, GGT, McA-RH7777 x 125.

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CANCER RESEARCH VOL. 44
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