Hepatocyte expression of tumor associated aldehyde dehydrogenase (ALDH-3) and p21 Ras following diethylnitrosamine (DEN) initiation and chronic exposure to di(2-ethylhexyl)phthalate (DEHP)


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Phthalate esters such as di(2-ethylhexyl)phthalate (DEHP) either promote or inhibit rat liver tumorigenesis depending on the carcinogenesis protocol. In this study, we examined the expression of two histochemical markers, the tumor associated isozyme of aldehyde dehydrogenase (ALDH-3) and the oncoprotein p21 Ras, in the livers of male F344 rats. The rats were initiated with DEN and further treated with either DEHP (a known inhibitor of hepatocarcinogenesis), phenobarbital (PB, a known promoter of hepatocarcinogenesis), or a combination of DEHP and PB. The studies were designed to examine the expression of these markers in both normal appearing liver and hepatic hyperplastic and neoplastic lesions and to correlate the early expression of the markers at 26 weeks in the normal appearing liver to later tumor incidence at 52 weeks. The expression of each marker was detected by immunohistochemical methods on formalin-fixed paraffin embedded sections of normal appearing liver or liver lesions. We found that ALDH-3 and p21 expression were significantly enhanced in rats receiving PB after DEN initiation at 26 weeks and that the incidence of hepatocellular carcinomas was likewise increased compared to control or DEN only treated animals. DEN initiation followed by a combination of PB and either 0.1 or 0.5% DEHP significantly reduced ALDH-3 but not p21 Ras expression at 26 weeks compared to DEN plus PB only. These treatment regimens also reduced the incidence of hepatocellular carcinomas at 52 weeks. DEN followed by any of the three doses of DEHP without PB resulted in ALDH-3 expression similar to DEN alone. However, p21 Ras expression was significantly increased after these treatments. For all treatment groups, both the early (26 weeks) expression of p21 Ras and ALDH-3 correlated with hepatocellular carcinoma incidence at 52 weeks. However, the correlation between hepatocellular carcinoma and ALDH-3 expression was better than p21 Ras or the other markers we have studied. We concluded that ALDH-3 expression is significantly downregulated after DEHP treatment, and that expression of the isozyme correlated with later hepatocarcinoma incidence and may indicate a significant relationship between ALDH-3 expression and hepatocarcinogenesis during DEHP treatment.

Introduction

The phthalate ester, di-(2-ethylhexyl)phthalate (DEHP*) is used widely as a plasticizer in the production of a variety of consumer products. Since the compound is not covalently linked to the plastic polymers, it can leach from the finished product with time and therefore present a potential risk for human exposure (1). Evidence that DEHP increases the incidence of hepatocellular carcinomas and neoplastic nodules in F344 rat liver has raised questions about its potential safety for human exposure (2,3).

The mechanism of DEHP induced carcinogenesis has not been established. However, DEHP is considered non-genotoxic and shares many biochemical effects with other compounds such as nafenopin and Wy-14643 that, like DEHP, induce proliferation of peroxisomes (3–5). DEHP treated rats develop gammaglutamyl transpeptidase (GGT) positive altered hepatic foci (AF) prior to development of neoplastic lesions and thus the compound appears to produce histopathological lesions in a multi-stage sequence similar to that observed with many other hepatocarcinogens (6).

Foci of hepatocellular alteration are induced by most rodent hepatocarcinogens and these foci are considered to be precursors to hepatic neoplasms (7,8). These AF are morphologically and biochemically heterogeneous, and their number, size, phenotype and conversion rate to hepatic neoplasms are changed by the carcinogenesis protocol. Cells within AF may be morphologically normal, but have alterations in tinctoral staining characteristics and/or enzyme and onco-protein expression. Thus, evaluation of biochemical changes in AF during hepatocellular carcinogenesis may be useful in elucidating possible mechanisms of carcinogenesis and in predicting relative carcinogenic potential.

Recent studies in which rats were initiated with diethylnitosamine (DEN) and treated with chronic exposure to DEHP indicated that DEHP inhibited rather than promoted the formation of GGT positive AF as well as hepatocellular carcinomas (CA), hyperplastic nodules (HN) and adenomas (4,5,9). This inhibition was observed with DEHP concentrations as low as 0.5%. These observations have suggested that DEHP may have some unique properties which inhibit the carcinogenic potential of a known hepatocarcinogen such as DEN (1,4,5,9,10).

Using immunohistochemical and computer enhanced quantitative image cytometry techniques, we have previously reported that DEHP inhibited the incidence of glutathione S-transferase (GST-P) as well as GGT positive AF in the livers of rats treated with DEN with and without PB promotion (11). These results were consistent with other reports that peroxisome proliferator compounds reduce the expression of these markers in rat liver as well as the incidence of AF, HN, hepatic adenomas (HA) and CA (12). We have also correlated the expression of GST-P positive AF observed in livers of rats treated with DEN with or without PB and three different concentrations of DEHP, with the tumor incidence at 52 weeks.
We found that the incidence of GST-P positive AF at 26 weeks did not correlate with the 52 week CA incidence (11). Here we report that DEHP affects expression of both another Phase Two drug metabolizing enzyme, the tumor associated isozyme of aldehyde dehydrogenase (ALDH-3), and the oncoprotein p21 Ras. We found that ALDH-3 is not only expressed in a significant percent of hepatic neoplastic lesions, but is also expressed in the hepatocytes of carcinogen treated animals prior to the appearance of neoplastic lesions. Like GGT and GST-P, ALDH-3 expression was inhibited by low doses of DEHP. Expression of the oncoprotein p21 Ras, however, was less affected by DEHP. When hepatocellular tumor incidence at 52 weeks following eight different carcinogenesis protocols was compared to early biomarker expression at 26 weeks as measured by computer enhanced image cytometry, ALDH-3 expression was found to be the marker most predictive of future CA, but not HA, incidence.

Materials and methods

Animals and carcinogen
Male F344 rats (Charles River Co., Raleigh, NC) weighing 101–125 g were sub-totally hepatectomized and 18 h later given a single i.p. injection of 30 mg/kg DEN or the saline vehicle. Ten days following DEN, the animals were divided into treatment groups and maintained on the various dietary regimens for up to 52 weeks (see Table 1). Four groups of animals received DEN alone and four groups of animals received DEN and 0.06% PB in their drinking water. DEHP at concentrations of 2.0%, 0.5% or 0.1% was added singly or to the diet of three of the four groups of animals as previously described (4). Groups of animals were sacrificed at 26 weeks for preneoplastic and neoplastic marker studies, while other animals were sacrificed at 52 weeks to determine tumor incidence. Histopathological diagnosis of lesions was provided by Pathology Associates, Inc. (Westchester, OH).

Immunohistochemistry

IHC methods were used to detect two histochemical markers for neoplastically transformed liver cells, ALDH-3 and p21 Ras. Sections of formalin-fixed paraffin embedded rat livers were de-paraffinized in xylene and hydrated to distilled water. A standard immunoperoxidase method (Vectastain ABC detection kit, Vector Laboratories, Burlingame, CA) was used. Appropriate dilutions of anti-ALDH-3 or p21 Ras antibodies were incubated with tissue sections for 12 h at 4°C. Anti-ALDH-3 was the generous gift of R.Lindahl (13). Anti-p21 Ras was obtained from Dupont (Boston, MA). Diaminobenzidine (DAP) was used as a chromogen to detect sites of antigen–antibody interaction. Details of the method, including antibody characteristics and dilutions, have been previously reported (11,14). The stained sections were examined microscopically and positively stained normal-appearing hepatocytes, AF, HN, HA or CA were recorded.

Image cytometry

The stained slides, after qualitative analysis, were evaluated by image cytometry with a Zeiss IBAS image cytometry system as previously described (11). Data were collected for three parameters: field area stained, reference area size and percent of area stained (i.e. field area/reference area×100%). By the principle of Delesse, area % = volume % of liver tissue with marker activity. Because there was an independent measurement of total liver volume (i.e. liver weight, assuming a density of unity), the grams of liver expressing the marker for preneoplastic or neoplastic transformation could be determined.
Table I. Qualitative ALDH-3 expression at 26 weeks

<table>
<thead>
<tr>
<th>Group treatment</th>
<th>Number of animals</th>
<th>Normal-appearing liver</th>
<th>Nodules</th>
<th>Adenomas</th>
<th>Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 DEN + PB</td>
<td>9</td>
<td>9/9 (100%)</td>
<td>1/1</td>
<td>1/1 (100%)</td>
<td>0/0</td>
</tr>
<tr>
<td>2 DEN + PB + 2% DEHP</td>
<td>10</td>
<td>0/10</td>
<td>2/9 (22%)</td>
<td>0/1</td>
<td>0/3</td>
</tr>
<tr>
<td>3 DEN + PB + 0.5% DEHP</td>
<td>10</td>
<td>10/10 (100%)</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>4 DEN + PB + 0.1% DEHP</td>
<td>10</td>
<td>0/10</td>
<td>1/1 (100%)</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>5 DEN</td>
<td>10</td>
<td>0/10</td>
<td>1/2 (50%)</td>
<td>1/2 (50%)</td>
<td>0/0</td>
</tr>
<tr>
<td>6 DEN + 2.0% DEHP</td>
<td>9</td>
<td>8/9 (89%)</td>
<td>0/2</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>7 DEN + 0.5% DEHP</td>
<td>9</td>
<td>9/9 (100%)</td>
<td>0/1</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>8 DEN + 0.1% DEHP</td>
<td>9</td>
<td>6/9 (67%)</td>
<td>0/3</td>
<td>0/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

*Results are reported as number of positive lesions/number of lesions examined.

bFor normal appearing hepatocytes, the results are expressed as the number of liver sections (one section per animal) showing multiple positive hepatocytes per number of liver sections examined.

Results

Hepatomegaly
Treatment with 0.5 or 2.0% DEHP or PB, after DEN initiation, produced significantly increased hepatomegaly compared to livers of rats treated with DEN alone (Figure 1A and B). Doses of 0.5 or 2.0% DEHP, combined with PB produced significantly greater hepatomegaly than PB alone. On the other hand 0.5 or 2.0% DEHP without PB produced hepatomegaly that was similar to that produced by PB alone.

ALDH-3 expression: qualitative results
ALDH-3 expression was detected in both normal appearing hepatocytes and hepatic lesions after 26 weeks of treatment as summarized in Table I. Multiple ALDH-3 hepatocytes were recorded in most livers of rats that received either DEN + PB, DEN + PB + 0.05% DEHP, and DEN + any of the three doses of DEHP alone. The positive hepatocytes were not typical altered hepatic foci, but were single positive cells scattered randomly across the hepatic lobules (Figure 2). Livers from rats treated with DEN alone, or DEN + PB + either 2.0 or 0.1% DEHP had either a few or no positive single hepatocytes scattered across lobules. All liver sections from all eight groups of rats did, however, show a weaker ALDH-3 expression in the centrolobular hepatocytes of nearly all liver lobules. This generally consisted of three or four layers of normal hepatocytes per lobule (Figure 3A). This stain pattern has been reported previously in rats treated with other hepatocarcinogenesis protocols (16-18) but has not been examined as a potential indicator of later hepatic neoplasm incidence. Although there were no untreated control livers in this study, our previous studies (15) have indicated no detectable centrolobular staining in non-carcinogen treated or untreated rodent liver.

Other than the normal appearing hepatocyte expression, ALDH-3 was also detected in a few hyperplastic nodules (Figure 4 and Table I). These lesions were observed in rats treated with DEN with or without PB or DEN + PB + either 2.0 or 0.1% DEHP. ALDH-3 negative HN and HA were observed in rats initiated with DEN alone or DEN + PB and 2.0% DEHP (Figure 5). No ALDH-3 positive carcinomas were observed at 26 weeks although, as described below, many were detected at 52 weeks.

ALDH-3 expression: quantitative results
Quantitative evaluation of ALDH-3 expression by computer enhanced image cytometry indicated significantly increased...
ALDH-3 expression in DEN initiated rat liver promoted with PB compared to DEN alone (Figure 6). In DEN + PB, 7.80 ± 0.68 g of tissue expressed ALDH-3 compared to 0.97 ± 0.13 g in livers of animals receiving DEN alone. DEN plus PB and either 0.1 or 0.5% DEHP resulted in significantly less tissue expression of ALDH-3 (4.21 ± 0.66 g and 3.51 ± 0.84 g, respectively) compared to DEN + PB. However, the addition of 2% DEHP to the diet of animals initiated with DEN and promoted with PB did not significantly alter ALDH-3 expression compared to rats treated with DEN + PB alone.

In the absence of PB promotion, significantly less tissue expressed ALDH-3 at any of the three DEHP doses (0.30 ± 0.07, 1.72 ± 0.50 and 1.34 ± 0.51 g at 0.1, 0.5 and 2% DEHP, respectively). Thus, although multiple single hepatocytes were observed qualitatively in rats initiated with DEN and receiving any of the three doses of DEHP, the overall ALDH-3 expression was significantly less than in animals receiving DEN and PB promotion. The results suggest that ALDH-3 expression is significantly decreased in DEN + PB treated rat liver also treated with either 0.1 or 0.5% DEHP. Interestingly the 2.0% DEHP dose did not produce this effect. Similar results were observed in rats treated with DEN + DEHP only, in which ALDH-3 expression was again not significantly altered in rats receiving the 2.0% DEHP dose compared to rats receiving DEN alone.

p21 Ras expression: qualitative results
Qualitatively, p21 Ras expression was detected in a few typical altered hepatic foci lesions in rats receiving either DEN + PB + 2.0% DEHP or in rats receiving any of the three doses of DEHP alone (Table II). Very few (<5) altered foci were detected per liver section. The pattern of p21 Ras expression differed considerably from that observed with ALDH-3 in that nearly all p21 Ras expression was confined to the few altered foci rather than randomly scattered normal appearing hepatocytes. However, p21 Ras expression was also observed surrounding the central veins of most hepatic lobules in livers from rats in all groups (Figure 7).

No negative HN or HC were observed. The HN from animals treated with DEN alone, DEN + PB or DEN + PB + 2.0% DEHP also co-expressed ALDH-3 (Figure 8A and B). However, p21 Ras positive HN that did not co-express ALDH-3 were recorded in livers of rats initiated with DEN and given any of the three concentrations of DEHP. p21 Ras expression was also observed in all lesions from rats treated with DEN alone. The three carcinomas detected in group No. 2 (DEN + PB + 2% DEHP) rats were positive for p21 Ras but negative for ALDH-3.

p21 Ras expression: quantitative results
Quantification of p21 Ras expression by image cytometry indicated that addition of DEHP had no effect on expression of this oncoprotein in rats initiated with DEN and promoted with PB (Figure 9). In rats receiving DEN + PB, 3.94 ± 0.59 g of tissue expressed p21 Ras, while in animals receiving 0.1 and 2% DEHP in addition to DEN + PB, 4.00 ± 0.62 and 2.62 ± 1.43 g of tissue expressed p21 Ras, respectively. In rats initiated with DEN but receiving no further treatment, only 0.06 ± 0.02 g of liver expressed p21 Ras. Treatment of DEN initiated rats with DEHP increased expression of p21 Ras at all dose levels (Figure 9). These results suggested that PB significantly increased the grams of tissue expressing p21 Ras in DEN initiated rats, and that addition of 2 or 0.5% DEHP to the PB protocol significantly reduced p21 expression. Generally, p21 Ras staining in hepatocytes was less intense and widespread than ALDH-3 (Figure 3B).

Tumor incidence at 52 weeks
The incidence (as lesions/group) and multiplicity (as lesions/animal) of CA was significantly increased by PB promotion compared to DEN alone (Table III). The incidence and multiplicity of CA was significantly reduced in rats receiving PB plus any of the three doses of DEHP compared to rats receiving DEN + PB promotion only. However, the CA incidence and multiplicity were not significantly altered in rats receiving any of the DEHP doses + DEN compared to DEN alone.

The HA incidence and multiplicity were not significantly altered by PB promotion alone. However, compared to DEN + PB, HA was significantly increased only after 2.0% DEHP. Likewise DEN + 2.0% DEHP significantly increased HA incidence and multiplicity compared to DEN alone.

These results suggested that DEHP + PB treatment generally decreases CA incidence and multiplicity, but increased the HA incidence and multiplicity. Rats treated with DEN plus any of the three DEHP doses generally showed no significant increased or decreased incidence or multiplicity compared to rats receiving DEN alone.

Correlation between marker expression at 26 weeks and tumor incidence at 52 weeks
To evaluate the correlation of marker expression with tumor incidence, the incidence of carcinomas, adenomas or any neoplasm in each of the eight treatment groups was compared with the grams of liver expressing the tumor marker, or with liver weight in those groups. The grams of liver expressing tumor associated ALDH-3 at 26 weeks was highly correlated.
Hepatocyte expression of ALDH-3 and p21 Ras

A. DEN + DEHP

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>DEN</th>
<th>DEN + 2.0% DEHP</th>
<th>DEN + 0.6% DEHP</th>
<th>DEN + 0.1% DEHP</th>
<th>DEN + 0.0% DEHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grams Liver Expressing ALDH-3</td>
<td>0.87</td>
<td>1.34</td>
<td>1.75</td>
<td>0.53</td>
<td>0.97</td>
</tr>
</tbody>
</table>

B. DEN + PB + DEHP

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>DEN</th>
<th>DEN + PB + 2.0% DEHP</th>
<th>DEN + PB + 0.5% DEHP</th>
<th>DEN + PB + 0.1% DEHP</th>
<th>DEN + PB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grams Liver Expressing ALDH-3</td>
<td>0.87</td>
<td>4.21</td>
<td>3.67</td>
<td>7.08</td>
<td>7.90</td>
</tr>
</tbody>
</table>

Fig. 6. Effect of dietary DEHP on the grams of liver expressing ALDH-3: (A) initiated with DEN; (B) initiated with DEN and promoted with PB. Asterisk indicates significantly different ($P < 0.05$) compared to DEN only (A) or DEN + PB (B).

The correlation between incidence of hepatic neoplasms at 52 weeks and grams of liver expressing p21 Ras after 26 weeks of treatment ($r = 0.770$) was less than with ALDH-3 as was the correlation with incidence of CA ($r = 0.852$). Adenoma incidence alone was not correlated with p21 Ras expression ($r = 0.080$).

Quantification of two other markers, GGT and GST, in these livers was previously reported (11). The grams of liver expressing these markers at 26 weeks also showed a good correlation with CA incidence at 52 weeks, but not with incidence of HA. The correlation coefficient for GGT expression with incidence of carcinoma, adenomas or any neoplasm was 0.853, 0.507 and 0.935, respectively. GST-p expression was more highly correlated with carcinoma incidence ($r = 0.870$) than with incidence of adenomas ($r = -0.174$) or any neoplasm ($r = 0.639$).

In contrast to marker expression, liver weight at 26 weeks was highly correlated with incidence of adenomas at 52 weeks ($r = 0.880$). A significant correlation was not found between carcinoma incidence at 52 weeks and liver weight at 26 weeks ($r = 0.344$).

**Discussion**

Previous studies have examined the carcinogenicity and carcinogenic mechanisms of the plasticizer, DEHP. Depending on the experimental protocol, DEHP appears either to promote or inhibit carcinogenesis in the rat liver (2-5). We have shown previously that DEHP appears to inhibit the formation of both altered hepatic foci and neoplastic lesions such as adenomas and carcinomas in the livers of rats initiated with a single dose of DEN and promoted with DEHP, with or without PB (4). These results were confirmed recently by examining GST-P and GGT expression in livers of rats receiving the same treatment protocol. In that study, we found that both GGT and GST-P expression were greatly reduced in animals receiving DEHP (11).

In this study, we have combined the methods of immunohistochemistry and computerized image cytometry to examine the expression of two other histochemical markers of transformed liver cells, the oncoprotein p21 Ras and ALDH-3 in the normal liver and hepatic lesions of rats after initiation with DEN and promotion with PB, PB + DEHP or DEHP alone. The objectives of this study were to examine the expression of these markers to determine if they were likewise reduced
Table II. Qualitative p21 Ras expression at 26 weeks

<table>
<thead>
<tr>
<th>Group treatment</th>
<th>Number of animals</th>
<th>Normal-appearing liver</th>
<th>Nodules</th>
<th>Adenomas</th>
<th>Carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 DEN + PB</td>
<td>6</td>
<td>0/6</td>
<td>0/0</td>
<td>1/1 (100%)</td>
<td>0/0</td>
</tr>
<tr>
<td>2 DEN + PB + 2% DEHP</td>
<td>6</td>
<td>2/6 (33%)</td>
<td>1/1 (100%)</td>
<td>1/1 (100%)</td>
<td>3/5 (100%)</td>
</tr>
<tr>
<td>3 DEN + PB + 0.5% DEHP</td>
<td>3</td>
<td>0/3</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>4 DEN + PB + 0.1% DEHP</td>
<td>5</td>
<td>0/5</td>
<td>0/0</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>5 DEN</td>
<td>9</td>
<td>0/9</td>
<td>1/1 (100%)</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>6 DEN + 2.0% DEHP</td>
<td>5</td>
<td>3/5 (60%)</td>
<td>2/2 (100%)</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>7 DEN + 0.5% DEHP</td>
<td>6</td>
<td>2/6 (33%)</td>
<td>1/1 (100%)</td>
<td>0/0</td>
<td>0/0</td>
</tr>
<tr>
<td>8 DEN + 0.1% DEHP</td>
<td>8</td>
<td>6/8 (75%)</td>
<td>3/3 (100%)</td>
<td>0/0</td>
<td>0/0</td>
</tr>
</tbody>
</table>

*See Table I for treatments.

**Number of positive altered hepatic foci, rather than individual hepatocytes as reported in Table I, recorded per number of sections examined (one section per animal).

*p21 Ras expression was done as a subset of randomly selected animals from the group used for aldehyde dehydrogenase expression.

Table III. Tumor incidence at 52 weeks

<table>
<thead>
<tr>
<th>Group treatment</th>
<th>Adenoma (%)</th>
<th>Adenoma/animal</th>
<th>Carcinoma (%)</th>
<th>Carcinoma/animal</th>
<th>Any neoplasm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 DEN + PB</td>
<td>3/17 (17.6)</td>
<td>0.176</td>
<td>16/17a (94.1)</td>
<td>1.412</td>
<td>94.1</td>
</tr>
<tr>
<td>2 DEN + PB + 2% DEHP</td>
<td>12/17 (70.6)</td>
<td>1.353</td>
<td>8/17 (47.1)</td>
<td>0.706</td>
<td>88.2</td>
</tr>
<tr>
<td>3 DEN + PB + 0.5% DEHP</td>
<td>7/17 (41.2)</td>
<td>0.647</td>
<td>4/17 (23.5)</td>
<td>0.235</td>
<td>58.8</td>
</tr>
<tr>
<td>4 DEN + PB + 0.1% DEHP</td>
<td>5/18 (27.8)</td>
<td>0.333</td>
<td>9/18 (50.0)</td>
<td>0.556</td>
<td>72.2</td>
</tr>
<tr>
<td>5 DEN</td>
<td>4/19 (21.2)</td>
<td>0.263</td>
<td>1/19 (5.3)</td>
<td>0.053</td>
<td>26.3</td>
</tr>
<tr>
<td>6 DEN + 2% DEHP</td>
<td>9/18 (50.0)</td>
<td>0.611</td>
<td>1/18 (5.6)</td>
<td>0.056</td>
<td>50.0</td>
</tr>
<tr>
<td>7 DEN + 0.5% DEHP</td>
<td>3/15 (20.0)</td>
<td>0.267</td>
<td>2/15 (13.3)</td>
<td>0.563</td>
<td>28.6</td>
</tr>
<tr>
<td>8 DEN + 0.1% DEHP</td>
<td>1/15 (6.7)</td>
<td>0.067</td>
<td>0/15 (0)</td>
<td>0.000</td>
<td>6.7</td>
</tr>
</tbody>
</table>

*Number of animals with lesion/total number of animals.

in DEHP treated animals. We also correlated the expressions of each of these histological markers with the overall liver neoplasm incidence after 52 weeks of treatment, to determine if the expression of any of the markers at 26 weeks was predictive of tumor incidence at 52 weeks. The correlation between our previously reported quantitation of GGT, GST-P and tumor incidence indicated that GGT expression was nearly equivalent to ALDH-3 in predicting the occurrence of all neoplasms in this model. GST-P expression at 26 weeks was highly correlated with carcinoma incidence but showed no correlation with adenoma incidence. Quantitatively, both ALDH-3 and p21 Ras expression were increased after promotion with PB. However, only ALDH-3 expression was reduced by DEHP. While all four markers were correlated with subsequent carcinoma incidence, we found that ALDH-3 expression at 26 weeks correlated best with overall tumor incidence.

The appearance of centrolobular ALDH-3 positive normal-appearing hepatocytes has been reported in previous studies (15–18) by ourselves and others. This staining pattern is consistently more intense and involves a greater number of hepatocytes in carcinogen treated compared to control livers. Studies by Lindahl *et al.* (reviewed 13), in which early appearing centrolobular staining was compared in the normal liver of rats during hepatocarcinogenesis induced by a number of different protocols, have reported that although centrolobular staining occurred early in the hepatocarcinogenesis process, the stain pattern was generally not observed at the time of appearance of hepatic adenomas or carcinomas. Also, although
Hepatocyte expression of ALDH-3 and p21 Ras Marker Expression vs. Carcinoma Incidence

Marker Expression vs. Carcinoma Incidence

Marker Expression vs. Adenoma Incidence

Fig. 10. Correlation between marker expression or liver weight at 26 weeks and carcinoma or adenoma incidence at 52 weeks. Tumor incidence at 52 weeks (Table III) was compared to expression of ALDH-3 (Figure 6) or p21 Ras (Figure 9) or liver weight (Figure 1). $R^2$ values were calculated by EXCEL.

this centrolobular pattern was observed early, hepatic adenomas and carcinomas tended to vary in ALDH-3 expression depending on the carcinogenesis protocol.

In the present study, the hepatic adenomas and carcinomas, detected after 52 weeks of treatment, that were examined for ALDH-3 expression tended to be low. Only 6/32 (19%) adenomas and 7/19 (37%) carcinomas were positive for ALDH-3 suggesting cellular remodeling within lesions during tumor progression. Consistent with cellular remodeling during tumor progression, cellular heterogeneity in stain intensity for ALDH-3 is found in large HN (Figure 4). Nearly all neoplasms were present in the non-DEHP-treated rats. Thus, although ALDH-3 expression indicated a likelihood for later neoplasms, the later appearing neoplasms were not consistently ALDH-3 positive.

Rat liver expresses several cytosolic and mitochondrial isozymes of aldehyde dehydrogenase (13, 15-24). Reports that ALDH-3 expression is localized in the putative preneoplastic and neoplastic stages of liver by a number of different protocols suggests that ALDH-3 is a tumor associated isozyme of the liver. Although the exact role or roles of ALDH-3 in the liver is unknown, at least two possible functions of the isozyme are relevant to the results reported here. First, all known
carcinogenesis protocols indicate that cell transformation must occur for ALDH-3 expression to occur (13). Second, peroxisome proliferators are known to produce a number of aldehyde substrates from the peroxidation of lipid membranes (24,25). Some of these aldehydes can be quite cytotoxic, inducing amino acid cross-linking, inhibition of cell proliferation and/or direct effects on DNA metabolism (25,26). Based on these functions, initiated cells that can express ALDH-3 may be less affected by lipid peroxidation than neighbor cells and therefore gain a growth advantage in the liver (27). Also, since ALDH-3 is inducible by xenobiotic agents as described above, ALDH-3 may play a role in the detoxification of these agents as has been described for other phase one and phase two enzymes, however the induction of ALDH-3 appears to be a stable alteration in initiated hepatocytes, and preneoplastic and neoplastic lesions. This observation implies that ALDH-3 expression is not due solely to xenobiotic exposure but is part of the overall adaptive response of initiated cells during carcinogenesis.

Both GGT and GST-P are also phase two xenobiotic metabolizing enzymes that are expressed during liver carcinogenesis (11,12). The expression of GGT can be influenced by a number of factors other than xenobiotics such as diet, sex, strain and age of the animal. Thus GGT expression may be less well correlated with tumor incidence than other markers. Likewise GST-P, another phase two enzyme, is better correlated with early stages of liver carcinogenesis such as AF and thus has been suggested as a better marker for neoplastic changes than GGT. However, both of these enzymes are known to be significantly reduced by peroxisome proliferator agents, and their significance in long-term carcinogenesis protocols is unclear (12). The p21 Ras oncoprotein expression has been described in a number of carcinogen-induced rat liver hepatocarcinogenesis protocols (28). Generally, p21 Ras, as either the Harvey or Kirsten strains, is induced early after carcinogen exposure (29). Although mutations in wild-type p21 Ras have been recorded at either the 12th, 13th, or 61st codons, some studies have found that wild type p21 Ras is also effective for cell transformation (30). Although our antibody to p21 Ras did not differentiate Harvey from Kirsten or wild-type from mutated, we were able to demonstrate p21 Ras expression in either putative preneoplastic or neoplastic liver tissue. p21 Ras was expressed in 100% of neoplasms at 52 weeks consistent with other reports. Expression of p21 Ras may be an early event in carcinogenesis and may be necessary but not sufficient for malignant conversion (28).

Our results suggest that unlike the expression of isozymes of drug metabolizing enzymes usually reported in peroxisome proliferator treated liver, the oncoprotein p21 Ras is not inhibited. However the expression of this oncoprotein was not as well correlated with later neoplasm incidence as ALDH-3.

In summary, the results indicate that expression of each marker after 26 weeks of treatment in this DEN initiation + PB promotion model is correlated with carcinoma incidence at 52 weeks, p21 Ras expression was preferentially correlated with subsequent carcinoma incidence, while ALDH-3 expression at 26 weeks was better correlated with overall tumor response. Of the four markers evaluated, ALDH-3 is the most highly correlated with carcinoma and overall tumor response. The data also indicate that, as has been suggested for the peroxisome proliferator nafenopin (30), the mechanism of DEHP modification of hepatic tumorigenesis may involve promotion of hepatic lesions with a different phenotype than those promoted by PB.

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References

Hepatocyte expression of ALDH-3 and p21 Ras


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