Persistently Increased Expression of a 3-Methylcholanthrene-inducible Phenol Uridine Diphosphate-Glucuronosyltransferase in Rat Hepatocyte Nodules and Hepatocellular Carcinomas

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ABSTRACT

Increased UDP-glucuronosyltransferase in rat hepatocyte nodules and hepatocellular carcinomas produced by feeding 2-acetylaminofluorene or N-nitrosomorpholine was studied using isozyme-selective substrates, antibodies, and DNA probes. UDP-glucuronosyltransferase (UDP-GT) activities toward 4-methylumbelliferone, 1-naphthol, and benzo[a]pyrene-3,6-quinol were reversibly increased by short term feeding of 2-acetylaminofluorene but were persistently increased in hepatocyte nodules and differentiated hepatocellular carcinomas. Immunoblot analysis revealed that short term feeding of 2-acetylaminofluorene increased a M, 55,000 polypeptide corresponding to the previously characterized UDP-GTI or phenol UDP-GT. However, in some hepatocyte nodules and hepatocellular carcinomas either the M, 55,000 or a new M, 53,000 polypeptide was preferentially increased, suggesting heterogeneous UDP-GT forms in liver nodules and carcinomas. Northern blot hybridization with a synthetic DNA probe to phenol UDP-GT demonstrated increased levels of mRNA in liver nodules. The results suggest persistently increased expression of at least two phenol UDP-GT enzyme forms in hepatocyte nodules, which may contribute to the toxin-resistance phenotype frequently observed at cancer prestages.

INTRODUCTION

Exposure to various carcinogens results in the appearance of hepatocyte clones showing the 'toxin-resistance phenotype' during early stages of carcinogenesis (1, 2). This phenotype includes enhanced excretion of drugs including chemotherapeutic agents (3, 4) and an altered pattern of drug-metabolizing enzymes, such as decreased P450-dependent monoxygenase activities (5) and increased UDP-GTs to in hepatocyte nodules (6, 7) and hepatocyte foci (8, 9). Since UDP-GT represents an enzyme family, it is interesting that increased UDP-GT activities can be ascribed to a 3MC-inducible isozyme, which has been operationally termed UDP-GTI, phenol UDP-GT (6, 10), or 4-nitrophenol UDP-GT (11). Comparative studies ascertained the similarity of this isozyme characterized in different laboratories (10). Its amino acid sequence has recently been deduced from its cDNA (12). A similar UDP-GT cDNA has been detected in extrahepatic tissues such as kidney and in human liver (13). Substrates of UDP-GT are various phenolic and polyphenolic metabolites of planar polycyclic aromatics. Glucuronidation of BP-3,6-quinol, for example, prevents quinone/quinol redox cycles which generate reactive oxygen species and semiquinones (14, 15). UDP-GTI is also involved in the inactivation of certain aromatic amines, such as 1- and 2-naphthylamine (16), and converts N-hydroxy-2-naphthylamine to a N-glucuronide, which is transported to the urinary bladder where it decomposes to the ultimate carcinogen (17). In the latter case, glucuronidation plays a role in determining the target of carcinogenicity. Persistently increased UDP-GTI may explain increased excretion of glucuronides of 2AAF metabolites in 3MC-treated and nodule-bearing rats (18). Hence, increased glucuronidation contributes to toxin resistance in the Solt-Farber model, which uses 2AAF as a mitoinhibitory agent (19). Because of their toxin resistance, nodular hepatocytes selectively proliferate in the presence of growth stimuli in this model.

In the present study, intermittent feeding of 2AAF was used to produce hepatocyte nodules and hepatocellular carcinomas (20). The properties of these liver nodules were compared to those produced by feeding N-nitrosomorpholine in a stop model (21). UDP-GTs were analyzed using (a) substrates for UDP-GT (such as 4-methylumbelliferone, 1-naphthol, and BP-3,6-quinol) and for UDP-GTI (such as 4-hydroxybiphenyl) as well as testosterone and bilirubin for the corresponding UDP-GT isozymes (22, 23), (b) antibodies to UDP-GTI for immunoblot analysis (10), and (c) a selective DNA probe for the analysis of UDP-GTI mRNA and DNA, synthesized according to its cDNA (12). It was found that the levels of at least two closely related UDP-GTI polypeptides (at M, 55,000 and 53,000) were enhanced in hepatocyte nodules and hepatocellular carcinomas. In addition, evidence was obtained that the enhanced enzyme level may be due to transcriptional activation.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained from the following sources: benzo[a]pyrene-3,6-quinone and 3-hydroxybenzo[a]pyrene from the Chemical Carcinogen Reference Standard Repository, National Institutes of Health (Bethesda, MD); 2AAF from Fluka (Buchs, Switzerland); Brij 58 (a condensate of hexadecyl alcohol with 20 mol of ethylene oxide/mol) from Atlas (Essen, FRG); and UDP-glucuronic acid, disodium salt, from Boehringer (Mannheim, FRG).

Hepatocyte Nodules and Hepatocellular Carcinomas. Male Wistar rats (120 g; Mollegaard Breeding Center, Ebel, Denmark) received a basal diet (Ewos, Södertälje, Sweden) supplemented with 0.05% (w/v) 2AAF. Rats were fed intermittently the basal diet containing the carcinogen and the basal diet alone, as suggested by Epstein et al. (20) and modified for Wistar rats by Eriksson et al. (24). The livers of all animals were perfused in situ with 0.25 M sucrose at 4°C. Large nodules (4-mm diameter) were carefully dissected from the surrounding tissue, immediately frozen in liquid nitrogen, and stored at ~80°C until used for enzyme assays. Control livers from untreated animals of the same age were prepared and stored under identical conditions.

When liver nodules were produced by feeding N-nitrosomorpholine, male Wistar rats (150 g) received a basal diet (Altromin, Lage, FRG) and N-nitrosomorpholine (160 mg/liter, in drinking water) for 7 weeks. Then the carcinogen was withdrawn for the following 10 weeks (21).

Culture of H4IIE Cells. Rat hepatoma H4IIE cells (25) were grown in 100- x 20-mm tissue culture plates, in Dulbecco's minimal essential medium (with L-glutamine) from Boehringer (Mannheim); Brij 58 (a condensate of hexadecyl alcohol with 20 mol of ethylene oxide/mol) from Atlas (Essen, FRG); and UDP-glucuronic acid, disodium salt, from Boehringer (Mannheim, FRG).

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2Abbreviations used: UDP-GT, UDP-glucuronosyltransferase (EC 2.4.1.17); 2AAF, 2-acetylaminofluorene; 3MC, 3-methylcholanthrene; BP-3,6-quinol, benzo[a]pyrene-3,6-quinol; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; cDNA, complementary DNA.


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diph of 5% CO₂ in air at 37°C. Cells were harvested at a density of 2–3 x 10⁶ cells/plate. Thereafter, they were washed with cold 0.01 M phosphate-buffered 0.9% NaCl, pH 7.4, and harvested by scraping the cells with the same solution. Washed cells were stored at −80°C.

UDP-GT Assays. Published methods were used to assay UDP-GT activities toward 1-naphthol (26), 4-methylumbelliferone (27), and testosterone (28). UDP-GT activity toward BP-3,6-quinol was determined as described (14), with some modifications. In brief, BP-3,6-quinone (0.5 mM, dissolved in hexamethyl phosphoric acid triamide) was reduced to the corresponding quinol in 1 M Tris/ascorbic acid buffer, pH 7.4, for 10 min under nitrogen at 37°C. Afterwards, microsomes (0.1 mg), 5 mM MgCl₂, Brij 58 (0.5%, w/v), and UDP-glucuronic acid (3 mM) were added, in a total volume of 0.1 ml. The reaction was started by addition of UDP-glucuronic acid. At zero time and after 1 and 2 min, 25-μl aliquots were removed from the incubation mixture. The aliquots were added to 50 μl methanol and 500 μl 1.6 mM glycine/NaOH buffer, pH 10.3, in a total volume of 750 μl. Relative fluorescence was determined at excitation and emission wavelengths of 460 and 520 nm for BP-3,6-quinol monoglucuronide and 285 and 433 nm for the corresponding diglucuronide, respectively, in a Perkin-Elmer LS-5B fluorescence spectrometer (slit, 2.5 and 2.5 nm). Relative fluorescence was calibrated (a) on the basis of the correlation between fluorescence and specific radioactivity of UDP-[¹⁴C]glucuronic acid, which was used for the formation of BP-3,6-quinol mono- and diglucuronide (9), and (b) on the basis of a known amount of BP-3,6-quinone, which was first completely converted to the corresponding quinol, then completely converted to BP-3,6-quinol monoglucuronide, and finally converted to the corresponding diglucuronide. Usually mono- and diglucuronide formations were determined simultaneously. Activities were calculated from initial rates. In some experiments, BP-3,6-quinol monoglucuronide was prepared biosynthetically in a UDP-GT assay mixture (from BP-3,6-quinol and liver microsomes of C57BL/6 mice; the latter converted the monoglucuronide to the diglucuronide in only negligible amounts (29)). Similar rates of diglucuronide formation were observed either when the monoglucuronide was used as substrate or when the two reactions were determined simultaneously.

P450-Dependent Monoxygenase Assays. Benzpyrene monooxygenase activity was determined by the method of Nebert and Gelboin (30), which measures the formation of fluorescent phenols. It was calibrated with 3-hydroxybenz(a)pyrene. 7-Ethoxyresorufin O-deethylase activity was determined as described elsewhere (31). Protein was determined according to the method of Lowry et al. (32).

Immunoblot Analysis. Analysis was carried out as previously described (10). Anti-rat UDP-GTI antibodies were obtained from rabbits immunized with cDNA sequence (12). Synthetic UDP-GTI DNA probes were labeled with 

**RESULTS**

Simple phenols, such as 4-nitrophenol and 1-naphthol, represent overlapping substrates for several UDP-GT isozymes (11). However, BP-3,6-quinol and its monoglucuronide appear to be preferential substrates for UDP-GTI (10). As shown in Table 1, glucuronidation of the quinol was markedly increased in hepatocyte nodules (monoglucuronide formation 4-fold and diglucuronide formation 8-fold). In the well differentiated hepatocellular carcinoma 1, mono- and diglucuronide formation was increased 10- and 38-fold, respectively. Similar increases in enzyme activities were found in 3MC-treated rats (10- and 40-fold, respectively) (14). P450-dependent monoxygenase activities were low in hepatocyte nodules and carcinomas, as expected. In the poorly differentiated carcinoma 10, UDP-GT activities were very low. UDP-GT activities in different nodules were found to be surprisingly uniform, whereas in 10 different hepatocellular carcinomas a wide range of enzyme activities was found (Fig. 1). Interestingly, UDP-GT activities toward 4-

<table>
<thead>
<tr>
<th>Enzyme and substrate</th>
<th>Liver</th>
<th>Nodules</th>
<th>Carcinoma</th>
</tr>
</thead>
</table>
| **Enzyme activity (nmol/min/mg protein)**
| UDP-GT | |
| 1-Naphthol | 22 ± 1.7 | 127 ± 10 | 130 ± 5 |
| BP-3,6-quinol | 4.6 ± 0.9 | 17.7 ± 1.7 | 47 ± 5 |
| BP-3,6-quinol monoglucuronide | 0.2 ± 0.1 | 1.5 ± 0.3 | 7.6 ± 0.9 |
| 4-Hydroxybiphenyl | 18 ± 1.1 | 28 ± 4 | 12 ± 4 |
| Bilirubin | 1.1 ± 0.4 | 1.4 ± 0.3 | 0.8 ± 0.9 |
| Testosterone | 7.0 ± 1.1 | 5.4 ± 1.3 | 4.4 ± 3.5 |

- Enzyme activities were determined in microsomal fractions.
- Means of 2-4 determinations are listed, means ± SD in the case of 4 determinations; ND, not determined.
alterations in the enzyme pattern were found in hepatocyte nodules produced by feeding N-nitrosomorpholine.

UDP-GT polypeptides present in hepatocyte nodules and carcinomas were analyzed by immunoblot analysis. Two kinds of UDP-GTI antibodies were used, (a) broad spectrum antibodies raised against the holoenzyme (Fig. 2I) and (b) selective antibodies raised against the \( M, 55,000 \) polypeptide eluted from sodium dodecyl sulfate-polyacrylamide gels (Fig. 2II). Besides the \( M, 55,000 \) UDP-GTI polypeptide, the broad spectrum antibodies recognized other polypeptides at \( M, 50,000, 52,000, \) and 56,000. The \( M, 50,000 \) and 52,000 polypeptides could be ascribed to testosterone and androsterone UDP-GT, respectively (10). The \( M, 56,000 \) polypeptide seen in livers from 3MC-treated rats (Fig. 2I) has not yet been identified. Feeding 2AAF for 2 weeks led to a slight increase of the \( M, 55,000 \) polypeptide (not shown). However, 2AAF administration for 4 weeks led to the appearance of a new polypeptide at \( M, 53,000 \) (Fig. 2I). The \( M, 53,000 \) polypeptide was preponderant in hepatocyte nodules. This new polypeptide was also recognized by the more selective antibody (Fig. 2II). In hepatocellular carcinomas 1 and 9, the \( M, 55,000 \) polypeptide was mainly present, whereas in carcinoma 8 the \( M, 53,000 \) polypeptide was preponderant. Similar results were observed with both antibodies (as shown for carcinoma 8). UDP-GT polypeptides were low in carcinomas 2 to 5 (not shown) and in carcinoma 10 (\( M, 55,000 \)), in agreement with low UDP-GT activities in these tissues (Fig. 1). Hepatocyte nodules and hepatomas produced by administration of N-nitrosomorpholine and the hepatoma H4IIE cell line revealed preferentially increased \( M, 55,000 \) UDP-GTI polypeptides, together with increased UDP-GT activities (not shown).

Levels of UDP-GTI mRNA were analyzed using a selective DNA probe, synthesized according to the UDP-GTI cDNA (12). UDP-GTI mRNA was found to be preferentially increased in 3MC-treated rat liver (10-fold) and only slightly increased (1.5-fold) in phenobarbital-treated animals (Fig. 3I). In hepatocyte nodules, UDP-GTI mRNA was increased 4-fold and, in H4IIE cells, 7-fold. DNA methylation analysis was carried out using the isoschizomeric restriction endonucleases HpaII/Mspl and BclI/NeII. Mspl cleaves at both methylated C-5-methyl-CGG and CCGG sequences, whereas HpaII cleaves only at nonmethylated CCGG sequences. Similarly, BclI cuts at both methylated C-5-methyl-C(C/G)GG and CCC(C/G)GG sequences, whereas NeII cleaves only at nonmethylated CCC(C/G)GG. According to the UDP-GTI cDNA (12), recognition sequences recognized by all the endonucleases used in these experiments were found beginning at nucleotide \(-116\) and at

### Table 2: Adaptive responses and persistent alterations of UDP-GT activities in liver nodules

<table>
<thead>
<tr>
<th>Tissue and treatments</th>
<th>4-Methylumbelliferone</th>
<th>BP-3,6-quinol</th>
<th>BP-3,6-quinol monoglucuronide</th>
<th>4-Hydroxybiphenyl</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control livers</td>
<td>42 ± 2(^a)</td>
<td>8.4 ± 1.3</td>
<td>0.4 ± 0.1</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>2AAF (2 weeks)</td>
<td>133 ± 7</td>
<td>12.0 ± 2.7</td>
<td>0.7 ± 0.2</td>
<td>53 ± 13</td>
</tr>
<tr>
<td>2AAF (2 weeks) + control diet (4 weeks)</td>
<td>41 ± 3</td>
<td>7.3 ± 1.4</td>
<td>0.3 ± 0.1</td>
<td>23 ± 4</td>
</tr>
<tr>
<td><strong>Nodules</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2AAF</td>
<td>151 ± 5</td>
<td>17.4 ± 2.8</td>
<td>1.7 ± 0.4</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>2AAF (24 weeks) + control diet (4 weeks)</td>
<td>124 ± 7</td>
<td>17.1 ± 1.8</td>
<td>1.6 ± 0.4</td>
<td>31 ± 6</td>
</tr>
<tr>
<td>2AAF (24 weeks) + control diet (10 weeks)</td>
<td>128 ± 6</td>
<td>19.0 ± 2.4</td>
<td>1.6 ± 0.4</td>
<td>27 ± 6</td>
</tr>
<tr>
<td><strong>Control livers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(N)-Nitrosomorpholine (7 weeks) + control diet (10 weeks)</td>
<td>35 ± 4</td>
<td>13.4 ± 1.5</td>
<td>0.5 ± 0.1</td>
<td>33 ± 4</td>
</tr>
</tbody>
</table>

\(^a\) The treatment schedule is described in "Materials and Methods."

\(^b\) Means ± SD are listed (\(n = 4\)).
DISCUSSION

The present study of UDP-GT in liver nodules and carcinomas suggests persistently increased expression of one UDP-GT isozyme (termed UDP-GTI) of a large enzyme family. As noted previously (14), BP-3,6-quinol appears to be a selective and functionally interesting probe to identify UDP-GTI. Immunoblot analysis using two types of antibodies substantiated the presence of introns in regions adjacent to those recognized by the synthetic DNA probe.

Availability of selective DNA probes for UDP-GTI may allow the elucidation of mechanisms underlying the persistently increased expression of UDP-GTI in hepatocyte nodules. In the case of NAD(P)H:quinone reductase or DT-diaphorase (39) and the protooncogene c-myc (40), it has been suggested that persistently increased expression of these proteins may be due to hypomethylation of their genes. However, cleavage of UDP-GTI DNA with two pairs of isoschizomeric endonucleases did not reveal differential cleavage sites in DNA of livers from untreated rats, liver nodules, and hepatoma H4IIE cells (Fig. 3, II and III), suggesting that the cleavage sites were not methylated. The fragments obtained with PstI were slightly larger (by 500 base pairs) than with MspI. Moreover, the DNA fragments obtained were much larger (approximately 6000 base pairs) than expected from cDNA (approximately 600 base pairs), suggesting the presence of introns in regions adjacent to those recognized by the synthetic DNA probe.

The persistently increased expression of UDP-GTI, which can be studied at multiple levels including the gene level, may provide clues to the nature of the multiple protein alterations observed at cancer prestages and summarized as the toxin-resistance phenotype, for example in the Solt-Farber model, which uses 2AAF as the mitoinhibitory agent (19). The increased level of UDP-GTI may explain enhanced urinary excretion of glucuronides of 2AAF metabolites in nodule-bearing

2AAF appears to be a weak inducer of both 3MC- and phenobarbital-inducible UDP-GT isozymes. 2AAF has also been shown to be a 'mixed-type' inducer of 3MC- and phenobarbital-inducible P450 isozymes, probably as a result of differential inducing properties of some of its metabolites (37). This may be due to its metabolism to a number of metabolites with differing inducing properties. It has been shown in the present and previous investigations that reversible induction could be clearly distinguished from persistent alterations of UDP-GTI during initiation of hepatocarcinogenesis (Table 2).

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rats, in particular the percentage increase of the glucuronide of N-hydroxy-2-acetylaminofluorene. Together with decreased antibodies to UDP-GT, Dr. Friedrich Wiebel (GSF Neuherberg, Munich, FRG) for providing selective nodules (42).

REFERENCES


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