A Relative Deficiency of Cytochrome P-450 and Aryl Hydrocarbon [Benzo(a)pyrene] Hydroxylase in Hyperplastic Nodules Induced by 2-Acetylaminofluorene in Rat Liver

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Summary

The concentrations of cytochrome P-450 and the activities of aryl hydrocarbon [benzo(a)pyrene]hydroxylase (AHH) and reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase were measured in early (gray-white) and remodeled (brown) hyperplastic nodules induced in the livers of rats with 2-acetylaminofluorene and were compared to the values in control livers and in the liver surrounding the nodules.

Cytochrome P-450 content of early (14 weeks) hyperplastic nodules is 30% of the activity of untreated control livers and 48% of the activity of the surrounding liver. AHH activity of the early nodules is 10% of the control activity and 33% of the activity in the surrounding nonnodular liver. Nicotinamide adenine dinucleotide phosphate-cytochrome c reductase activity in the microsomes of early nodules is 76% of the control activity and 78% of the activity in the surrounding liver. In the late remodeled nodules, (22 and 25 weeks), the cytochrome P-450 content is 40% of that of controls and AHH activity is 15% of the control activity. In primary hepatomas induced by 2-acetylaminofluorene, cytochrome P-450 content is 21% of that of controls, AHH activity is 11% of the activity of controls, and reductase is 50% of the control activity. These results, indicating a relative nodule deficiency in some of the cellular components believed to be important in the activation of hepatocarcinogens and hepatotoxins, offer one possible explanation for the relative resistance to carcinogen cytotoxicity of hyperplastic liver nodules.

Materials and Methods

Male Fischer rats (Carworth Farms, New City, N. Y.) weighing 130 to 160 g were fed either basal diet (4) or 0.05% 2-AAF diet according to the pulsating regimen outlined by Epstein et al. (4) and Farber et al. (8). All animals were sacrificed by cervical fracture, following a 24-hr period of fasting. Nodules and nonnodular foci were obtained from the same liver. Where sufficient tissue (about 1 g) could be obtained, biochemical analysis was performed on tissue from each animal separately; otherwise, nodules and surrounding liver tissue from several animals were pooled. Careful histological study was performed on all tissues used for biochemical assay. A rim of nodular tissue was routinely left to ensure clean separation of nodular tissue from the surrounding nonnodular tissue. The tissue was fixed in Carnoy’s solution for histological examination and stained to the necrogenic effects of 2 hepatotoxins, CCl₄ and DMN, as compared to the surrounding nonnodular liver. In addition, the hyperplastic nodules show a considerable reduction in the labeling of their DNA, RNA, and protein with [³H]DMN, as compared to normal or surrounding liver, without any significant decrease in the uptake of the DMN. The changes seen when [9-¹⁴C]-2-AAF was used instead of labeled DMN were much more complex and difficult to delineate, since the uptake of radioactive 2-AAF was decreased by at least 80% in the nodules as compared to control liver.

In an attempt to understand the possible basis for the relative resistance of nodules to cytotoxicity and for the decrease in macromolecular labeling with DMN, the 1st hypothesis was that the hepatocytes in the hyperplastic nodules had less ability to activate hepatotoxins or hepatocarcinogens than did normal or nonnodular liver. Such a lesion could account for the decreased toxicity of the hepatotoxins and the decrease in the level of labeling of DNA, RNA, and protein.

The present study was undertaken to test this. Three known components of the microsome mixed-function oxygenase system were examined: cytochrome P-450, AHH, and the flavoprotein NADPH-cytochrome c reductase (20, 21, 31, 38). The large decrease in the concentration of cytochrome P-450 and in the activity of AHH in nodules of various ages induced by 2-AAF (2) is the subject of this paper.
with hematoxylin and eosin and with periodic acid-Schiff with or without prior incubation with diastase.

Gray-white nodules appear as early as 8 weeks on the pulsating regimen of 2-AAF and are sufficiently large by 14 weeks for gross isolation for discrete biochemical and morphological study. When followed in vivo, these nodules are seen to "mature," i.e., show areas that are liver colored, and, finally, by the end of the regimen (21 weeks), most of the nodules have become liver colored (4, 6, 7, 9).

All livers were perfused with cold 0.9% NaCl solution via the portal vein for 2 min to flush out RBC. The nodules and hepatomas perfused as readily as did the grossly uninvolved liver, as judged by visual inspection.

Tissue was homogenized in a Tris-KCl buffer (0.05 M Tris-HCl, pH 7.5-0.1 M KCl) with 10 strokes of a Thomas homogenizer. The homogenate was centrifuged at 2,600 rpm (800 x g) for 10 min (Sorvall centrifuge with a SS-34 rotor) to pellet nuclei. The supernatant was then centrifuged at 8,500 rpm (9,000 x g) for 20 min to pellet mitochondria. An aliquot of the supernatant was taken for AHH determination, and the remaining supernatant was centrifuged at 33,000 rpm (105,000 x g) for 60 min to pellet the microsomes (International IEC B60 centrifuge with Rotor A169). The microsomal pellet was resuspended in Sorenson's 0.1 M phosphate buffer, pH 7.4. Total DNA of the nucleus was determined by the method described previously (4), and protein was determined according to the method of Lowry et al. (22).

NADPH, cytochrome c, benzopyrene, quinine sulfate, and bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals were from other commercial sources except as specified.

Cytochrome P-450 Assay. Aliquots (0.7 ml) of dilute whole-liver homogenate or of a microsomal suspension were added to sample and reference cuvets (10 x 4 mm) and mixed with a solution of dithionite. After mixing, the cuvets were placed in a Hitachi Perkin Elmer Model 356 dual-wavelength spectrophotometer operating in the difference mode (5). Specimens were scanned at an ordinate expansion of either 0.03, 0.1, or 0.3 A, full-scale deflection from 500 to 430 nm. After a baseline had been recorded, carbon monoxide was bubbled for 10 to 20 sec into the cuvet without removal from the spectrophotometer, and immediately following this a 2nd scan was made. The extinction coefficient $E_{max}$ at 450 to 490 nm = 91 cm$^{-1}$ for conventional difference spectroscopy was used as originally described by Omura and Sato (25).

NADPH-Cytochrome Reductase. The reductase assay was performed on microsomal suspensions by the method of Williams and Kamin (43), modified for the dual-wavelength spectrophotometer. Aliquots (0.25 ml) of 3 components of the reaction mixture, namely, the microsomal suspension, 3 mm KCN, and 0.15 mm oxidized cytochrome c were pipetted in turn into a 10-mm path length x 4-mm cuvet. The reference beam of the spectrophotometer was set at 565 nm and the sample beam at 550 nm. Absorbance (0.1 A, full-scale deflection) was displayed on the ordinate of a Rikadenki 10-inch fast response (0.1 sec for 90% full-scale deflection) recorder run at a paper speed of 150 mm/min.

To initiate the reaction, 25 μl NADPH (50 mM in phosphate buffer, pH 7.4) was added using a specially fabricated plunger carefully machined to fit the microcuvet but with two 1.5-mm holes drilled through it into which the NADPH solution could be placed. A millimolar extinction coefficient for a change from oxidized to reduced cytochrome c of 18.6 cm$^{-1}$ was used.

Assay for AHH. The AHH assay was modified from the method of Poland and Glover (28). The assay using benz(a)pyrene was performed in a reaction mixture containing a total volume of 1.0 ml which included 62.5 μM Tris-HCl buffer, pH 7.2; 3.75 μM MgCl₂; bovine serum albumin (1.25 mg/ml); 0.1 ml postmitochondrial supernatant suspension (equivalent to 5 mg of liver); and 50 μl of 10 μM NADPH in Tris-HCl buffer, pH 7.2 (7.67 mg/ml), with 50 μl of 2 μM benz(a)pyrene in methanol (0.504 mg/ml) added to initiate the reaction. The reaction was incubated for 10 min in a covered, shaking water bath at 37° and was terminated by adding 1.0 ml acetone and 3.25 ml hexane. The mixture was shaken vigorously in the dark to extract the benzo(a)pyrene and metabolites. A 1-ml sample of the organic phase was extracted with 3.0 ml of 1 N NaOH, and the alkali-extractable metabolites were examined in an Amico-Bowman Model 4-8202 SPF spectrophotofluorometer. Fluorescence corresponding to 3-hydroxybenzo(a)pyrene has an activation peak at 395 nm and an emission maximum at 520 nm. The fluorescence of a sample blank, to which the benzo(a)pyrene was added, immediately before the acetone and hexane, was subtracted from the fluorescence of each experimental sample. The fluorometer was calibrated with a standard solution of quinine sulfate and 3-hydroxybenzo(a)pyrene, kindly supplied by Dr. H. Gelboin (11). One unit of AHH was defined as the amount of enzyme catalyzing the formation per min at 37° of hydroxylated product causing fluorescence equivalent to that of 1 pmole 3-hydroxybenzo(a)pyrene.

RESULTS

The hyperplastic nodules in general show a considerable reduction in the cytochrome P-450 content in microsomes and in the AHH activity of the 9000 x g supernatant, compared to control liver (Table 1; Charts 1 and 2). In every instance, the early (14 week) nodules show a larger decrease in each measurement when compared to control or surrounding liver than do nodules at later time periods. Grossly, the early nodules are much more uniform in appearance, being gray-white and sharply demarcated from the surrounding red-brown liver. Later nodules are much more variable, some (a minority) being gray-white but the majority being red-brown and less well demarcated from the surrounding liver. Histologically, the gray-white nodules consist of fairly uniform, large hepatocytes with abundant eosinophilic cytoplasm, due to hypertrophied smooth endoplasmic reticulum and large pale nuclei with large nucleoli (6, 7). The later, liver-colored nodules show a much more varied cell population with many areas of remodeling or maturation to normal-appearing liver arranged in single-cell plates.

The values for the hepatomas were again quite uniformly
Table 1

Cytochrome P-450 and AHH activity in hyperplastic nodules, surrounding nonnodular liver, and hepatomas of 2-AAF-treated rats and in control livers

<table>
<thead>
<tr>
<th>Diet*</th>
<th>Nodule** or hepatoma</th>
<th>Surrouding</th>
<th>Control***</th>
<th>Nodule</th>
<th>Surrouding</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AAF (14 wk)</td>
<td>0.51 ± 0.02*</td>
<td>1.05 ± 0.17*</td>
<td>1.68 ± 0.19*</td>
<td>4.9 ± 1.1*</td>
<td>15.0 ± 4.2*</td>
<td>48.7 ± 2.3*</td>
</tr>
<tr>
<td>2-AAF (21 wk)</td>
<td>0.23 ± 0.03</td>
<td>0.31 ± 0.04*</td>
<td>0.56 ± 0.05</td>
<td>8.9 ± 1.0</td>
<td>11.8 ± 1.2</td>
<td>50.1 ± 10.2</td>
</tr>
<tr>
<td>Purina Chow (1 wk)</td>
<td>0.37 ± 0.09</td>
<td>0.48 ± 0.07*</td>
<td>0.95*</td>
<td>2.0 ± 0.2</td>
<td>4.3 ± 0.8</td>
<td>15.2*</td>
</tr>
<tr>
<td>2-AAF (21 wk)</td>
<td>0.20 ± 0.08</td>
<td>0.96 ± 0.03</td>
<td>1.2 ± 0.5*</td>
<td>10.6 ± 1.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The rats were fed 0.05% 2-AAF in basal diet for 14 or 21 weeks, according to the pulsating regimen outlined in the text, followed by Purina Chow. Control rats were fed basal diet for up to 21 weeks followed by Purina Chow.

** The measurements on 14, 21 and 1, and 21 and 4 weeks were on hyperplastic nodules, whereas those at 21 and 23 weeks were on hepatomas.

*** The values represent the mean ± S.D. of 4 separate experiments. Each experiment represents the liver of 1 rat. In the case of early nodules, occasionally, the nodules of 2 or 3 rats were pooled.

1 The values for nodules were statistically highly significant when compared to controls (p < 0.001). Nodule values compared to surrounding values were statistically significant at p < 0.05 except the 2 values marked f.

2 In these 2 experiments, the surrounding liver contained microscopic nodules (6, 9). The values for surrounding liver compared to nodules at this stage were different only at the p < 0.2 level of significance (not significant).

3 These values involved only 1 experiment.

Chart 1. Cytochrome P-450 in nmoles/mg protein of the microsomes of nodules and hepatoma expressed as a percentage of either control values or values for the surrounding nonnodular liver. Each bar graph plot represents the mean and standard deviation of the values from 4 experiments (4 control rats or 8 to 12 rats with nodules).

Chart 2. AHH in units/mg liver of 9000 x g supernatant of nodules and hepatoma expressed as a percentage of control activity and as a percentage of the activity of the surrounding nonnodular liver. The measurements were made on the same tissues as were the cytochrome P-450 (Table 1).

low, in the same range as were the early nodules. The surrounding liver falls for the most part between the values for control and nodules or hepatomas. Early, the surrounding liver is reasonably homogeneous with only a few very small nodules seen histologically. However, at later time periods, the surrounding liver is much more heterogeneous, consisting of liver-colored nodules as well as ductular tissue and nonnodular liver. This makes comparison between the nodules or the hepatomas and the surrounding liver less exact.

Since the hepatocytes in hyperplastic nodules are generally much larger than are normal liver cells and since much of the enlargement is due to abundant smooth endoplasmic reticulum (6), it became important to determine to what extent the decreases in cytochrome P-450 content and in AHH activities were due to “dilution” with more protein and fewer cells per unit weight of liver. In Table 2, the differences in AHH activities between nodules and nonnodular liver tissue remain large, even when expressed as units per mg DNA. The results with cytochrome P-450, not shown, are similar. Thus, it appears that the differences in these parameters between hyperplastic nodules and surrounding or control liver are not due to dilution.

It must be pointed out that the values for cytochrome P-450 and for AHH activity in the control animals vary as a function of time on the control diet. The basis for such variation is not clear at this time. However, the level of cytochrome P-450 and for AHH activity, relative to the control, is consistently less, regardless of this variation.

NADPH-cytochrome c reductase activity in the microsomes of the early (gray-white) nodules is decreased by 20% as compared to the surrounding and control livers (Table 3; Chart 3). This value increases to 30 to 40% in the late (liver...
AHH activity in hyperplastic nodules, surrounding nonnodular liver of 2-AAF-treated rats, and in control livers, expressed per weight of liver, protein, or DNA

<table>
<thead>
<tr>
<th></th>
<th>AHH activity (Units/mg DNA)</th>
<th>Total nuclear DNA (mg/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Units/mg liver</td>
<td>Units/mg protein</td>
</tr>
<tr>
<td>Nodule*</td>
<td>4.9 ± 1.1</td>
<td>1.160 ± 270</td>
</tr>
<tr>
<td>Surrounding</td>
<td>15.0 ± 4.2</td>
<td>1.850 ± 440</td>
</tr>
<tr>
<td>Control†</td>
<td>48.7 ± 2.3</td>
<td>12.380 ± 1,120</td>
</tr>
</tbody>
</table>

* Total DNA in nodules was significantly different from surrounding (p < 0.001) but not different from controls.

† The AHH activities in nodules compared to controls were highly significant at p < 0.001. Nodule values compared to surrounding were significant at p < 0.05.

‡ Most of the values represent the mean ± S.D. of 4 separate experiments. Each experiment represents the liver of 1 rat (controls) or 1 g of tissue (nodule and surrounding) obtained from pooling tissue from 2 or 3 rats.

§ These values involved 3 separate experiments.

‖ Control rats were fed basal diet for 14 weeks (4).

NADPH-cytochrome c reductase of microsomes in hyperplastic nodules, surrounding nonnodular liver, and hepatomas of 2-AAF-treated rats and in livers of untreated control rats

<table>
<thead>
<tr>
<th>Diet</th>
<th>NADPH-cytochrome c reductase of microsomes (µmoles cytochrome c reduced/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nodule or hepatoma</td>
</tr>
<tr>
<td>2-AAF (14 wk)</td>
<td>0.260 ± 0.06</td>
</tr>
<tr>
<td>2-AAF (21 wk)</td>
<td>0.220 ± 0.02</td>
</tr>
<tr>
<td>Purina Chow (1 wk)</td>
<td>0.200 ± 0</td>
</tr>
<tr>
<td>2-AAF (21 wk)</td>
<td>0.090 ± 0.04</td>
</tr>
<tr>
<td>Purina Chow (4 wk)</td>
<td></td>
</tr>
<tr>
<td>2-AAF (21 wk)</td>
<td></td>
</tr>
<tr>
<td>Purina Chow (23 wk)</td>
<td></td>
</tr>
</tbody>
</table>

* These values represent the mean ± S.D. of 4 separate experiments. The reductase was measured on the same microsomal preparations on which the P-450 was measured (Table 1).

† Nodule values were significantly different from controls at p < 0.05. When compared to surrounding liver, 14-week nodule values are not significantly different (p < 0.1).

‡ These measurements were on hepatomas. Hepatoma values were significantly different from their controls (44 weeks Purina Chow) at p < 0.02.

DISCUSSION

The results of this study on some components of the liver microsomal monooxygenase system in hyperplastic nodules and hepatomas induced by 2-AAF are in good agreement with those of Gravela et al. (13) with ethionine-induced nodules and hepatomas, which were published while this study was in progress. Thus, it appears that, with 2 chemically quite different hepatocarcinogens, putative premalignant hepatocyte populations and hepatomas show remarkably similar patterns with respect to the microsomal activation system. In the study of Gravela et al. (13), cytochrome P-450 and NADPH-cytochrome c reductase activity were measured in common with ours. However, instead of AHH activity, they measured another enzyme in the system, amidopyrine demethylase activity.

Our results and those of Gravela et al. (13) are in agreement with the few published reports showing low levels of cytochrome P-450 in various hepatomas (3, 14, 26). Other cytochromes, including microsomal cytochrome b5 and mitochondrial cytochrome a + a3 are also lower in hepatomas than in normal liver. Cytochrome P-450, unlike these other cytochromes, is decreased in the liver as a whole at 22 weeks during carcogenesis with 3'-methyl-4-dimethylaminoazobenzene (26). In this study, the level in hyperplastic nodules separated from nonnodular liver was not measured.

The results of our study with 2-AAF and those of Gravela et al. (13) with ethionine offer a possible theoretical explanation for the relative resistance of the putative premalignant hepatocytes, hyperplastic nodules, to the cytotoxic effects of hepatotoxins and hepatocarcinogens, as described in the preceding publication (8). Although the exact enzymological components for activation and inactivation of 2-AAF and DMN as well as CCI₄ have yet to be delineated it appears that all 3 of these hepatotoxins and many others (10, 11, 16, 24, 29, 36) are metabolized by liver microsome systems, probably part of the mixed-function monooxygenase system (see Refs. 3, 12, 21, 30, 34, and 37). Since it is probable that some portion of the system is concerned with...
the activation of 2-AAF, DMN, and CCl₄, to hepatotoxic derivatives, the decrease in levels and activities of some components of the system in hyperplastic nodules could account for the relative resistance to the necrogenic effects of CCl₄ and DMN and to the decrease in binding of DMN to macromolecules. Naturally, further in-depth studies are required before such a hypothesis can be considered to be other than suggestive.

The results of this study offer some additional support for the hypothesis that the induction, by carcinogens, of carcinogen-resistant hepatocytes may be important in the selective growth and evolution of preneoplastic and premalignant hepatocytes in liver carcinogenesis (see Refs. 6, 8, and 18). This conceivably may be part of a larger construct in which the levels of carcinogen-activating and -inactivating enzymes may play a major determinant role in liver carcinogenesis (17, 23, 27, 35, 39).

Cytochrome P-450 content in hepatocytes shows regional variations in normal and induced livers histochemically (1, 32, 39). There are also striking age, species, and sex differences in the content of the cytochrome P-450 complex and in the presence of certain forms of the P-450 cytochrome (15, 19, 33, 41). If the microsomal monooxygenase system and its contained cytochrome P-450 group are as important to the normal functions of the liver as is now believed, it becomes important to understand their role in maintenance of normal homeostasis and in the genesis of both acute and chronic liver cell injury. The present observations and those of Gravela et al. (13) indicate that the hepatocytes in hyperplastic nodules induced by at least 2 carcinogens offer an interesting new model for the study of the relationship between the functioning of the monooxygenase system and the biological behavior of hepatocytes.

REFERENCES


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