Patterns of Ligand Binding to Normal, Regenerating, Preneoplastic, and Neoplastic Rat Hepatocytes

Leonard Harris, Veronique Preat, and Emmanuel Farber

ABSTRACT

The binding of epidermal growth factor, asialoorosomucoid, and apoprotein E-rich lipoproteins to isolated hepatocytes was investigated at various time intervals during the step-by-step development of liver cancer in rats. The degree of binding of the three ligands showed a progressive reduction in early persistent and late persistent putative preneoplastic hepatocyte nodules. This was further decreased in hepatocytes isolated from unequivocal hepatocellular carcinomas. Regenerating liver hepatocytes bound lesser amounts of epidermal growth factor and asialoorosomucoid than did hepatocytes from control resting liver but increased amounts of apoprotein E-rich lipoproteins. The progressive decrease in ligand binding during the precancerous phase of hepatocarcinogenesis, the nodule-to-cancer sequence, may render nodules less responsive to the influences of their external environments.

INTRODUCTION

A new pattern of phenotypic changes, characteristic of a new state of differentiation, is seen in hepatocyte nodules and in the small subset of persistent nodules which are proximate and ultimate precursor populations for hepatocellular carcinoma (1, 2). These changes include altered morphology and cellular architecture (3, 4), altered hemodynamics (5–8), a characteristic biochemical pattern (9–11), increased cell proliferation (12, 13), and a possible change in growth regulation (13). These phenotypic changes are associated with the acquisition of a characteristic resistance of nodules to cytotoxicity including inhibition of cell proliferation by many xenobiotics. This resistance is the basis for the formation of nodules during promotion in at least one model of hepatocarcinogenesis (14–18). The large majority of resistant nodules redifferentiate to normal-appearing liver (3, 19, 20), but a few continue to proliferate, and these persistent nodules are the sites of cancer development at a later stage (1).

Since many phenotypic expressions of cells are considered to be modulated by external signals acting on the plasma membrane (21–26), it is possible that variations in the binding of different ligands may have a role to play in the biological behavior of different cell populations during the carcinogenic process (27–30).

The aim of the present study was to determine whether hepatocytes isolated from discrete hepatocyte nodules at two different steps or stages during progression in hepatocarcinogenesis would show alterations in the binding of 3 different ligands. These are EGF, ASOR, and apo-E-rich rat serum lipoproteins. Three reports have appeared in the literature in which desialylated glycoproteins (31), asialofetuin (32), and ASOR (33) displayed decreased binding to hepatocytes isolated from whole liver or from liver nodules during liver carcinogenesis. Because of the current availability of a model of liver carcinogenesis, the resistant hepatocyte model, in which at least 7 discrete and synchronized steps can be identified (1, 34), it became of interest to study several of these steps with respect to binding of ligands to isolated hepatocytes. Two of the ligands, EGF and apo-ELP, have not been reported to have been used previously in binding studies during hepatocarcinogenesis. For comparison, hepatocytes isolated from livers during liver regeneration and from hepatocellular carcinomas were also used. The distinctive patterns of ligand binding to the various hepatocytes and some implications of the findings are the subjects of this paper.

MATERIALS AND METHODS

Materials. Diethylaminoethylamine was from Kodak, Rochester, NY; 2-AAF, from Aldrich Chemical Co., Milwaukee, WI; human orosomucoid and murine EGF, from Sigma Chemical Co., St. Louis, MO; 125I-EGF (180 Ci/Mg), from New England Nuclear, Boston, MA; IODOGEN, from Pierce Chemicals, Rockford, IL; and Na125I and carrier-free and 125I-Protein A, from Amersham Canada, Oakville, Ontario, Canada.

Asialoorosomucoid was obtained by mild hydrolysis of human orosomucoid in 0.1 N H2SO4 at 80°C for 60 min (35), and the extent of desialylation was monitored by agarose gel electrophoresis. apo-E-rich lipoproteins were isolated by centrifugal flotation as described by Havel et al. (36). Briefly, 30 to 60 ml of fresh rat serum were adjusted to a density of 1.06 by the addition of solid KBr. This was overlaid with 0.01% EDTA solution in 0.01 M Tris (pH 7.0) at a density of 1.006. The serum was centrifuged at 100,000 × g (average) for 18 h, and the interface was collected, readjusted to a density of 1.060, and centrifuged as before. The d < 1.060 layer was collected and desalted by passage through a column of Sephadex G-25. The lipoprotein fraction was subjected to polyacrylamide gel electrophoresis and found to consist mainly of apo-E with some contaminating apo-B and apo-C (37).

Animals. Male Fischer 344 rats (Charles River Breeding Laboratories, Kingston, NY), initially weighing 150 to 180 g, were treated by a modification of the regimen originally described by Solt and Farber (15). Animals were initiated with a single dose of diethylnitrosamine (200 mg/kg, i.p.,) and 2 week later were given 2-AAF (20 mg/kg) by gavage for 3 consecutive days and followed by a ½ partial hepatectomy and one 5-mg/kg dose of 2-AAF 4 days later (38). Early nodules were isolated 4 mo after initiation, and late nodules and cancers were isolated 12 to 14 mo after initiation.

Isolation of Hepatocytes. Hepatocytes were isolated as described previously with minor modifications (39). Rats were anesthetized with sodium pentobarbital (0.1 ml/100 g, body weight) and remained viable when perfusion was started. Cells from untreated and regenerating (24 h post-PH) livers were isolated by perfusing the liver through the portal vein with Hanks' balanced salt solution (without Ca2+ or Mg2+) containing ethyleneglycol bis(β-aminoethyl ether)-N,N',N''-tetraacetic acid (0.01 m) and HEPES (0.1 m) for 5 min, followed by collagenase type I (90 units/ml in Williams E medium containing 0.01 m HEPES, 42°C) until the liver was soft (usually 4 to 7 min). The capsule was removed, and the hepatocytes were shaken into fresh medium. Parenchymal cells were enriched by repeated sedimentation in MEM. Nodule and cancer hepatocytes were similarly isolated, but collagenase type IV
was used, and perfusion was via the inferior vena cava because of the decrease in portal vein blood supply to nodules (5-8). Collagenase perfusion was continued until the nodules were soft, and they were freed from the firmer surrounding hepatocytes. Viability was determined by trypan blue exclusion, and preparations with fewer than 70% viable cells were discarded.

Preparation of Labeled Ligands. ASOR was iodinated using 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril (IODO-GEN) to a specific activity of 1 to 2 × 10^6 cpm/μg. IODO-GEN (35 μg) was coated in glass vials and reacted with 300 μg of ASOR and 1 μCi of Na^125I in 50 μl of NaPO_4 buffer (pH 7.5) at 0°C. The reaction was continued for 15 min, and labeled ASOR was separated from free iodide by gel filtration (40). Lipoproteins were iodinated to a specific activity of 4 to 6 × 10^6 cpm/μg as described by Shepherd et al. (41).

Binding Assay. All binding assays were performed within 30 min after cell isolation. Aliquots of hepatocytes were incubated in binding buffer (Eagle’s minimal essential medium—20 mM HEPES, pH 7.5) at 4°C for various amounts of time with iodinated ligands. After incubation, the cells were centrifuged, and the pellet was washed with Williams E medium. This was repeated 3 times. The pellets were cut, and radioactivity was measured with a gamma counter (ILKB CompuGamma 1282). All assays were performed in duplicate. To determine EGF binding, 250,000 viable cells were incubated at 4°C in 0.5 ml of MEM containing 0.1% albumin and 0.05 ng/ml to 100 ng/ml of ^125I-EGF for 4 h. Nonspecific binding was determined in the presence of 0.5 μg of unlabeled EGF. ASOR binding was determined using 250,000 to 500,000 viable hepatocytes at 4°C containing 0.5 to 15 μg/ml of ^125I-ASOR in a final volume of 0.5 ml of MEM for 90 min. Nonspecific binding was determined in the presence of a 50-fold excess of unlabeled ligand. apo-E lipoprotein binding was determined using 250,000 to 500,000 viable hepatocytes at 4°C in 0.8 ml of MEM containing 1.4 mM CaCl_2 and 2 to 20 μg/ml of ^125I-apo-ELP for 120 min. Nonspecific binding was measured in the presence of a 50-fold excess of unlabeled ligand.

In Vivo Binding of ^125I-ASOR to Hepatocytes. Animals, either untreated or with nodules 4 mo after initiation, were given injections through the inferior vena cava with approximately 10 μg of ^125I-ASOR (6 × 10^6 cpm in 0.05% bovine serum albumin-0.9% NaCl solution). After 5 or 20 min, the livers were perfused with 250 ml of ice-cold 0.25 M sucrose through the inferior vena cava with drainage through the portal vein. The animals had been anesthetized with sodium pentobarbital (0.1 ml/100 g body weight) and remained viable until 4 h. Nonspecific binding was determined in the presence of 0.5 μg/ml of bovine serum albumin-0.9% NaCl solution. After 5 or 20 min, the mice were killed by an overdose of pentobarbital, and the liver was excised and weighed. The livers were fixed in 10% buffered formaldehyde, sectioned, and stained with hematoxylin and eosin.

Immunodetection of ASOR Receptors. A total membrane fraction was prepared by homogenizing 1-g aliquots of tissue isolated from 3- to 4-mo-old rats treated control or with nodules 4 mo after initiation, were given injections through the inferior vena cava with approximately 10 μg of ^125I-ASOR (6 × 10^6 cpm in 0.05% bovine serum albumin-0.9% NaCl solution). After 5 or 20 min, the mice were killed by an overdose of pentobarbital, and the liver was excised and weighed. The livers were fixed in 10% buffered formaldehyde, sectioned, and stained with hematoxylin and eosin.

RESULTS

Generation of Nodules and Cancers. The livers of treated rats contained discrete grayish-white nodules. Animals sacrificed at 4 mo had many small nodules in their liver, 0.3 to 0.7 cm in diameter, which stained histochemically for γ-glutamyltransferase. Animals sacrificed at 14 mo typically had large (2 to 4 cm) neoplasms irregular in outline, showing umbilication, variability in color, and consistency. These were typical carcinomas (1-4). Microscopically, these neoplasms were typical trabecular hepatocellular carcinomas. These livers also contained a small number of γ-glutamyltransferase-positive positive hepatocyte nodules, grayish-white and discrete, 1 to 2 cm in diameter, which showed no signs of malignancy either grossly or microscopically.

Isolation of Hepatocytes. Retrograde perfusion of nodular liver with collagenase type IV allowed the isolation of a relatively “clean” population of nodules with good viability (70 to 90%). To ensure that retrograde perfusion with type IV collagenase was not modifying receptor sites, control hepatocytes were similarly isolated, and the bindings of EGF and ASOR were compared to the binding to hepatocytes isolated by perfusion with collagenase type I through the portal vein. The time of perfusion with collagenase was also varied between 4 and 15 min. No significant difference in ligand binding was observed in control hepatocytes isolated by any of these procedures (Table 1).

Binding of ^125I-ASOR. Binding curves and a Scatchard plot of ^125I-ASOR binding to control hepatocytes in the standard assay are presented in Fig. 1 as representative for all three ligands. The binding capacity of isolated hepatocytes from different lesions for the iodinated ligands is shown in Table 2. Control hepatocytes possessed 1170 × 10^3 ± 437 × 10^3 binding sites/cell for ASOR (Table 2). This was decreased in early and late nodules and in hepatocellular carcinoma with relative binding values of 50%, 28%, and 21% of control values, respectively (Fig. 2). Cells isolated from regenerating liver 24 h after PH bound only 40% of control values.

Binding of ^125I-EGF. There were a decrease in binding in regenerating liver and a progressive decrease in nodules and cancer during hepatocarcinogenesis (Table 2). Regenerating liver 24 h after PH showed a 79% decrease, as compared to that in control liver hepatocytes (Fig. 2), while the binding in early and late nodules and hepatocellular carcinomas was decreased to 46%, 6.5%, and 7.5% of the control value, respectively.

Binding of ^125I-apo-E-rich Lipoproteins. Based on an average molecular weight of 500,000 for the lipoproteins, control cells had 1678 × 10^3 ± 643 × 10^3 binding sites/cell (Table 2). This

Table 1 Effects of perfusion with two different collagenases on EGF binding to hepatocytes

<table>
<thead>
<tr>
<th>Collagenase</th>
<th>No. of animals</th>
<th>ng bound/10^6 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>3</td>
<td>1.34 ± 0.12*</td>
</tr>
<tr>
<td>Type IV</td>
<td>1</td>
<td>1.25 ± 0.31†</td>
</tr>
</tbody>
</table>

* Mean ± SD of duplicate assays on cells from each animal. † Not significantly different from mean of results with type I collagenase, using Student’s t test.
Table 2 Saturation binding of ASOR, EGF, and apo-ELP to hepatocytes isolated from different lesions during hepatocarcinogenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>ASOR ng bound/1 x 10^6 cells</th>
<th>ASOR No. of binding sites/cell × 10^3</th>
<th>EGF ng bound/1 x 10^6 cells</th>
<th>EGF No. of binding sites/cell × 10^3</th>
<th>apo-ELP ng bound/1 x 10^6 cells</th>
<th>apo-ELP No. of binding sites/cell × 10^3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>85.7 ± 32.1* (5)</td>
<td>1170 ± 437</td>
<td>1.64 ± 0.19 (4)</td>
<td>164 ± 19</td>
<td>1393.8 ± 534.9 (3)</td>
<td>1678 ± 643</td>
</tr>
<tr>
<td>Regenerating (24 h)</td>
<td>34.3 ± 6.2* (3)</td>
<td>468 ± 84</td>
<td>0.36 ± 0.03* (3)</td>
<td>36 ± 3</td>
<td>2338.0 ± 318.70* (3)</td>
<td>2815 ± 96</td>
</tr>
<tr>
<td>Early nodules (4 mo)</td>
<td>43.2 ± 7.9 (2)</td>
<td>589 ± 107</td>
<td>0.73 ± 0.13* (2)</td>
<td>74 ± 13</td>
<td>216.8 ± 79.4* (2)</td>
<td>261 ± 95</td>
</tr>
<tr>
<td>Late nodules (14 mo)</td>
<td>24.0 ± 0.3* (2)</td>
<td>327 ± 4</td>
<td>0.11 ± 0.02* (2)</td>
<td>11 ± 2</td>
<td>83.4 ± 2.4* (2)</td>
<td>100 ± 3</td>
</tr>
<tr>
<td>Cancer</td>
<td>17.9 ± 5.6* (3)</td>
<td>244 ± 76</td>
<td>0.13 ± 0.03*</td>
<td>13 ± 3</td>
<td>22.5 ± 9.9* (3)</td>
<td>27 ± 12</td>
</tr>
</tbody>
</table>

* Calculated using 500,000 as average molecular weight of lipoproteins.

Fig. 2. Saturation binding of ASOR, EGF, and apo-E to regenerating hepatocytes and hepatocytes isolated from nodules during hepatocarcinogenesis as percentage of control values. ü, 3-mo control; ü, regenerating (24 h); 0, 4-mo nodules; Ü, 14-mo nodules; •, cancer.

Table 3 In vivo binding of 125I-ASOR to control liver and nodules

<table>
<thead>
<tr>
<th>Population</th>
<th>Treatment duration (min)</th>
<th>Cpm/mg protein</th>
<th>Relative binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5 (1)*</td>
<td>3515</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20 (4)</td>
<td>4000 ± 500</td>
<td></td>
</tr>
<tr>
<td>Nodules</td>
<td>5 (1)</td>
<td>980</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 (4)</td>
<td>1827 ± 710</td>
<td>0.43</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of animals.

was decreased to 16 and 6% in early and late nodules, and to 2% of control binding in hepatocellular carcinomas. However, unlike the other two ligands, the binding of 125I-apo-E-rich lipoproteins was greatly increased (by about 70%) in hepatocytes isolated from regenerating liver (Fig. 2).

In Vivo Binding of 125I-ASOR. Nodules isolated from animals 4 mo after initiation bound only 43 ± 15.5% of control values (20-min incubation). Four determinations were made from each animal, and the assay was repeated 3 times for both control and nodular hepatocytes (Table 3).

Immunodetection of ASOR Receptors. As shown in Fig. 3, there is a decrease in the level of ASOR receptors detected by the Western blot technique similar to the pattern seen in binding of 125I-ASOR to isolated hepatocytes. Old (10 mo) untreated animals also show a decrease in detected receptors, although this is not as striking as the pattern seen in nodules and cancers.

DISCUSSION

This is the first report demonstrating a sequential decrease in ligand binding to discrete new cell populations at three distinct time points during hepatocarcinogenesis. Several reported studies have shown a decreased binding for desialylated glycoproteins after liver regeneration (46) and early after carcinogen treatment (31-33). The results presented in this paper are consistent with these earlier reports and indicate that the observed decrease in ASOR binding is probably due to a decrease in receptor protein. Recently, it has been reported that phenobarbital can modulate the number of asialoglycoprotein receptors in the liver (47). The receptor for EGF has also been demonstrated to be decreased during liver regeneration (48, 49) and after exposure to carcinogens (50, 51). EGF binding is also decreased in microsomes prepared from several Morris hepa-
In several different carcinomas, the receptors for low density lipoproteins are also decreased (53–55). The increase in apo-E lipoprotein binding to regenerating liver indicates that the sequential decrease in nodule and carcinoma hepatocytes is not simply an association with rapidly proliferating cells. This decrease is not likely due to the acute effects of carcinogen treatment, given the long interval after exposure to the carcinogen. The sequential decrease observed may be due to a natural enrichment of nodules with persistent proliferating hepatocytes (13, 19, 20). As nodules remodel, the more persistent lesions continue to proliferate, and a decrease in ligand binding may reflect a “persistent phenotype.”

The observed decrease in ligand binding could be attributed to a number of factors. There may be a decrease in receptor number due to down-regulation of receptors (22, 23, 25, 31). Changes in phosphorylation (22, 46, 56–58) as well as glycosylation (28, 29) of receptors may also affect ligand binding. The terminal sialic acid portion of the ASOR receptor is essential for binding (59), and various changes in glycosylation have been observed in a variety of carcinomas that involve sialic acid residues (60–65). Huber et al.4 have data suggesting the asialoglycoprotein receptor gene remains transcriptionally active in regenerating, preneoplastic, and neoplastic rat hepatocytes. The decreased expression of asialoglycoprotein receptors may therefore involve a posttranslational event, such as altered packaging, transport, and/or recycling of the receptor to the plasma membrane.

The expression of several oncogenes is altered during liver regeneration and hepatocarcinogenesis (66), and this may also regulate receptor binding. There is great structural homology between the v-erb-B oncogene and a truncated form of the EGF receptor that lacks a ligand binding site but still maintains a protein kinase activity (67). Oncogenes may also affect receptor binding through phosphorylation and glycosylation (27–29, 68–70).

It is interesting to note that orosomucoid and the receptors for EGF and apo-E-rich lipoproteins all have considerable sequence homology and may be under similar regulatory control (71–73).

The biological significance of a decrease in receptor binding during hepatocarcinogenesis is not clear. Persistent nodules are able to respond to a proliferative stimulus in vivo (13). However, persistent nodules display a higher basal labeling index compared to surrounding liver (3, 4, 13, 20). Later persistent nodules, unlike surrounding liver, do not return to their basal rates of cell proliferation after a proliferative stimulus (14).

Recently, it has been shown that hepatocytes from persistent nodules are less responsive to the mitogenic effects of EGF in vitro than are hepatocytes isolated from untreated animals.5 In contrast, hepatocytes isolated 24 h after partial hepatectomy are still able to proliferate in vitro in response to EGF, although EGF receptors are undetectable (74). This may indicate fundamental differences in regulatory control between cells proliferating rapidly in response to a strong mitogenic stimulus, such as PH, and the proliferating hepatocytes in a persistent nodule. Thus, decreased ligand binding may play a role in the altered response of persistent nodules to external factors that regulate cell proliferation and other physiological cell functions. This could conceivably be associated with a presumed acquisition by hepatocyte nodules of new patterns of control, such as autocrine control (75), if such a phenomenon does occur in precursor cells and in cancer cell populations in the liver.

REFERENCES

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