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

Leonard Harris, Veronique Preat, and Emmanuel Farber
Departments of Pathology [L. H., V. P., E. F.] and Biochemistry [E. F.], Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada MSS IA8

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 later 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 stage 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 to ultimate precursor populations for hepatocellular carcinoma. These changes include altered morphology and cellular architecture, altered hemodynamics, altered biochemical pattern, increased cell proliferation, and a possible change in growth regulation. 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. Since many phenotypic expressions of cells are considered to be modulated by external signals acting on the plasma membrane, it is possible that variations in the binding of different ligands may have a role in the biological behavior of different cell populations during the carcinogenic process.

MATERIALS AND METHODS

Materials. Diethylnitrosamine 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 μCi/μg), 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 a layer of 10% Ficoll-Hypaque, and the d < 1.060 layer was collected and desalted by passage through a column of Sephadex G-25. The lipoprotein fraction was subjected to polycrylamide 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 followed by a 1/2 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',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. Nodules 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-3,6-diphenyldifluorobenzene (IODO-GEN) to a specific activity of 1 to 2 x 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^225I in 50 µl of Na_2HPO_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 x 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 (LKB 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 1^25I-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 1^25I-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 1^25I-apo-ELP for 120 min. Nonspecific binding was measured in the presence of a 50-fold excess of unlabeled ligand.

In Vivo Binding of 1^25I-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 1^25I-ASOR (6 x 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 perfusion with sucrose. The livers were excised, and 1-g nodules were prepared by homogenizing 1-g aliquots of tissue isolated from 3- to 10-mon controls and regenerating liver, as well as from nodules and hepatocellular carcinomas. Each point represents the mean of duplicate values.

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 y-glutamyltransferrase. 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 y-glutamyltransferase-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 1^25I-ASOR. Binding curves and a Scatchard plot of 1^25I-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 ± 437 x 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 1^25I-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 1^25I-apo-E-rich Lipoproteins. Based on an average molecular weight of 500,000 for the lipoproteins, control cells had 1678 ± 437 x 10^3 x 10^3 binding sites/cell (Table 2). This
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</th>
<th>EGF</th>
<th>apo-ELP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng bound/ 1 x 10^6 cells</td>
<td>No. of binding sites/cell</td>
<td>ng bound/ 1 x 10^6 cells</td>
</tr>
<tr>
<td>Control</td>
<td>85.7 ± 32.1*(5)</td>
<td>1170 ± 437</td>
<td>1.64 ± 0.19 (4)</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>
</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>
</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>
</tr>
<tr>
<td>Cancer</td>
<td>17.9 ± 5.6° (3)</td>
<td>244 ± 76</td>
<td>0.13 ± 0.03°(3)</td>
</tr>
</tbody>
</table>

* Calculated using 500,000 as average molecular weight of lipoproteins.
° Mean ± SD of duplicate assays in each experiment.
* Numbers in parentheses, number of rats.
° p < 0.05.
• p < 0.001.
/ P < 0.01.

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. 1, 3-mo control; 2, regenerating (24 h); 3, 4-mo nodules; 4, 14-mo nodules; 5, cancer.

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-
tomomas (52). 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 oromucoid 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 vivo than are hepatocytes isolated from untreated animals.5 In contrast, hepatocytes isolated 24 h after partial hepatectomy are still able to proliferate in vivo 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


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

Leonard Harris, Veronique Preat and Emmanuel Farber


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/47/15/3954

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.