Effect of Heterogeneity of Carcinoembryonic Antigen on Liver Cell Membrane Binding and Its Kinetics of Removal from Circulation

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ABSTRACT

Carcinoembryonic antigen (CEA) is a glycoprotein metabolized primarily by the liver. Subcellular fractions of rat liver were examined for CEA binding activity. Hepatocyte plasma membrane and microsome fractions bound CEA, and this binding shared the calcium requirement, neuraminidase sensitivity, and carbohydrate specificity of the hepatocyte asialoglycoprotein receptor. CEA had previously been shown to react with this galactose-specific receptor, in vivo, only following neuraminidase treatment.

Galactose receptor binding of CEA was measured in three different purified CEA preparations. The fraction of CEA capable of binding to excess levels of galactose receptor on membranes varied (46.5%, 40.2%, and 4.7% for CEA-1, -2, and -3, respectively). These CEA were shown to be 2.3%, 7.9%, and 0.7% as effective, respectively, as asialo-α1-acid glycoprotein in inhibiting the binding of radiolabeled asialo-α1-acid glycoprotein to liver cell membranes.

Each of the three CEA preparations showed different clearance kinetics from the circulation of mice. Coinjection of asialo-α1-acid glycoprotein with the CEA revealed differing inhibition of the clearances. These results show that differences in the carbohydrate components of purified CEA preparations affect their rate of removal from circulation and thus possibly the relationship between CEA production and observed plasma levels in patients. The possible origin of these CEA differences is discussed with their clinical implications.

INTRODUCTION

CEA* is a heavily glycosylated glycoprotein, comprised of 55 to 60% carbohydrate by weight. It has a molecular weight of approximately 200,000; is produced by embryonic gut tissue, colon carcinoma, and normal gut epithelium (1); and is identified by its reaction with antisera. Biochemical characterization of the antigen has been reported by several groups, and a consensus has been reached on most of its chemical properties (2, 3). There is a large variation in the carbohydrate composition of molecules purified from a single tumor and also between antigenic molecules isolated from different tumors. Heterogeneity of CEA has been demonstrated by electrophoresis and by isoelectric focus-
tions from the circulation were determined in 20- to 25-g male CD-1 mice (Charles River). A 0.3-ml solution of radiolabeled glycoprotein in 0.9% NaCl solution (saline) was injected into the tail vein of ether-anesthetized mice, and 25-μl blood samples were taken from the retroorbital sinus beginning within 10 s of injection. The samples were collected in heparinized capillary tubes and were rinsed into 1 ml of 15 mm EDTA for measurement of $^{125}$I using a well-type gamma counter.

Glycoprotein Reagents. CEA was prepared from liver metastases of colon carcinomas by a modification of the method of Krupen et al. (19). Tumors were homogenized in saline, extracted with 1 M perchloric acid, and fractionated on Sepharose 4B and Sephadex G-200. CEA-bearing ascites fluids were extracted and chromatographed by the same procedure. The purification was monitored using a modified Roche radioimmunoassay procedure (20). These preparations gave a single diffuse band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis corresponding to a molecular weight of 180,000 to 200,000. Carbohydrate analyses were performed by the method of Reinhold (21). AG was prepared from pooled human serum using the method of Whitehead and Sammons (22), as modified by Van Lenten and Ashwell (23). ASAG was prepared from this material by incubation with V. cholerae neuraminidase (Calbiochem, LaJolla, CA) using 1 IU/20 mg for 18 h at 37°C followed by dialysis and lyophilization. No residual sialic acid was detectable upon carbohydrate analysis (21). Neuraminidase activity was not present after this treatment. Yeast mannan was obtained from Sigma Chemical Co., and fetuin was obtained from Grand Island Biological Co. (Grand Island, NY).

Binding Assay. The procedure of Van Lenten and Ashwell (15) was followed using radiolabeled ASAG or radiolabeled CEA as the test ligand. Inhibition experiments were performed as described above with all glycoprotein inhibitors being compared on a dry weight basis. Membrane protein concentrations were determined by the method of Lowry et al. (24). Glycoproteins were radiiodinated by the chloramine T method (25) and separated from free iodine by gel filtration on Sephadex G-50 (Pharmacia, Piscataway, NJ). Specific activities of 6 to 10 mCi/mg were obtained.

RESULTS

Membrane Binding of CEA in Vitro. In preliminary experiments, a binding assay developed by Ashwell et al. was modified to test subcellular membrane fractions for CEA binding activity. Hepatocyte plasma membranes isolated by the method of Neville (16) were tested for CEA and ASAG binding activity. The binding of both ligands to the membrane was time, temperature, calcium, and concentration dependent with respect to ligand and receptor, and saturable with unlabeled test ligand. When the binding of either ASAG or CEA to hepatocyte membranes was examined in the presence of unlabeled ASAG, CEA, AG, or yeast mannan, only ASAG and CEA had inhibitory activity (Chart 1A). The ability of ASAG, but not AG, to inhibit CEA binding suggests that this binding is probably due to the well-characterized hepatic galactose receptor.

Because the hepatic galactose receptor is known to be sensitive to neuraminidase treatment (15), the microsomal membrane CEA binding activity was also examined for this sensitivity. Chart 1B shows that neuraminidase treatment effectively eliminated most CEA and ASAG binding activity in the membranes, while the small amount of residual activity retained galactose specificity (i.e., saturability with ASAG but not AG). These experiments indicate that the in vitro test detects CEA binding to liver membranes via the galactose receptor. Particularly, binding of CEA to the hepatic galactose receptor had been observed, in vivo, only after neuraminidase treatment of CEA (10). The observation that purified labeled CEA used in this study expressed exposed galactose residues was unexpected. To examine the extent of this phenomenon, other CEA preparations were examined.

CEA Heterogeneity. CEA was purified from 2 different liver metastases of colon carcinomas and from the ascites of a patient with an exceptionally high circulating CEA level associated with liver metastases of colon carcinoma (26). The purified CEAs (labeled 1 to 3) were tested with a modification of the Roche CEA assay (10) and found to be 49%, 100%, and 67% as active by weight, respectively, as the Roche standard. All preparations gave a single diffuse band corresponding to a molecular weight of 180,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The carbohydrate compositions of the 3 CEAs are shown in Table 1.

Heterogeneity of In Vitro Membrane Binding. To determine the percentage of CEA molecules expressing sufficient galactose residues for hepatic galactose receptor recognition, the CEAs were radiolabeled, and then 10 ng of labeled glycoprotein were incubated with increasing amounts of microsomal membranes until a large excess of hepatic galactose receptor activity was

*P. Thomas, unpublished observations.
Heterogeneity of in Vivo Clearance from the Circulation.

The kinetics of removal of the different CEAs from the circulation of mice was examined. In order to validate our in vivo clearance model system, radiolabeled CEA-2 was injected into mice, blood samples were taken and chromatographed on prepacked Pharmacia G-50 columns, and the ratios of radioactivity in the V₀ and Vᵢ fractions of the column were compared. Initial blood samples were over 95% excluded by Sephadex G-50, and the ratio remained over 90% up to 20 min postinjection. At 60 min, the excluded fraction contained 85% of the recovered radioactivity. The fraction of ¹²⁵I-CEA precipitable with anti-CEA antibody was determined by diluting blood samples containing 50,000 cpm to 6.5 ml with EDTA buffer, adding a saturating amount of antibody, and incubating overnight under the conditions used for the CEA radioimmunoassay (20). For CEA-2, 63% of the ¹²⁵I was immunoprecipitable before injection, and this percentage varied from 50% to 66% during the 1-h clearance experiment. Syringes used for injection were measured for radioactivity before and after injection, and based on the radioactivity observed in the initial blood samples, the average volume of dilution was 2.55 ± 0.75 ml (SD) for 20 mice.

The 3 different radiolabeled CEA preparations were injected i.v. into mice, and blood samples were taken to measure the radioactivity remaining in the circulation. The clearance curves obtained (Chart 4) were not simple exponential or log-linear decays but more closely followed a biexponential curve (27). The possibility that early rapid clearance represents equilibration of the radioligand into a relatively large pharmacological space was excluded by a sequential clearance experiment (Chart 5). Radiolabeled CEA-2 (5 μg) was injected into a mouse, and after the initial rapid clearance phase, a blood sample was taken and injected into a second animal. The slow clearance observed in the second animal supports the hypothesis that the initial sample contained at least 2 kinetically distinct species (28).

Inhibition experiments were performed to test the possibility that the rapid portion of the curves reflects clearance of asialo-CEA, while the slower portion represents clearance of native or sialylated CEA. When the 3 radiolabeled CEA preparations were injected separately into mice, in the presence or absence of an inhibitory dose of ASAG, a different amount of inhibition of CEA clearance by ASAG was observed for each preparation (Chart 6).

Discussion

The liver is the major organ involved in CEA metabolism (8) both in animals and in humans (29). When the CEA binding activity of rat liver was examined on a subcellular level, the

Table 1

<table>
<thead>
<tr>
<th>Sugars (mol/100 mol)</th>
<th>CEA-1</th>
<th>CEA-2</th>
<th>CEA-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fucose</td>
<td>14.0</td>
<td>18.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Mannose</td>
<td>13.7</td>
<td>15.3</td>
<td>16.3</td>
</tr>
<tr>
<td>Galactose</td>
<td>21.4</td>
<td>19.3</td>
<td>23.2</td>
</tr>
<tr>
<td>N-Acetylglucosamine</td>
<td>40.2</td>
<td>38.3</td>
<td>33.6</td>
</tr>
<tr>
<td>Sialic acid</td>
<td>9.7</td>
<td>8.2</td>
<td>25.0</td>
</tr>
</tbody>
</table>
binding detected was to membrane fractions and was apparently mediated by the hepatocyte galactose receptor. The failure to detect a CEA-specific receptor in the membrane fractions may be due to: (a) the inactivation or loss of the Kupffer cell membrane receptor during the liver membrane fractionation procedure; (b) the relative preferential purification of hepatocyte membrane components by these procedures; or (c) disruption of the architecture of the liver, which provides direct contact between the bloodstream and the Kupffer cell.

The chemical characteristics of the CEA used to quantify receptor binding clearly have an important bearing on the interpretation of the results. In earlier studies, Thomas et al. (30) reported an average of approximately 0.5 exposed galactose units per molecule in their CEA preparations. With these 3 preparations, using a sensitive membrane receptor binding assay, we observed that our purified CEAs had a significant but variable amount of exposed galactose residues. According to the structures proposed by Chandrasekaran et al. (31), most of the carbohydrate chains in their CEA preparations were free of sialic acid. The occurrence of terminal galactose residues on CEA is thus a complex question.

The variation among the 3 purified CEA preparations used in this study could also be caused by the method used for their purification. This is unlikely, however, as all 3 preparations were isolated by identical procedures. The initial purification step, extraction with 1 M perchloric acid, can result in loss of sialic acid from glycoproteins when performed at elevated temperature. At 4°C, however, it does not cause detectable hydrolysis. The remarkably high sialic acid content of CEA-3 presented in this paper clearly indicates that this procedure does not eliminate sialic acid residues from the antigen. Because there are no quantitative methods of carbohydrate analysis that can adequately characterize the carbohydrate component of CEA before it is first purified, we are unable, at present, to conclusively exclude the possibility that our purification procedures have altered the carbohydrate components we are trying to examine.

The in vivo experiments show an apparent biphasic clearance of CEA in xenogeneic animals similar to that reported by Shuster et al. (9). The reason for the biphasic curve is not known, but simple equilibration has been excluded as an explanation. While previous studies showed no inhibition of CEA clearance from the circulation by coinjected doses of asialoglycoproteins (11), with these CEAs, we observed inhibition that varied markedly between preparations. These differences in clearance kinetics may be due to differences between oligosaccharide structures of different CEA preparations (31). The biphasic clearance kinetics may be explained by a 2-component model in which the rapid phase has a galactose-dependent component, the size of which varies among CEA preparations, and a galactose-independent component that represents the Kupffer cell binding activity reported previously (11, 12). Because there is not a complete inhibition of the rapid phase by asialoglycoproteins (Chart 6), the situation may be more complex, and additional components may be involved.

The clearance of patient plasma CEA containing levels exceeding 10,000 ng of CEA per ml from the circulation of rats has been examined. We have reported that different CEA-containing samples can exhibit different clearance kinetics (26, 32). These animal model results are consistent with the clinical observations of Lokich et al. (33) in colon cancer patients.

If 2 types of CEA are produced by tumors, one cleared rapidly and one cleared by another slower mechanism, then determination of both the quantity and type of CEA produced by a patient may be important when interpreting increases or decreases in CEA levels caused by tumor progression or treatment. Variations
in carbohydrate structure can influence clearance and hence levels of circulating CEA. This depends on sialic acid content, the amount of terminal galactose, and the interaction between both Kupffer cell and hepatocyte receptors. CEA production by colorectal cancer cells is influenced by cellular differentiation; to what extent differentiation influences the variation in carbohydrate structure is not known. Interestingly, CEA-3 came from a patient with a well-differentiated adenocarcinoma, and the slower clearance rate of his CEA presumably accounts for the fact that his circulating CEA level was extraordinarily high (26). We are developing a more sensitive in vitro test for CEA galactose receptor binding in order to explore the possible clinical significance of this heterogeneity.

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REFERENCES


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