Receptor-mediated Endocytosis of Carcinoembryonic Antigen by Rat Liver Kupffer Cells

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ABSTRACT

In vivo, carcinoembryonic antigen (CEA) is removed from the circulation by the liver Kupffer cells. Immunologically identifiable CEA is transferred from these macrophages to the hepatocytes, where degradation is completed. Circulatory clearance of CEA is specific, rapid (t1/2 = 3.7 ± 0.9 (S.D.) min), and saturable. In vitro, Kupffer cells take up CEA by a saturable process which is time/temperature dependent and colchicine sensitive. Isolated Kupffer cells endocytose CEA with an apparent Km of 6 × 10⁻⁸ m. There are approximately 16,000 CEA binding sites per cell. Nonspecific cross-reacting antigen (NCA), a glycoprotein structurally similar to CEA, is recognized with lower affinity by the same receptor. Endocytosis is independent of the nonreducing terminal sugars on the molecule: CEA modified by Smith degradation inhibits Kupffer cell recognition of native CEA. Since performic acid oxidized CEA also inhibits endocytosis, receptor binding is similarly independent of intact protein conformation. Isolated Kupffer cells have mannose and/or N-acetyl glucosamine receptor activity but do not internalize CEA by that mechanism.

Galactose-terminated glycoproteins impede CEA and NCA clearance in vivo but not Kupffer cell endocytosis in vitro. Radiolabeled CEA released from isolated Kupffer cells following endocytosis shows no apparent molecular weight change. However, the released CEA contains species with higher isoelectric points, suggesting that perhaps the removal of sialic acid and the resulting exposure of galactose residues mediate the subsequent transfer to the hepatocyte.

INTRODUCTION

CEA⁵ is a glycoprotein with a molecular weight of 200,000 excreted by human gut epithelium and various adenocarcinomas (6, 7). CEA, a normal constituent of the human digestive tract, is present in the fetus in higher concentrations than in the adult (3). Galactose-terminated glycoproteins impede CEA and NCA clearance in vivo but not Kupffer cell endocytosis in vitro. Radioiodinated CEA released from isolated Kupffer cells following endocytosis shows no apparent molecular weight change. However, the released CEA contains species with higher isoelectric points, suggesting that perhaps the removal of sialic acid and the resulting exposure of galactose residues mediate the subsequent transfer to the hepatocyte.

MATERIALS AND METHODS

CEA. CEA was isolated by perchoric acid extraction of hepatic metastases of a colorectal adenocarcinoma. Purification was achieved by separation with Sepharose 4B and Sephadex G-200 chromatography (11). Purity was determined by radioimmunoassay and electrophoresis on 7.5% SDS-polyacrylamide gels.

Chemical Modifications of CEA. Asialo-CEA was produced by neuraminidase (Vibrio cholerae) digestion of CEA (35). Sialic acid content of modified CEA was determined by the thiobarbituric acid assay (34) and

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3 Supported by Research Grant CA-16750 from the National Large Bowel Cancer Project, National Cancer Institute, NIH.
4 Supported by Research Grant CA-04486 from the National Cancer Institute, NIH.
5 The abbreviations used are: CEA, carcinoembryonic antigen; NCA, nonspecific cross-reacting antigen; HBP, hepatic binding protein.
by gas-liquid chromatography (18).

The terminal galactose residues of asialo-CEA were modified by exposure to galactose oxidase (Dactylium dendroides) (Sigma Chemical Co.). Disulfide bridges on CEA were oxidized by performic acid treatment (38). Removal of terminal carbohydrates from CEA was accomplished by multiple cycles of the Smith degradation, involving sequential sodium periodate oxidation, sodium borohydride reduction, and mild acid hydrolysis (2). Complete carbohydrate analysis of the modified CEA preparations was accomplished using the method of Reinhold (18).

Non-specific Cross-Reacting Antigen. NCA was extracted by perchloric acid from the liver metastases of a colon adenocarcinoma. Further separation was accomplished by Sepharose 4B and Sephadex G-200 molecular sieving columns. Final purification was by DEAE-cellulose chromatography and acetone precipitation. The purity of the resulting preparation was determined by radioimmunoassay and polyacrylamide gel electrophoresis (37).

α1-Acid Glycoprotein. α1-Acid glycoprotein was isolated from pooled human serum (39). Purification was accomplished by DEAE-cellulose chromatography and (NH₄)₂SO₄ precipitation. Isolated α1-acid glycoprotein ran as a single M, 44,000 band on SDS-polyacrylamide gels. Asialo α1-acid glycoprotein was prepared by the method described for asialo-CEA.

Other Glycoproteins. Fetuin (Sigma) was desialylated by neuraminidase treatment. Galactosyl bovine serum albumin was purchased from Sigma Chemical Co.

Radio-labeling of Glycoproteins. Glycoprotein iodination was accomplished using chloramine-T and sodium [125I]iodide (New England Nuclear) (8).

In Vivo Assay: Hepatic Clearance of Circulating CEA. Male Sprague-Dawley rats (250 to 300 g) were used to determine the clearance of CEA. Animals were lightly anesthetized with diethyl ether followed by sodium pentobarbital (50 mg/kg). An initial blood sample was taken, and CEA (250 μg) in 0.5% NaCl solution (saline) with or without a potential competitive inhibitor was injected into the femoral vein; this results in a CEA concentration of 100 ng/10μl sample of blood. Sequential blood samples (10 to 20 μl) were drawn from the tail at 1-min intervals for 10 min and then at 5-min intervals for 1 hr and placed in EDTA buffer (0.037 M, pH 7.6). CEA content was determined by radioimmunoassay (Roche) in duplicate. Clearance rates of [125I]-CEA were determined in the same way and assessed by counting 25-μl blood samples for [125I].

Immunohistochemistry. BALB/c mice were given injections of asialo-CEA or galactose oxidase-treated asialo-CEA (250 μg) via the tail vein and killed by cervical dislocation. Livers were fixed (10% formalin), and CEA or galactose oxidase-treated asialo-CEA was rapidly removed from the circulation at least 30 min after injection (3.9 min); 10 min after injection, it is present only in the Kupffer cells, and CEA is promptly removed from the circulation (mean t½ = 3.9 min). Doses greater than 300-μg dose of CEA (i.e., blood levels of 12 to 16 μg/ml), inhibited clearance occurred with a 300- to 400-μg dose of CEA. Potential inhibitory doses of fetuin and α1-acid glycoprotein had no effect on the rate of CEA clearance. After treatment with neuraminidase, clearance of CEA was impeded, indicating the involvement of terminal galactose residues in the process. A third glycoprotein with terminal galactose residues, galactosyl bovine serum albumin, also had a similar effect. The inhibitory effect of asialo α1-acid glycoprotein had not affected the clearance of CEA (Table 1). When asialo-CEA was co-injected with

RESULTS

In Vivo Studies. We have reported previously that in the adult rat, intravenously injected CEA was removed from the circulation with a half-life of 3.7 ± 0.9 min; in this respect, both unlabeled and [125I]-labeled CEA behaved identically, and NCA was cleared more slowly (mean t½ = 8.5 min) (30). These studies show saturation of clearance occurred with a 300- to 400-μg dose of CEA (i.e., blood levels of 12 to 16 μg/ml), doses greater than these result in prolonged circulatory half-lives.

Previous work has shown that CEA is taken up by Kupffer cells in vivo, and immunologically identifiable CEA is then transferred to the hepatocytes. However, these studies show that asialo CEA is rapidly removed from the circulation at least partially by the hepatocyte, presumably by the HBP, a receptor for asialoglycoproteins (1). Ten min after i.v. injection of asialo CEA, immunoperoxidase staining shows CEA reactivity in both the hepatocytes and Kupffer cells. HBP recognition of asialoglycoproteins can be eliminated by treatment of asialoglycoproteins with galactose oxidase (1). Galactose oxidase-treated asialo-CEA is promptly removed from the circulation (mean t½ = 3.9 min); 10 min after injection, it is present only in the Kupffer cells, as determined by the immunoperoxidase method. When galactose oxidase-treated CEA is co-injected with CEA, circulatory clearance is delayed (t½ = 10.3 min).

Table 1 shows the effects of asialoglycoproteins and their derivatives on the in vivo clearance rates of CEA. Potential inhibitory doses of fetuin and α1-acid glycoprotein had no effect on the rate of CEA clearance. After treatment with neuraminidase, clearance of CEA was impeded, indicating the involvement of terminal galactose residues in the process. A third glycoprotein with terminal galactose residues, galactosyl bovine serum albumin, also had a similar effect. The inhibitory effect of asialo α1-acid glycoprotein was not changed following galactose oxidase treatment. However, galacto α1-acid glycoprotein did not inhibit CEA clearance (Table 1). When asialo-CEA was co-injected with

CANCER RESEARCH VOL. 45 JANUARY 1985
Table 1

<table>
<thead>
<tr>
<th>Potential inhibitor</th>
<th>Molar excess of cold inhibitor</th>
<th>Mean circulatory half-life (min) of $^{125}$I-CEA</th>
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<tr>
<td>None (16)</td>
<td></td>
<td>3.7</td>
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<tr>
<td>$\alpha_1$-Acid glycoprotein (3)</td>
<td>1500</td>
<td>2.8</td>
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<tr>
<td>Asialo $\alpha_1$-acid glycoprotein (8)</td>
<td>1500</td>
<td>14.6</td>
</tr>
<tr>
<td>Galactose oxidase-treated asialo $\alpha_1$-acid glycoprotein (2)</td>
<td>1500</td>
<td>13.9</td>
</tr>
<tr>
<td>Fetuin (2)</td>
<td>1300</td>
<td>2.9</td>
</tr>
<tr>
<td>Asialofetuin (6)</td>
<td>1500</td>
<td>12.7</td>
</tr>
<tr>
<td>Galactosyl bovine serum albumin (2)</td>
<td>400</td>
<td>12.2</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, number of animals.

**CEA ENDOCYTOSIS BY RAT KUPFFER CELLS**

Cellular uptake of CEA by Kupffer cells is concentration dependent (Chart 2). Data points were adjusted to exclude nonspecific binding, which was determined by measuring $^{125}$I-CEA uptake in the presence of large amounts of unlabeled CEA. Nonspecific binding accounted for less than 30% of the total cell-associated ligand. Analysis of concentration dependent CEA uptake data by double reciprocal plot, and Michaelis-Menton kinetics indicated an approximate $K_{\text{max}}$ of $6 \times 10^{-8} \text{ M}$. After 30 min at saturating concentrations of CEA, the Kupffer cells endocytosed amounts of label equivalent to 30,000 CEA molecules per cell.

Since internalization by endocytosis does not occur at 4°C, this temperature and 60-min incubations were used to study the concentration dependence of the specific binding of CEA by Kupffer cells. Linear regression analysis of a double reciprocal plot of the data showed each cell bound label equivalent to approximately 15,000 CEA molecules. By this estimation, the putative receptor for CEA exhibited a $K_{\text{binding}}$ of $1.3 \times 10^{-7} \text{ M}$. The saturation of the system and the temperature and colchicine dependence indicate that the process is receptor mediated.

At 37°C isolated Kupffer cells took up labeled NCA in a concentration-dependent manner. A double reciprocal plot of the concentration-dependent data was analyzed by linear regression analysis and Michaelis-Menton kinetics. The calculable $K_m$ for the process was approximately $2 \times 10^{-7} \text{ M}$. Forty-one percent of labeled CEA uptake was inhibited by a 500-fold molar excess of cold NCA. However, endocytosis of labeled CEA at the same concentration was inhibited 71% by a 113-fold molar excess of unlabeled CEA. Thus, CEA binds more tightly to Kupffer cells than does NCA, and the receptor affinity in vitro for NCA was
lower than that for CEA.

CEA, following various chemical modifications, retained the ability to inhibit Kupffer cell uptake of labeled CEA. However, none of the modified substances were as potent inhibitors as native CEA (Chart 3). Asialo CEA inhibited cellular uptake by 45% after 60 min, compared to 75% inhibition with the same amount of CEA. The same quantity of performic acid-oxidized CEA inhibited CEA uptake by 37% and CEA from the first cycle of the Smith degradation was less effective as an inhibitor of Kupffer cell uptake.

The uptake of yeast mannan by the mannose:N-acetylglucosamine:fucose receptor of Kupffer cells in vitro has been reported previously (15). In this study, Kupffer cells took up yeast mannan in a saturable manner. However, CEA uptake was not affected by inhibitory doses of unlabeled yeast mannan. In contrast to the in vivo results, asialo α1-acid glycoprotein or asialofetuin did not affect the rate of uptake of CEA by isolated Kupffer cells (Chart 4).

To study the nature of the transfer of the CEA molecule from the Kupffer cell to the hepatocyte, isolated Kupffer cells were incubated for 1 hr with radiolabeled CEA (20 μg/ml). Cells were washed repeatedly to remove unbound ligand and resuspended in fresh CEA-free medium. The medium was sampled for 1 hr, and the cells released the labeled CEA in a time-dependent manner. After 1 hr, approximately 14,000 CEA molecules were released into the medium per cell. When the CEA was examined by SDS-polyacrylamide gel electrophoresis (7.5%) and compared with the original CEA, no change in molecular weight was seen. Analysis by agarose isoelectric focusing (pH 3 to 9.5) showed that the CEA released by the Kupffer cells had a greater amount of subspecies with higher π than of the original radiolabeled CEA (Fig. 1).
**DISCUSSION**

Hepatic metabolism of CEA is a process involving 2 distinct cell populations. Immunoperoxidase localization and electron microscopic observations showed that CEA is removed from the circulation by the Kupffer cells and subsequently transferred to the hepatocytes (25, 30). Hepatic clearance was rapid, saturable, and specific for CEA and NCA. After i.v. administration of a 250-µg dose of CEA, rat liver took up CEA at a rate of 45 µg/min, implicating a mechanism with specific affinity for CEA, and a finite number of binding sites. Isolation of CEA from the circulation and examination by gel filtration on Bio-Gel A-1.5m failed to demonstrate an increase in molecular weight that would be expected with immune complex formation (24). In addition, endocytosis by Kupffer cells in vitro was a serum-independent process.

In vitro Kupffer cells isolated by collagenase perfusion endocytosed CEA in a specific and saturable manner. Endocytosis was concentration dependent, with an apparent \( K_m \) of \( 6 \times 10^{-8} \text{ M} \). Binding studies at 4° showed that isolated Kupffer cells have approximately 15,000 surface receptor sites for CEA with a calculable \( K_{endo} \) of \( 1.3 \times 10^{-7} \text{ M} \). In the rat in vivo, the receptor system was saturated by a dose of approximately 350 µg of CEA, resulting in a blood concentration of \( 7 \times 10^{-8} \text{ M} \), a value similar to the in vitro binding constant.

In vivo, NCA is cleared from the rat circulation by the same mechanism as CEA (30) with a half-life twice that for CEA. In vitro, Kupffer cells endocytos NCA with a \( K_{plate} \) of \( 1.4 \times 10^{-7} \text{ M} \). Greater concentrations of NCA than CEA are required to inhibit CEA uptake in vivo and in vitro. NCA is found in the circulation of healthy adults at a 50-fold higher concentration than CEA (33). While NCA and CEA are closely related, small structural changes around the binding site to the Kupffer cell could account for the higher affinity of CEA. Complete structural studies are not available on either glycoprotein to confirm this hypothesis.

Chemical and enzymatic modifications of CEA were used to determine the specificity of binding to the Kupffer cell in vitro. Asialo-CEA and Smith degraded CEA [sequential applications of periodate oxidation borohydride reduction and mild acid hydrolysis results in the removal of about one-half of the carbohydrate from CEA without affecting its antigenic activity (21)] both inhibited endocytosis of native CEA, thus ruling out terminal carbohydrate groups as the recognition site for the receptor.

Modification of CEA by performic acid oxidation resulted in breakage of the intrachain disulfide bonds, accompanied by loss of tertiary conformation as determined by circular dichroism (38). Performic acid-oxidized CEA inhibited in vitro endocytosis, indicating that receptor binding is independent of protein conformation. These in vitro results are in agreement with our previously published data on the inhibition of CEA clearance in vivo in the rat (30).

In vitro, the specificity of Kupffer cell recognition of CEA was similar to that in vivo, except that there was no detectable inhibition of in vitro uptake by asialoglycoproteins. In vivo, clearance of both CEA and NCA was inhibited by glycoproteins with terminal galactose groups. It is likely that the transfer of CEA and NCA to the hepatocytes may involve recognition of galactose. However, the in vivo capability of asialoglycoproteins to inhibit CEA clearance was not affected by galactose oxidase treatment.

Asialoglycoproteins treated with this enzyme are not endocytosed by the hepatocyte. Thus, the role of the hepatocyte asialoglycoprotein receptor HBP in this transfer needs further study (1).

Previous studies showed that the Kupffer cell did not change the molecular weight or immunological reactivity of CEA before it was transferred to the hepatocyte (30). The isolated CEA lost approximately 20% of its sialic acid content (24). In vitro, Kupffer cells released CEA into the medium after endocytosis. The CEA exocytosed by the Kupffer cells showed no apparent molecular weight change by SDS-polyacrylamide gel electrophoresis. When the isoelectric focusing pattern of CEA released by the cells was compared to that of the CEA originally added to the incubation medium, the exocytosed CEA was enriched with CEA molecules of higher isoelectric points. Two possible explanations can be made for this observation: (a) the cells preferentially endocytose those subspecies of CEA with high isoelectric points; or (b) the increased ratio of high-pl subspecies in the exocytosed CEA may reflect removal of sialic acid by the Kupffer cell. The latter explanation is consistent with the observation of 20% decrease in sialic acid content of CEA isolated from hepatocytes in vivo (24). This modification may result in the unmasking of galactose residues on the CEA molecule, allowing interaction with the hepatocyte receptor. While in vivo the Kupffer cell is responsible for CEA clearance from the circulation, it is not the only macrophage capable of endocytosing CEA. Rat lung alveolar macrophages show similar properties, though the rates of exocytosis of CEA by the cells may differ (31).

These observations improve our understanding of the mechanism by which the liver and Kupffer cells in particular clear CEA, NCA, and other glycoproteins and elucidate the regulation of their circulating levels. In other studies, we have shown that the sialic acid content of CEA circulating in patients with metastatic cancer appears to have important practical application in determining the circulating levels, even though sialic acid is not required for recognition by the Kupffer cell. It is likely that a high sialic acid content can mask the binding site on CEA, and we have found that CEA molecules with a high sialic acid content are cleared less readily in vivo (28). A clinical test to transiently impede CEA clearance by the liver and increase its plasma levels may make possible earlier detection of CEA-producing cancers and improved monitoring of therapy.

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