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

Carol A. Toth,1 Peter Thomas,2 Selwyn A. Broitman,3 and Norman Zamcheck4

Gastrointestinal Research Laboratory, Mallory Institute of Pathology Foundation, Boston City Hospital [C. A. T., P. T., N. Z.]; Department of Medicine, Harvard Medical School [P. T., N. Z.]; and Departments of Microbiology [C. A. T., S. A. B.] and Pathology [S. A. B., N. Z.], Boston University School of Medicine, Boston, Massachusetts 02118

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 \((t_1/2 = 3.7 \pm 0.9 \, \text{S.D.} \, \text{min})\), 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 \(K_m\) of \(6 \times 10^{-8}\) 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. Radio-labeled 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

CEA\(^5\) 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). The glycoprotein has a single polypeptide chain intralinked by 6 disulfide bridges (36). Branched oligosaccharides composed of fucose, sialic acid, galactose, \(N\)-acetylglucosamine, and mannose comprise over 50% of the CEA molecule by weight (35). When tumor production results in abnormal plasma levels of CEA, serial determinations function as an indicator of prognosis and a monitor of therapy and provide postoperative surveillance in the treatment of carcinoma of the colon, breast, lung, and pancreas (4, 5, 22, 40, 41). Small increases in plasma CEA also occur in some patients with benign liver disease (13).

Carcinoembryonic antigen is primarily metabolized by the rat liver, with traces taken up by the spleen and lungs (26). Rapid circulatory clearance occurs in other species, including the dog, rabbit, hamster, mouse, rhesus monkey, and baboon (17, 21, 27, 29). Hepatic metabolism of CEA is a multistep process involving both the macrophages and the parenchymal cells (25). Within 15 min following i.v. injection, at which time the bulk of CEA has been cleared from the systemic circulation, most of the CEA is found in the Kupffer cells, with little or none in the hepatocytes. Immunologically identifiable CEA is subsequently transferred to the hepatocyte, where degradation is completed (25, 30). An understanding of degradation mechanisms for CEA has been helpful in explaining clinically elevated values in both benign and malignant diseases as described in a recent review of the role of liver metabolism in the clinical assessment of CEA plasma levels (28).

In vivo, NCA, a structurally related glycoprotein (32), is cleared from the circulation by the same mechanism (30). Immunofluorescence staining has shown both CEA and NCA on the surface of normal adult human Kupffer cells (10). Substances with known affinity for the hepatic receptors for mannose, \(N\)-acetylglucosamine, fucose, and galactose all fail to inhibit circulatory clearance of NCA by the liver. Kupffer cell uptake in vivo is not mediated by the terminal carbohydrate groups present on the glycoproteins, nor is uptake dependent on the tertiary conformation of the molecule (30).

This paper further describes in vivo clearance and in vitro uptake experiments with isolated rat Kupffer cells. Isolated sinusoidal cells (approximately 70% Kupffer cells) were used to study the molecular specificity of the Kupffer cell recognition site separate from hepatocyte transfer. This methodology permits the manipulations of the extracellular environment in vitro to facilitate kinetic assessment of cellular uptake and detection of endocytosis.

MATERIALS AND METHODS

CEA. CEA was isolated by perchloric acid extraction of hepatic metastases of a colorectal adenocarcinoma. Puriﬁcation was achieved by separation with Sepharose 4B and Sephadex G-200 chromatography (11). Puriﬁcation was determined by radioimmunoassay and electrophoresis on 7.5% SDS-polycrylamide 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...
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 (36). 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).

Nonspecific Cross-Reacting Antigen. NCA was extracted by per- chloric acid from the liver metastases of a colonic 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 polyclaryl- amide 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 E. Y. Laboratories. Yeast mannan was purchased from Sigma Chemical Co.

Radiolabeling of Glycoproteins. Glycoprotein iodination was accomplished using chloramine-T and sodium [¹²⁵]I (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 pentobartital (50 mg/kg). An initial blood sample was taken, and CEA (250 µg) in 0.9% NaCl solution (saline) with or without a potential competitive inhibitor was injected into the femoral vein; this results in a 95% CEA content by radioimmunaoassay. When galactose oxidase (1) was added (8 min after injection), CEA clearance was significantly delayed (t½ = 10.3 min).

Kupffer Cell Isolation. Kupffer cells were harvested from the livers of anesthetized fasting male Sprague-Dawley rats (250 to 300 g) using the isolation buffers described by Seglen (20). After i.v. administration of sodium heparin (100 units), the livers were excised and stained in situ by portal vein perfusion with Ca²⁺-free buffer (0.1 M N-2-hydroxyethylpipera-azine-N'¢-2-ethanesulfonic acid with 0.3% NaCl; pH 7.4). Livers were excised, minced, and subsequently incubated with 0.05% collagenase in buffer (0.1 M N-2-hydroxyethylpipera-azine-N'¢-2-ethanesulfonic acid with 0.39% NaCl; 0.05 M KCl; pH 7.4). These cells were then suspended, pelleted, and reincubated with fresh collagenase buffer for an additional 20 min. Hepatocytes and cell clumps were removed from the suspension by low-speed centrifugation (50 x g, 2 min). Remaining cells were washed several times with Gey’s balanced salt solution. Final purification was achieved by centrifugation (1400 x g) for 15 min at room temperature in a 17.5% solution of metrizamide in Gey’s balanced salt solution. More than 85% of the aminosialo cells harvested from the top layer were viable, as determined by trypsin blue dye exclusion. On average, this resulted in 2 x 10⁶ isolated cells per liver, of which 70% stained positively for nonspecific esterase activity. Unlike the esterase activity of neutrophils, monocyte nonspecific esterase activity is inhibited by fluoride, and only 5% of the isolated cells stained in the presence of sodium fluoride (33±9%). Thirty-four % of the isolated cells phagocytosed colloidal carbon. The preparation contained less than 1% hepatocytes, as counted microscopically; hepatocytes are easily distinguished from the other cells by their larger size. Thus, approximately 75% of the viable cells were Kupffer cells.

In Vitro Determination of Glycoprotein Uptake by Isolated Cells. Glycoprotein uptake by Kupffer cells was monitored using the method of Stahl et al. (23). Cells suspensions were incubated with the radiolabeled glycoprotein in buffered RPMI 1640 (1% bovine serum albumin), and the incubation mixture was sampled in duplicate at various times. Cells were separated from the incubation medium by centrifugation at 11,000 rpm in an Eppendorf microcentrifuge for 5 min through an oil phase [dibutyrylphosphatide-dioctylphosphatide (3:1)]. The resulting supernatant and cell pellet were counted to determine the amount of cell-associated glycoprotein.

Electrophoresis. Radiolabeled samples were pretreated with mercaptoethanol and SDS. Polyacrylamide (7.5%) slab gels were used for the electrophoresis. The gel was fixed, stained, dried, and exposed to Kodak X-Omat XR-5 film (14).

Isoelectric Focusing. Isoelectric focusing was performed in agarose (0.5%) gels (ampholyte range, 2.5 to 10.0, LKB) according to the method of Saravis et al. (19). Gels were fixed, dried, and exposed to X-ray film.

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 [¹²⁵]I-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 cojncetti with CEA (28), 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 cojncetti with...
Table 1

<table>
<thead>
<tr>
<th>Potential inhibitor</th>
<th>Molar excess of cold inhibitor</th>
<th>Mean circulatory half-life (min) of 125I-CEA</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (16)</td>
<td></td>
<td>3.7</td>
</tr>
<tr>
<td>α1-Acid glycoprotein (3)</td>
<td>1500</td>
<td>2.8</td>
</tr>
<tr>
<td>Asialo α1-acid glycoprotein (8)</td>
<td>1500</td>
<td>14.6</td>
</tr>
<tr>
<td>Galactose oxidase-treated asialo α1-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.

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 125I-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 \( K_{\text{m}} \) of \( 6 \times 10^{-8} \) 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°, 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 \( K_{\text{m}} \) of \( 1.3 \times 10^{-7} \) M. The saturability of the system and the temperature and colchicine dependence indicate that the process is receptor mediated.

At 37° 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 \( K_{\text{m}} \) for the process was approximately \( 2 \times 10^{-7} \) M. Forty-one \% 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

asialo α1-acid glycoprotein, immunoperoxidase staining of the liver revealed CEA only in the Kupffer cells. Circulatory clearance of labeled NCA (5 μg) was also impaired by asialofetuin (1.4 mg), resulting in a prolonged circulatory half-life of 40 min.

In Vitro Studies. Uptake of 125I-labeled CEA and NCA by isolated Kupffer cells was studied in suspension assay. In time-dependent experiments, uptake of 125I-CEA was saturable and temperature dependent (Chart 1). The uptake value at Time 0 was adjusted to 0 to compensate for the inclusion of free ligand in the cell pellet. Pretreatment of macrophages in vitro with colchicine inhibits microtubule formation and partially inhibits receptor mediated endocytosis of lysosomal enzymes (12). Pretreatment of isolated Kupffer cells with colchicine (75 μg/ml) for 1 hr inhibited CEA internalization by 43\% but did not affect cell viability.

Chart 1. Uptake of 125I-CEA by isolated Kupffer cells. O, 125I-CEA at 10 μg/ml of medium (37°); ●, 125I-CEA at 10 μg/ml of medium plus 100-fold molar excess of unlabeled CEA (37°); □, 125I-CEA at 10 μg/ml of medium (4°). Data points are the averages of duplicate samples for 2.5 × 10⁶ cells.

Chart 2. Double reciprocal plot of the effect of CEA concentration on CEA uptake by isolated Kupffer cells. Data points are the averages of replicate samples for 7 × 10⁶ cells incubated for 30 min at 37°. (From this plot, \( K_{\text{m}} \) = \( 6 \times 10^{-8} \) M; \( r = 0.99 \).)
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 $^{125}$I 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 mannann 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 mannann in a saturable manner. However, CEA uptake was not affected by inhibitory doses of unlabeled yeast mannann. 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 pi 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 Kₐ of 6 x 10⁻⁸ M. Binding studies at 4°C showed that isolated Kupffer cells have approximately 15,000 surface receptor sites for CEA with a calculable Kₐ of 1.3 x 10⁻⁷ 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 x 10⁻⁸ 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 vivo, Kupffer cells endocytose NCA with a Kₐ of 1.4 x 10⁻⁷ 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 asialo-glycoproteins. 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 hepatocytoma may involve recognition of galactose. However, the in vivo capability of asialo-glycoproteins 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 vivo, Kupffer cells released CEA into the medium after endocytosis. The CEA endocytosed 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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