Carcinoembryonic Antigen Has a Different Molecular Weight in Normal Colon and in Cancer Cells due to N-Glycosylation Differences

M. Garcia, C. Seigner, C. Bastid, R. Choux, M. J. Payan, and H. Reggio

Laboratoire de la Biologie de la Differenziation Cellulaire, Faculète des Sciences de Marseille-Luminy, UA CNRS 179, Case 901, 163 Avenue de Luminy, F-13 288 [M. G., C. S., H. R.], and Service d'Hépato Gastroentérologie, Hôpital Sainte Marguerite, 270 Chemin de Sainte Marguerite BP 29, F-13 273 [C. B., R. C., M. J. P.], Marseille, Cédex 9, France

ABSTRACT

Carcinoembryonic antigen, an apical membrane glycoprotein expressed in normal human colonic epithelial cells, colonic polyps, tumor, and tissue culture cell lines originating from colon adenocarcinomas, is generally considered to have a molecular weight of 180,000. Using sodium dodecyl sulfate-polyacrylamide gel electrophoresis associated with immunoprecipitation or immunoblotting with both monoclonal (Mab 517 and Mab 601) and polyclonal antibodies, we observed that carcinoembryonic antigen was actually expressed as two discrete apparent molecular weight forms in normal tissues: a broad band averaging at \( M, \) 200,000 and a sharp band at \( M, \) 130,000. This constituted the phenotype of the normal colon. In cancer cells we detected a single band at \( M, \) 170,000 or lower. This variation was mainly the consequence of a modification of the glycosylation pattern of the molecule since deglycosylation by \( \beta - \) mannosidase or by \( \alpha - \) fucosidase did not change the apparent molecular weight of the antigen. The structure of the molecule has been extensively studied. It is an apical membrane protein of the colonic cells (18–21) which is anchored in the plasma membrane by a glycosylphosphatidylinositol linkage (22–25). Its primary structure indicates that it belongs to the immunoglobulin-like superfamilly, forming seven highly conserved loop domains (16, 26, 27). It is highly glycosylated; more than 50% of the molecule consists of \( N - \) linked oligosaccharides on 28 potential sites of \( N - \) glycosylation (28). \( O - \) Glycosylations are absent or quantitatively not important (15, 26, 29).

The primary structure of the CEA core protein has been determined by cloning the complementary DNA isolated from a human colon malignant tissue (28) and the human colon adenocarcinoma cell lines LS 180 and LS 174T (30). In these cells, the CEA core protein is a polypeptide of 702 amino acids corresponding to a molecular weight of 72,800 (30).

Molecules similar to the CEA have been characterized in different tissues as a class of proteins presenting immunological cross-reactivity (for review see Ref. 31) including a transmembrane form detected in human colonic tumor cell lines (27). Several of these proteins have been cloned and sequenced (32–35). Their implications in cancer research have been discussed (for review see Refs. 15 and 17). A certain confusion may arise from the fact that CEA-like proteins have been studied in normal tissues, in tumors, and in cancer cells of various organ origins. Among these some molecules are more interesting since they are not or are very weakly expressed in normal colon and appear during carcinogenesis, such as the NCAs (27) or TEK (38). It has been proposed that NCA-55 and TEK are probably produced by the induction of the expression of the same mRNA and differential posttranslational processing since they share the same core protein (39). In colonic tumors, a new mRNA, smaller but recognized in Northern blot analysis by CEA complementary DNA is actually produced and could code for this NCA core protein (40).

Many antibodies to CEA and related molecules have been raised and characterized in several laboratories. Data obtained in cancer research using these antibodies were somewhat difficult to interpret and compare due to the differences in the recognized epitopes. Therefore, a classification was recently proposed to define antibodies based on their cross-reactivities, their competitions, and their tissue specificity (41).

We have previously described a monoclonal antibody (Mab 517) that recognized a membrane glycoprotein specific for the apical membrane of human colonic epithelial cells (19). This protein was expressed with a different molecular weight in normal human colon (\( M, \) 200,000 and 130,000) and in tissue-cultured cell lines (HT-29) originating from human colon adenocarcinoma (\( M, \) 170,000) (20). Using purified CEA, we discovered during the course of this work that Mab 517 recognized a polypeptide epitope common to the CEA and NCA-95 molecules.

This led us to investigate the possibility of using the shift in molecular weight of CEA as a marker of colonic cancer instead of...
of using its level of expression or its presence in serum as it is usually done (15–17). We have systematically used Mab 517 to compare the molecular weight of the CEA molecule in normal human colon, in colonic polyps, in cancer tissues, and in tissue culture cell lines originating from human colonic adenocarcinomas. We have shown a variation in molecular weight which was essentially a consequence of a difference in the N-glycosylation pattern of the molecule. CEA thus appears to be an interesting model for the study of the modifications of N-glycosylations associated with carcinogenesis.

MATERIALS AND METHODS

Reagents

Cell culture reagents were purchased from Gibco BRL (Cergy Pontoise, France). Protein A-Sepharose was from Pharmacia (Uppsala, Sweden). SO4-NHS-biotin was from Pierce (Rockford, IL). N-Glycanase was from Genzyme, (Boston, MA). Tunicamycin was from Boehringer-Mannheim (Manneheim, Germany). 125I-Streptavidin (20–40 mCi/mg) and L-[4,5-3H]leucine (120–190 Ci/mmol) were from American (Amersham, Buckinghamshire, United Kingdom). CEA was purified from LS 174 cells, a gift from Dr. F. Jean Immunotech S. A. (Marseille, France). All other chemicals were reagent grade.

Antibodies

Mab 517 was described previously (19), Mab 601 (42) was purchased from Biosys (Compiègne, France), polyclonal anti-CEA was from Dakopatts (Glostrup, Denmark), goat anti-mouse IgG coupled with alkaline phosphatase was from Promega (Madison, WI); monoclonal restricted to NCA (N 33) was a gift from Dr. P. Burtin, (ISRC, Villejuif, France).

Tissue Preparation

Tissues were obtained from normal colon or tumors during surgical resection. Polyps were dissected during fiberscopy examination, split into two parts, and examined for both pathological routine diagnosis and immunoblotting analysis. The fragments for biochemical analysis were used immediately or frozen and stored at -20°C. Polyps were boiled with 10% of 0.5% SDS, 100 mM 0-mercaptoethanol, 50 mM Tris-HCl, pH 8.0, for 5 min. The denatured, biotinylated antigens were digested with N-glycanase according to the method of Plummer et al. (49). The reaction was performed in a final reaction volume of 60 μl with or without enzyme during 36 h at 37°C. Enzyme (0.3 unit) was added twice during the digestion period. The digestion was stopped by adding 30 μl of gel sample buffer 3-fold concentrated. The mixture was boiled for 5 min, spun for 5 min in an Eppendorf centrifuge, and directly analyzed as described later.

Membrane Preparation

Membranes from HT-29-18 cells or from normal colon were purified from intestinal tissues or cultured cells according to the method of Gratecos et al. (50). The first pellet was used as a total cellular membrane fraction. Membrane proteins were extracted with 1% Triton X-100.

After thawing, the polyps were rinsed 3 times with cold PBS. Proteins were solubilized by adding 4 μl of 10% SDS/mg of fresh tissue. After 20 min incubation at room temperature, the volume was adjusted to 1 mg/10 μl with 100 mM Tris-HCl buffer, pH 6.8, supplemented with a cocktail of protease inhibitors (47). The polyps were homogenized in a Potter-Elvehjem apparatus (clearance, 10–15 mm) driven by a Janke & Kunkel Polybroyer at 2000 rpm. The homogenate was sonicated 3 times for 10 s each with a 100-W MSE ultrasonic disintegrator with an amplitude of 4 μm. One volume of gel sample buffer twice concentrated and prepared without SDS and reducing agent was then added and extraction was achieved by boiling the mixture for 10 min.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was carried out as described by Laemmli (51). Sample buffer was 100 mM Tris-HCl pH 6.8–2% SDS-5% β-mercaptoethanol-2.5% glycerol-0.1% bromphenol blue. The proteins were transferred from the gel to nitrocellulose according to the method of Burnette (52).

Detection of Radiolabeled Antigens. Immunoprecipitated, labeled antigens were recovered by boiling the beads in SDS gel sample buffer for 5 min and were centrifuged (10,000 x g for 10 min) in an Eppendorf centrifuge. The supernatant was analyzed by SDS-PAGE 6–15% under reducing condition. Gels were processed for fluorography using Amersham amplify and Hyperfilm 3H.

Detection of Biotinylated Antigens. The biotinylated antigens were analyzed by SDS-PAGE 6/15% under reducing conditions and transferred to nitrocellulose. The antigens were detected with 125I-streptavidin (10 x 106 cpm in 40 ml PBS-1% bovine serum albumin-nitrocellulose sheet) according to the method of Sargiacomo et al. (46) and revealed by autoradiography with Hyperfilm MP Amersham.

Detection of Antigen 517 in Polyps. Homogenates prepared and boiled as above, were centrifuged at 10,000 x g for 10 min in an Eppendorf centrifuge. Five μl of supernatant were loaded on a SDS-PAGE 8%
constant gel without reduction. After transfer to nitrocellulose, antigen
517 and NCA were detected with the relevant antibodies and revealed
by the alkaline phosphatase method.

RESULTS

Mab 517 Recognizes a CEA Epitope. CEA purified from LS
174 cells was analyzed by SDS-PAGE, blotted on nitrocellulose
sheets, and stained with Mab 517. A broad band was ob-
Serves at a molecular weight of 180,000. The same pattern was ob-
served with the polyclonal and the monoclonal 601 antibodies
(not shown). In addition, CEA immunoprecipitated from HT
29-18 cells with the polyclonal anti-CEA was recognized by
Mab 517 by immunoblotting and reciprocally. This demon-
strated that the Mab 517 recognized an epitope on the CEA
molecule. Its cross-reactivity is probably somewhat restricted
since it was not found in several tissues known to contain CEA
cross-reacting antigens (19). Mab 517 was not compared sys-
tematically to other anti-CEA antibodies and therefore was not
classified according to the system of Hammarstrom et al. (41).
We refer, later in this paper, to the 517 epitope of the
CEA. Major results were confirmed, however, with other CEA-
specific antibodies.

Epitope 517 Bearing CEA Has Different Apparent Molecular
Weights in Normal Colon, in Colonic Tumors, or in Adenocarcinoma
Tissue-cultured Cell Lines. We analyzed the proteins
from various sources by SDS-PAGE using Mab 517 to monitor
the apparent molecular weight modifications of the CEA. In
normal colon, a broad band averaging at M, 200,000 and a
sharp one at M, 130,000 were observed (Fig. 1, Lane d; Ref.
19). However, in HT-29-18 cells lines, a unique band averaging
at M, 170,000 was observed (Fig. 1, Lane b; Ref. 20). In tumor
cells, a major, very strong band was observed averaging at M,
170,000 or lower, but the labeling was much more intense and
the band was broader (Fig. 1, Lane f), consistent with an
increase of CEA expression in cancer. Another band was ob-
served at M, 95,000 but it was also specifically recognized by
NCA-specific antibody (Fig. 1, Lane g) and, therefore, could be
ascribed to NCA-95 molecules. This also indicated that
the normal molecular weight bands recognized by Mab 517 were
CEA molecules. At least 20 tumors and more than 50 specimens
of normal colon mucosa were screened and displayed such phe-
notypes without exception (Table 1). Similar patterns were
obtained using Mab 601 and polyclonal antibodies to CEA. We
concluded that the apparent molecular weight of CEA was
consistently decreased in tumor cells.

The Variation in the Apparent Molecular Weight of CEA,
Detected with Mab 517, Was Generally Consistent with Cancer
Clinical Diagnosis of Colonic Polyps. The variation of the ap-
parent molecular weight of CEA was investigated on a set of
human colon polyps obtained from clinical resection during
diagnosis using Mab 517 and the peroxidase-antiperoxidase tech-
34 benign polyps displayed a small shift in the molecular weight
of CEA (Fig. 30), an
where a unique band was observed at M, 170,000; (d) tumor
phenotype, where a broad band at M, 170,000 or lower was
observed in the absence of the M, 130,000 band and with the
appearance of a band at M, 95,000. Fifty-one polyps were
 analyzed and compared to the corresponding clinical diagnosis
(Table 1). Although the results were generally consistent, 10 of
34 benign polyps displayed a small shift in the molecular weight
of CEA (Table 1, Column 1, intermediary phenotype). This
indicated that the CEA phenotype partially shifted while the
morphological changes used in diagnosis were not yet observed.
On the other hand, a normal phenotype was never observed in
cancerous polyps or in surgical tumors. Moreover, the shift of
molecular weight was observed in polyps where NCA-95 was
not yet apparent (see Fig. 2, lane g). In diagnosis of carcinoma,
the shift of molecular weight was observed with the presence
of NCA-95. We concluded that the shift of the CEA molecular
weight precedes the morphological changes and the appearance
of NCA-95.

Epitope 517-bearing CEA Was Expressed Intracellularly in
Moderately Transformed Polyps. Paraffin sections from polyps
analyzed in the preceding study were stained for CEA localiza-
using Mab 517 and the peroxidase-antiperoxidase tech-
nique. In a benign polyp displaying a normal colon phenotype
(Fig. 3a), the labeling was apical as previously described (18,
19). In a moderately dysplastic polyp presenting an HT-29
phenotype for the molecular weight of CEA (Fig. 3b), an
additional labeling was also observed in the apical region of the
cytosol at the exclusion of the terminal web domain (Fig.
The Difference in Apparent Molecular Weight of CEA Is a Consequence of Differences in Glycosylations of the Molecule.

We presumed that the various forms of the normal colon and the cancerous phenotypes were the consequence of differences in glycosylation as has been shown, for instance, for TEX and NCA-95 (39). Actually, differences in glycosylation have been described in colonic cancer (29, 54); we decided to test the hypothesis that such an effect could explain the molecular weight variation of CEA, as recognized by Mab 517. CEA was immunoprecipitated with Mab 517 from normal colon, from tissue-cultured cells, and from tumors, after SO4-NHS-biotin immunoprecipitation with Mab 517 from normal colon, from tumor phenotype (4), presence of one band at M, 170,000 or lower and appearance of a M, 95,000 band.

3b, arrowheads). This is consistent with an increased synthesis of CEA (53) and is probably due to molecules associated with newly synthesized membranes (18).

The results presented in this paper show that CEA, an apical membrane glycoprotein from human colonic epithelial cells, is expressed with a different molecular weight in normal colon, in tumor tissues, and in tissue-cultured cell lines originating from human colonic adenocarcinoma. We have found a simple pattern of discrete molecular forms of the CEA, specific for the normal colon: M,

<table>
<thead>
<tr>
<th>Clinical diagnosis</th>
<th>Dysplastic</th>
<th>Benign</th>
<th>Moderate</th>
<th>Severe</th>
<th>Carcinoma</th>
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<tr>
<td>Normal colon phenotype (1)*</td>
<td>23 3 0 0</td>
<td>0 0 0 0</td>
<td></td>
<td></td>
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<tr>
<td>Intermediary phenotype (2)</td>
<td>10 1 2 2</td>
<td>0 0 0 0</td>
<td></td>
<td></td>
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<tr>
<td>HT-29 phenotype (3)</td>
<td>1 2 0 3</td>
<td>0 0 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor phenotype (4)</td>
<td>0 0 0 4</td>
<td>0 0 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgical tissues</td>
<td>Normal colon phenotype (1)</td>
<td>50 0 0 0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor phenotype (4)</td>
<td>0 0 0 20</td>
<td></td>
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</tbody>
</table>

*T normal colon phenotype (1), presence of two bands at M, 200,000 and 130,000; intermediary phenotype (2), the M, 200,000 band partially shifted to lower molecular weight; HT-29 phenotype (3), presence of one band at M, 170,000; tumor phenotype (4), presence of one band at M, 170,000 or lower and appearance of a M, 95,000 band.

DISCUSSION

The results presented in this paper show that CEA, an apical membrane glycoprotein from human colonic epithelial cells, is...
DECREASE OF CEA MOLECULAR WEIGHT AND GLYCOsylation IN CANCER

Fig. 3. Immunolocalization of antigen 517 in colon polyp tissues. Paraffin sections of benign and moderately dysplastic polyps were stained with Mab 517 using the peroxidase-antiperoxidase method. a, benign polyp, labeling restricted to the apical plasma membrane; b, dysplastic benign polyp, with an important labeling observed intracellularly in addition to the apical one. Arrowheads, unlabeled area of the apical domain which is probably the terminal web.

Fig. 4. Action of \( N \)-glycanase on the molecular weight of CEA immunoprecipitated with Mab 517. Membranes isolated from different tissues and cultured cells were labeled using \( \text{SO}_4 \)-NHS-biotin and immunoprecipitated with Mab 517. They were then incubated for 36 h at 37°C in the absence (Lanes a,b,c) or in the presence (Lanes d,e,f) of \( N \)-glycanase. The hydrolysis products were separated on SDS-PAGE (6-15%) and revealed with \( ^{125} \text{I} \) streptavidin followed by autoradiography: Lanes a,d, normal colon; Lanes b,e, tumoral tissue; Lanes c,f, HT-29-18 cells. Molecular weight markers were the same as in Fig. 1.

Fig. 5. Inhibition of CEA glycosylation by tunicamycin. Tissues or cultured cells were preincubated in the presence of tunicamycin and then labeled for 3 h with \( l-[4,5-^3 \text{H}] \)leucine in the presence (Lanes b,d) or in the absence (Lanes a,c) of tunicamycin. CEA was immunoprecipitated with Mab 517, analyzed by SDS-PGE (6-15%), and processed for fluorography. Lanes a,b, normal colon; Lanes c,d, HT-29-18 cells. Molecular weight markers were the same as in Fig. 1.

We found that the major CEA band averaged at \( M, 200,000 \) the cross-reacting antigens peculiar to each normal or malignant tissue. The original approach of the present work was to follow the pattern of expression in parallel on normal and cancerous cells of a single molecule already expressed in normal tissue, using appropriate antibodies. Similar studies might be performed on other organs when it is possible to follow the expression of a single known molecule.

We found that the major CEA band averaged at \( M, 200,000 \)
in normal colon as has also been described by Ahnen et al. (18). This band was shifted in cancer cells where it was found mainly at \( M, 170,000 \). This was also true for several cell lines originating from human colonic adenocarcinoma, e.g., HT-29, Caco-2, while other lines (SKCO15, LS 174 and GEO) displayed a higher molecular weight (48, 59). In tumor tissues, the molecular weight was always shifted to \( M, 170,000 \) or, occasionally, lower.

The presence of the band detected by immunoblotting at \( M, 130,000 \) deserves some comment. It was already mentioned by Neumaier et al. (33, 60) but in carcinoma tissues. We found it in normal colon, where it is probably associated with the microvilli of the differentiated cells (19). We did not find it by immunoblotting in cancer tissue or in cancer cell lines.

Differences in glycosylation have been shown previously in cancer studies (15, 17) but the sources of the material analyzed were also heterogeneous. A complete analysis of the polysaccharide core of CEA was performed on a liver metastasis from a primary colon cancer (29). It has been shown recently that two glycoforms of the NCA family designated as TEX (\( M, 75,000 \)) and NCA (\( M, 45,000 \)) extracted from a single liver metastasis can be deglycosylated producing a \( M, 35,000 \) peptide which was identical as shown by peptide mapping (39). We show here, using deglycosylation with N-glycanase and synthesis in presence of tunicamycin, a similar mechanism for CEA, a molecule which has the advantage of being expressed in nontumorous colonic cells. It cannot be excluded, this far, that the polypeptide backbone or other posttranslational processes could also be modified but this would have a minor affect on the molecular weight, as shown by our data on deglycosylation and synthesis in the presence of tunicamycin. We concluded that the appearance of discrete molecular weight species specific of the normal colon phenotype (\( M, 200,000/130,000 \)) and, with a certain variability, the cancer phenotype (\( M, 170,000 \)) were the consequence of a modification of the glycosylation pattern of CEA. Intermediary phenotype may represent the progressive passage of one form to another and/or may result in a mixed population of normal and malignant cells.

The significance of this variation in glycosylation is not yet known. It is different from that previously described for sucrase-isomaltase upon differentiation and polarization of HT-29 cell (61, 62) since CEA is expressed with the same molecular weight in both conditions whether or not they express the polarized phenotype. Although the polarization of HT-29 cells is apparently linked to a differentiation process (63–65), it is not associated with a reversion of the cancer phenotype for CEA.

Our study was further documented by screening a bank of human colonic polyps with the possibility of comparing the results to those obtained in classical diagnosis. It is shown that the variation of the molecular weight of CEA can be used in cancer diagnosis. Actually it seems that the decrease of the molecular weight of CEA could be related to the degree of transformation of resected tissues (see Figs. 1 and 2). It may be of particular interest to monitor serum CEA molecular weight. Such a variation may be related to the decreased glycosyltransferase activity which has been described in cancer (66, 67). In addition, we demonstrate here that this modification of CEA glycosylations is probably a very early event in cancer biogenesis since it was clearly detectable in polyps where no striking sign of transformation could be observed by morphology. Furthermore, Mab 517 allowed demonstration that the shift in the molecular weight of CEA precedes the detection of NCA-95/97 by immunoblotting on moderately transformed polyps. Actually this seems to be the earliest molecular event detectable during carcinogenesis.

Such a process may actually be relatively fast. In this respect, one should recall that the expression of CEA can be modified by incubation of colonic epithelial sheets for 48 h in vitro (68). The authors pointed out that CEA expression was greatly enhanced as in cancer. The molecular weight of the molecule was found to be 180,000, it would be of interest to follow its variation throughout the incubation. When we take this together with our data we think that the variation of CEA expression and glycosylation are very early events and may be of major interest in the understanding of the mechanism of dedifferentiation associated with colonic cancer.

In the future it would be important to determine, by chemical analysis similar to that of Yamashita et al. (29), the exact type of modifications involved and to investigate the possibility of raising antibodies specific for the cancerous form which would be of considerable interest for cancer diagnosis.

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