Mucin-like Carcinoma-associated Antigen Defined by Three Monoclonal Antibodies against Different Epitopes

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ABSTRACT

Three monoclonal antibodies (MAb), b-8, b-12, and b-15, have previously been shown to react with mammary carcinomas and with a restricted set of cells in normal human tissues [C. Stähli et al., Experientia (Basel), 41: 1377-1381, 1985; H. R. Zenklusen et al., Virchows Arch. A Pathol. Anat., 413: 3-10, 1988]. They are shown here to recognize the same high molecular weight acid soluble glycoprotein antigen. Lectin binding, biolabelling, and deglycosylation experiments demonstrate that it contains O-linked carbohydrate side chains with sialic acid and hexoses including fucose, galactose, and/or galactosamine but little if any mannose. These properties, typical of mucin-like glycoproteins, agree with the antigen expression on mucin-secreting epithelial surfaces (H. R. Zenklusen et al., Virchows Arch. A Pathol. Anat., 413: 3-10, 1988). The antigen is thus named mucin-like carcinoma-associated antigen (MCA). The three MAb are shown to bind to three different epitopes on MCA. Two of these epitopes (MCA-b-8 and MCA-b-15) are O-linked carbohydrates, and one (MCA-b-12) contains sialic acid. The epitope MCA-b-12 is of peptide nature. Of various two-site sandwich enzyme immunoassays composed of different combinations of the three MAb, the one with MAb b-12 in both positions is selected for a serum assay. Analyses of tumor patients' sera demonstrate that this MCA enzyme immunoassay can be used as a tumor marker assay for mammary carcinomas. The parameter MCA enzyme immunoassay is shown to differ from other parameters described in the literature.

INTRODUCTION

In a previous publication (1) a collection of MAb against antigens on the surface of mammary carcinoma cells had been described. Three of these MAb, named b-8, b-12, and b-15 (all γ/α), which in immunoblot experiments gave double bands at the same position (Mf, approximately 350,000), showed similar reactivity patterns with a variety of normal and cancerous human tissues and some selectivity for tumor tissue. The distribution of the epitope recognized by MAb b-12, which had given the strongest signals of the three MAb in various assays, was then investigated extensively by immunohistological analysis of tissue thin sections (2). The b-12 epitope was found to be restricted to epithelial cells. A majority of mammary carcinomas of all histological subtypes displayed a pattern of strong staining in all tumor cells. In the remaining mammary and most ovary and uterus carcinomas only a fraction of cells were b-12 positive and in gastrointestinal carcinomas few cells were stained. The epitope was also found to be expressed in some normal mucinous epithelia, such as the ductuli of the breast or the distal tubuli of the kidney. In these epithelia expression was strictly apical, i.e., limited to the luminal side of epithelia. Thus, although the epitope was not tumor specific, its consistent high expression in mammary carcinomas and its rather restricted, focal expression in normal tissues suggested that it might serve as tumor marker. Here, the biochemical and immunochemical nature of the antigen and the epitopes were studied. To investigate its potential as a tumor marker, a serum EIA was developed and sera from carcinoma patients and from healthy control persons were analyzed. In accordance with the results the antigen was designated MCA.

MATERIALS AND METHODS

Culturing of Carcinoma Cells and Preparation of Antigen. The estrogen-dependent mammary carcinoma cell lines ZR-75-1 (1, 3) and MCF-7 (1, 4) and the estrogen-independent line MDA-MB-231 (5) were cultured in RPMI 1640 supplemented with 10% fetal calf serum. As described before (1) antigen was extracted by incubation of cells on their culture vessel with a lysis buffer which included 1% Nonidet P-40 or Triton X-100 and protease inhibitor (1 mm phenylmethylsulfonyl fluoride). The lysate was centrifuged for 10 min at 10,000 × g and the supernatant was kept frozen. For biolabeling experiments 2-mL cultures containing between 1 and 5 × 10⁶ mammary carcinoma cells were grown in complete RPMI 1640 supplemented with 10% fetal calf serum and 50 μCi of the respective ¹²⁵I-labeled hexoses for 2 to 5 days.

SDS-PAGE (12.5% Acrylamide), Immunoblot, and Autoradiography. These were performed as described earlier (1, 6).

Deglycosylation of MCA. MCA derived from human milk was deglycosylated with TFMS as described earlier (7). Freeze dried samples were added to an anisol TFMS mixture and stirred at room temperature for 0.5 and 2 h. The deglycosylated material was dialyzed, separated on SDS-PAGE (5% polyacrylamide), and submitted to Western blot analysis (1, 6). In another set of experiments ZR-75-1-derived MCA was treated: (a) with Vibrio cholerae neuraminidase (Behring Institut, Marburg, Federal Republic of Germany); 1000 units/ml of MCA were incubated with 1 unit/ml of neuraminidase for 1 h at 37°C in 50 mM sodium acetate, pH 6.0; (b) with periodate (20 IHM sodium periodate in 50 mM sodium acetate, pH 4.5) for 30 min at room temperature (8); (c) with N-glycanase (Genzyme Corp., Boston, MA) which hydrolyzes asparagine-linked oligosaccharides [according to manufacturer's instructions (9); prior to α-l-fucosidase treatment, MCA was boiled in 0.5% SDS]; (d) with O-glycanase (Genzyme Corp.) which hydrolyzes the galactosyl-(1,3)-N-acetylgalactosamine core disaccharide linked to either serine or threonine residues of glycoproteins [according to manufacturer's instructions (10)].

Solid Phase Antibody Binding Assays. Solid phase antibody binding assays were performed as described before (1, 11).

EIA. Sandwich enzyme immunoassays were developed essentially as described for other antigens (12, 13) using either microtiter plates or polystyrene beads as solid phase matrix which were coated with b-8, b-12, or b-15. Antigen and horseradish peroxidase-labeled MAb b-12 were added either simultaneously for a one-step immunological incubation (Fig. 2; Table 1) or in two steps (Fig. 4), followed (after a washing step) by incubation of substrate (o-phenylenediamine).

RESULTS

Biochemical Characterization of the MCA Molecule. When antigen obtained from cell extracts of different mammary carcinoma cell lines (ZR-75-1, MCF-7, MDA-MB-231) was analyzed by Western blotting under reducing or nonreducing conditions, the same high molecular weight bands located in the Mf, 350,000 position reported earlier (1) were always observed (results not shown). Human milk gave a band of higher apparent molecular size (approximately 450,000). A series of biolabeling experiments were performed using a variety of ¹⁴C-labeled monosaccharides. Antigen was extracted from the cells and...
analyzed by SDS-PAGE followed by autoradiography. In ZR-75-1 cells a large fraction of the [14C]glucosamine label was found incorporated into a double band in the MCA position near the top of the gel (Fig. 1, Lane 1). Before this analysis the 14C-labeled cell extract was affinity purified (b-12-Affi-Gel), the double band was retained (Fig. 1, Lane 2). It eluted at low pH (Fig. 1, Lane 3) confirming that the glucosamine was integrated primarily into MCA. When during the [14C]glucosamine uptake the ZR-75-1 line was grown in the presence of a high dose of tunicamycin (4 µg/ml), an inhibitor of N- but not of O-linked glycosylation (14), uninhibited incorporation of [14C]glucosamine into MCA was observed. Some of the lower bands disappeared (Fig. 1, Lane 4 versus Lane 6), providing a control for tunicamycin activity. When these extracts were treated with neuraminidase, the MCA bands disappeared, while at least some of the others remained (Fig. 1, Lane 5). Thus, in MCA [14C]glucosamine was integrated exclusively into O-linked carbohydrate [presumably as sialic acid (14)].

This interpretation was supported by the pattern of incorporation of other hexoses. MCA from cultures of ZR-75-1 cells with 14C-labeled fucose, galactose, and glucose but not with [14C]-mannose gave bands in autoradiographs (not shown). These properties of mucin-like glycoproteins (14) suggested high acid solubility of MCA. When (20× concentrated) ZR-75-1 cell culture supernatant containing 10% fetal calf serum was incubated for 30 min at 4°C with 0.6 M trichloroacetic acid, >95% of the protein was precipitated (by A280), whereas >70% of MCA remained in the supernatant (by b-12/b-12-EIA).

One- and Two-Site Assays with the Three MAbs; One Antigen Molecule with Three Epitopes; Partial Characterization of the Epitopes. MCA purified from ZR-75-1 cell culture supernatant containing 10% fetal calf serum was incubated with TFMS. After staining sharp bands were obtained. The experiment confirmed that MCA contained peptides. The experiment confirmed that MCA was present as sialylated MCA analogous to those shown in Fig. 2 were analyzed, asialo-MCA analogous to those shown in Fig. 2 were analyzed, indicating that sialic acid was contained in the MCA-b-15 epitope.

To study the nature of the epitopes recognized by the MAb b-8, b-12 and b-15, the effect of deglycosylation of MCA on the two-site EIAs described above was investigated. Treatment of MCA with neuraminidase had effects on all of these sandwich EIAs (Fig. 2, dashed lines). The signals in the b-12/b-12 and b-8/b-12 assays increased, suggesting a shielding effect of sialic acid. In the b-15/b-12 assay a strong decrease of signal was observed, indicating that sialic acid was contained in the MCA-b-15 epitope.

Table 1 MCA activity after enzymatic deglycosylation and periodate treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity (A405) in two-site EIA</th>
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<tbody>
<tr>
<td>None</td>
<td>b-8/b-12</td>
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<tr>
<td></td>
<td>b-12/b-12</td>
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<tr>
<td></td>
<td>b-15/b-12</td>
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<tr>
<td>Periodate</td>
<td>1.76</td>
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<td></td>
<td>2.82</td>
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<td></td>
<td>3.05</td>
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<tr>
<td>O-Glycanase</td>
<td>0.30</td>
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<td></td>
<td>2.98</td>
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<tr>
<td></td>
<td>0.01</td>
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<tr>
<td>Neuraminidase + O-glycanase</td>
<td>0.27</td>
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<tr>
<td></td>
<td>2.93</td>
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<td></td>
<td>0.01</td>
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<tr>
<td>N-Glycanase</td>
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<td></td>
<td>2.51</td>
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Fig. 2. Standard curves in 4 two-site EIAs with MCA (——) and asialo-MCA (— — — —). Beads were coated with the reagent [MAB or wheat germ lectin (WGL)] listed on the right side of the curves and assayed with b-12 enzyme conjugate.
MUCIN-LIKE CARCINOMA ANTIGEN

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DISCUSSION

Three MAb named b-8, b-12, and b-15 were shown here to recognize three different epitopes on a mammary carcinoma cell-derived antigen molecule designated MCA. By immunoblotting with the three MAb, MCA isolated from two estrogen-dependent and an estrogen-independent mammary carcinoma cell line consistently showed an apparent molecular weight of 350,000 with a narrowly spaced double band. The molecular size and the spacing of the double band were insensitive to the presence or absence of reducing agents. MCA was soluble in 0.6 M trichloroacetic acid. Biolabeling experiments showed that MCA contained large amounts of sialic acid, galactose, and fucose but no mannose. Lectins specific for glucosamine or N-acetylgalactosamine bound MCA in sandwich assays but lectins specific for mannose did not. Tunicamycin did not inhibit incorporation of 14C into sialic acid nor did it affect the migration of MCA in SDS-PAGE. These observations suggested that MCA is a mucin-like (14) glycoprotein with O-linked rather than N-linked carbohydrate side chains, which carry a high number of charged groups. A molecule immunochemically related to MCA was found in milk. It expressed the epitopes defined by the three MAb but exhibited a higher molecular size (Mr, 450,000). Of the three epitopes one (MCA-b-15) was found to contain sialic acid, without being one of the sialyl-Lewis epitopes (MAb b-15 did not react with partially purified sialyl-Lewis-a or sialyl-Lewis-x antigen preparation). The other two epitopes were partially masked by sialic acid. While the epitopes recognized by MAb b-8 and b-15 were composed of O-linked carbohydrates, the MCA-b-12 epitope was found to be proteinaceous. Because carbohydrate epitopes may be attached to different carrier molecules (15) and the carbohydrate composition of glycoproteins may vary between individuals (16), generally less variable protein epitopes such as MCA-b-12 may be preferable in assays for the quantification of highly glycosylated serum antigen.

The analysis of MCA levels in sera of normal individuals and cancer patients with the MCA EIA (b-12/b-12 EIA) showed significant elevations in breast carcinoma patients with distant metastasis. Patients in early stages of disease displayed MCA levels, which on the average were elevated over those of the control groups, suggesting that longitudinal MCA determination (monitoring) of early stage patients might reveal therapeutic success or failure. Immunohistology has shown that MCA was expressed on breast cancer cells independently of the histological subtype (2). MCA was not expressed by nonepithelial analyzed and found to contain high MCA levels in the fat and in the aqueous phase. Sera from (TNM-staged) breast cancer patients were divided into three groups: patients (staging T1-a) without [B(N–)] or with [B(N+)] local lymph node involvement or with distant metastases [B(M+)], respectively. There was no information regarding therapeutic measures possibly initiated before taking serum samples. In a majority of mammary carcinoma patients with distant metastases (T1-a, N+, M+ staging) MCA levels were found to be significantly elevated above the values of the control groups. In the other two groups few values above the controls were observed, but the group averages were elevated. Sera from unstaged ovarian cancer patients also showed significant elevation. Few of the sera of colon carcinoma patients were elevated, although the majority of these patients were reported to have advanced disease, a staging confirmed by CEA determinations on these sera (not shown). The results (expressed in arbitrary units) are shown

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ACKNOWLEDGMENTS

Several research groups have described MAbs recognizing mucin-like glycoproteins released by mammary carcinomas (17-24) that furthermore also reacted with components in milk (19-22). Some of these antibodies differ from MCA in their published molecular sizes and/or reactivity patterns (see also “Discussion” in Ref. 1). Thus, the antigens recognized by the MAb B72.3 (TAG-72, Mr >10^6), F36/22 (Mr <100,000), and HMFG-2 (several bands with molecular weights from 80,000 to 400,000) have molecular weights clearly different from those of MCA (21, 23, 24). For the antigen recognized by the MAb DF3 (Mr 300,000-400,000) (19), 115D8 (antigen named MAM-6, Mr >400,000) (20, 25, 26), Cal (antigen named CA, Mr 350,000 and 390,000) (17, 18) HMFG-1 (Mr 250,000-400,000), and W1 (Mr 300,000) (22), identity with MCA could not be excluded on the basis of molecular size alone. Thus, reactivity patterns were compared. Unlike MCA the CA antigen recognized by MAb Cal was reported to display an almost restricted to a subset. Thus, sera of colon carcinoma patients including late stages (with high CEA values) showed practically no MCA elevations. Unlike CEA, MCA was not elevated in smokers. MCA but not CEA was elevated in pregnant women starting with the second trimester.

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