ABSTRACT

Human mammary tumor glycoprotein (MTGP) antigen has recently been identified and purified from human breast tumors. In the present study, the incidence and concentrations of MTGP in breast tumors were analyzed. By quantitative immunoelectrophoresis with a sensitivity of 100 units/ml (approximately 200 ng/ml), MTGP was demonstrated in the ultracentrifugally soluble fraction in all seven metastatic breast tumors tested at concentrations of 480 to 3125 units/mg glycoprotein. When the ultracentrifugally sedimentable fractions of three breast tumors were solubilized in sodium dodecyl sulfate, MTGP was demonstrated at 140 to 323 units/mg total protein. MTGP was demonstrated at 140 to 323 units/mg total protein. Breast carcinomas (11, 12). This MTGP of molecular weight circa 19,500 was identified in both ultracentrifugally soluble and sedimentable forms. In addition, the soluble MTGP occurs in 2 distinct types that consistently differ in isoelectric point, buoyant density, carbohydrate composition, and an apparent amino acid substitution. Antisera to MTGP have been observed to react with a cross-reacting antigen in some spontaneous murine mammary tumors; however, the human MTGP molecules are antigenically unrelated to the glycoprotein with a molecular weight of 52,000 of murine mammary tumor virus (12) and thus do not appear to be the same human breast tumor-associated antigen described by Keydar et al. (9), Mesa-Tejada et al. (17), and Teramoto et al. (22). In view of the association of MTGP with breast tumors, we have now examined (a) the frequency with which this candidate marker is present or associated with the histological type, differentiation, and other features of breast tumors; (b) the association between MTGP and other selected tumor markers; and (c) the degree of specificity for tumors of breast origin.

MATERIALS AND METHODS

Cytosols. The aqueous soluble and nonsedimentable fractions (cytosols) of biopsies from breast carcinomas were prepared by homogenization of 1.0 g of frozen tissue in 5 ml of 10 mM Tris-HCl-1.5 mM EDTA-1 mM diithiothreitol, pH 7.4, at 4° according to the method of Johnson et al. (8). The homogenate was centrifuged at 100,000 x g for 30 min to obtain the clear lipid-free supernatant. The samples were adjusted to 2 mg protein per ml by Lowry assay (13).

PCA Extracts. Two-g samples of neoplastic or normal tissues were homogenized at a volume of 12 ml in 0.6 N PCA at 4°, and the supernatants were recovered. After centrifugation for 30 min at 100,000 x g, the supernatants containing the PCA-soluble glycoproteins were extensively dialyzed, lyophilized, redissolved, and adjusted to 5 mg glycoprotein per ml by the assay of Lowry et al. (13).

Particulate Fractions. Tissues were homogenized as above and centrifuged at 100,000 x g for 30 min. The precipitate was washed 3 times in 5 ml of 10 mM Tris-HC1, 1.5 mM EDTA, and 1 mM diithiothreitol, pH 7.4, at 4° and then solubilized by incubating the pellets overnight at 37° with equal volumes of 0.5% SDS (12). After centrifugation at 10,000 x g for 30 min at 20°, the supernatant was dialyzed at 22–24° against 0.05% SDS, concentrated, and adjusted to 2.5 mg protein per ml by modified Lowry assay (14).

Antisera. Antisera were produced by immunizing goats and rabbits with 150 μg partially purified MTGP or with 10 μg highly

INTRODUCTION

In the search for molecules uniquely restricted to neoplastic cells of the breast, i.e., candidate tumor-specific antigens, we have recently identified, isolated, and characterized a new glycoprotein present in trace quantities in human breast ca...
purified MTGP in complete adjuvant (12). The animals were boosted s.c. each month with 10 μg MTGP to maintain the titer of antibody as tested by gel double diffusion. Goat antiserum (G-300) was absorbed with lyophilized homogenates of a panel of normal tissues, after which 4-ml aliquots were passed at 4° through 200 μg of immobilized PCA-soluble glycoprotein from normal bowel and lung coupled to glutaraldehyde-activated Biogel P-6 (Bio-Rad Laboratories, Richmond, Calif.) (23) or cyanogen bromide-activated Sepharose 4B (2, 12). The free protein peak was eluted with 0.14 M NaCl solution, the γ-globulin was precipitated at 45% ammonium sulfate saturation at 4°, and the precipitate was dissolved and dialyzed against 0.14 M NaCl, 0.01 M sodium phosphate, pH 7.2.

Radioassays. The estrogen receptor content of tumor cytosols was analyzed by the simplified Scatchard plot assay described by Johnson et al. (8). CEA-S and NCA were assayed by equilibrium competitive inhibition radioimmunoassays (3, 18).

Quantitative Electroimmunodiffusion. The concentration of MTGP in the cytosol fractions of tissues and in the PCA-soluble glycoprotein fractions was estimated by quantitative electroimmuno- diffusion in agarose at 1.5 V/cm for 16 hr as described by Zimmerman et al. (25) and adapted for MTGP (12). For MTGP solubilized in SDS, the procedure was modified as described by Converse and Papermaster (1) using electrophoretic migration through Lubrol to displace SDS from the sample and permit effective immunoprecipitation. A PCA-soluble fraction of a metastatic ductal cell carcinoma of the breast (T-124) at 218 μg protein per ml was assigned an arbitrary value of 1000 units/ml and used at serial dilutions in all assays as a secondary standard. Selected assays were performed with purified MTGP also present as a primary standard and to provide gravigmetric quantitation of the secondary standard (Chart 1). One unit was estimated as approximately 2.0 ng for both type I and type II MTGP with the current reagents. Quantitation of MTGP in the standard PCA-soluble extract of tumor T124 was linear from 125 to 1000 units MTGP per ml. Preparations of purified MTGP were linear over a similar unit range corresponding to 250 to 2000 ng/ml except for some deviation of type I at low concentrations. Unknown samples were assayed and calculated as units per mg total protein or glycoprotein, since the amount of material was usually insufficient for typing and assignment of MTGP mass values.

Histopathology. A representative section was removed from the center of each of the tumors fixed in 10% neutral buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. The histological classification of the breast carcinoma biopsies was based on criteria presented by Fisher et al. (4). Six histological features were systematically analyzed, including (a) histological pattern of growth and (b) degree of differentiation. Invasive ductal tumors were designated as Grade 3, poorly differentiated, if they consisted of solid cords and/or solid islands, sheets of cells with no ductal structures. Grade II, moderately differentiated tumors were composed of mixed solid and ductal areas. Grade I were well-differentiated tumors with predominant ductal differentiation. (c) Nuclear atypia was noted. Grade I nuclei were regular and vesicular with a delicate nuclear membrane, chromatin was finely distributed, and mitoses were frequent. Grade II nuclei were intermediate between Grade I and Grade III nuclei, which were hyperchromatic, pleomorphic, and sometimes giant or multiple.

Nucleoli were prominent and mitoses were frequent. (d) Cellularity was estimated from the relative proportion of the tissue occupied by tumor cells. (e) Necrosis was scored, as was the amount of fibrous or hyalinized stroma. (f) The inflammatory infiltrate was graded as to relative extent, and the composition was scored as predominantly lymphocytic, plasmacytic, or mixed.

Cell Culture. Human breast carcinoma line HS-578T [previously (12) designated AY-726] was grown in minimum essential medium supplemented with 20% fetal calf serum, 50 units penicillin-streptomycin per ml, 0.2 mM L-glutamine, and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Grand Island Biological Co., Grand Island, N. Y.) in stationary cultures. Cells were harvested by scraping.

Statistical Analysis. The Pearson product-moment correlation (6) was used for parametric analyses comparing the concentrations of estrogen receptor, NCA, CEA-S, and MTGP in the 83 breast carcinoma biopsies. The significance (p) and correlation (r) were obtained from Simpson et al. (21). The Spearman’s rank correlation was used for nonparametric analysis of the concentration of MTGP in breast tumor biopsy cytosols in relation to the ranking of tissue histopathology (20). Student’s t test was used to evaluate the significance of the correlation (21). The sampling distribution of MTGP concentrations was determined from a standardized normal distribution (20), and the probability that MTGP concentrations of <50 units occurred in the distribution was calculated by a 1-tailed test of significance (20). Distribution was also verified by probit analysis (5) using the log10 of MTGP concentration.

RESULTS

MTGP was identified and quantitated in 7 large metastatic ductal carcinomas of the breast in which relatively homogeneous areas of tumor tissue, devoid of inflammation and necrosis, could be confidently sampled (Table 1). Serial titrations of the PCA-soluble glycoprotein fractions of these tumors gave dilutional slopes parallel to that of the standard (data not shown). The mean concentration of MTGP was 1044 ± 927 (S.D.) (range, 480 to 3120) units/mg PCA-soluble glycoprotein, which is equivalent to a mean of 1.36 to 3.97 μg MTGP per mg glycoprotein, depending on whether the MTGP is of type I or II (12). The ultracentrifugally sedimentable (i.e., particulate) fractions of 3 tumors contained MTGP at 140 to 323 units/mg.

Chart 1. Representative standard curves for electroimmunodiffusion (E.I.D) analysis of MTGP. The standard (Std) is a PCA-soluble fraction of tumor T124, and MTGP is expressed in units/ml ( ). Purified MTGP-I ( ) and purified MTGP-II ( ) given in mg/ml. Shaded area, limit of sensitivity.
total protein. In contrast, in 54 samples of normal tissues and in 22 tumors of sites other than the breast (Table 2), no MTGP was demonstrable in the current assay with a limit of sensitivity of 20 units/mg glycoprotein.

To estimate the concentration of this molecule in breast carcinoma cells, we assayed tumor cell line HS-578T (7), previously shown by immunohistochemical techniques to contain MTGP within the cytoplasm and at the surface of viable cells (12). The concentration in the PCA-soluble fraction was 192 units/10⁶ cells. This cell line produced MTGP type I by reference to isoelectric point (pl 5.35). Since MTGP type I has a conversion of 1.3 ng/unit, the cells were estimated to contain 2.5 ng soluble MTGP per 10⁶ cells or about 80,000 molecules/cell in the cytoplasmic pool. MTGP has been undetectable in control cell lines.

The concentration of MTGP in the cytosol preparation of 83 histologically characterized biopsies of breast ductal carcinomas was analyzed. Of these, 66 (79.5%) contained more than 50 units MTGP per mg total protein [mean, 195 ± 114 (S.D.) units/mg total protein; range, 50 to 695] as illustrated in Chart 2. The concentrations of MTGP described a normal Gaussian distribution; the probability that samples with <50 units were a distinct subpopulation and were excluded from the normal distribution was p < 0.15. A number of these latter samples could have contained trace quantities of MTGP, and this probability was statistically supported by probit analysis.

Two other glycoprotein markers, i.e., CEA-S and NCA, as well as the estrogen receptor, were also assayed in the 83 biopsy-derived cytosols of the ductal carcinomas of the breast (Chart 2). Of the 83 biopsies, 71 (85.5%) were positive for estrogen receptor with a mean of 43.1 ± 115 (S.D.) (range, 1 to 901) fmol/mg total protein. The CEA-S content was greater than 14.5 units/mg total protein in 54 of the 83 biopsy cytosols (65.5%) with a mean of 40.8 ± 66.5 (S.D.) (range, 14.5 to 364) units/mg total protein. NCA was demonstrable in all biopsy cytosols varying from 34.4 to 166,000 units (0.24 to 1160 µg) NCA per mg total protein with a mean concentration of 3941 units (37.4 µg) and a S.D. of ±6088 units/mg total protein.

The presence and concentration of the 4 markers, i.e., MTGP, estrogen receptor, NCA, and CEA-S, were compared when positive (Table 3). The presence and concentration of each of the 4 segregated independently, and absence of MTGP did not correlate with the presence, absence, or concentration

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<th>MTGP antigen in 7 carcinomas of the breast metastatic to the liver</th>
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* ND, not determined.
of any other marker. For example, MTGP was found in both estrogen receptor-positive and -negative breast carcinomas. Marginally weak correlations were observed between NCA and CEA-S ($r = 0.266, p < 0.1$) for comparison of estrogen receptor and NCA ($r = 0.236, p < 0.1$).

When the concentration of MTGP was compared with the histological degree of differentiation, there was no significant correlation (Table 4). A similar lack of correlation was observed for the degree of cellularity and for the degree of nuclear atypia. Additional data (not shown) indicated that there was no correlation with the extent of the inflammatory infiltrates, the type of inflammatory cells present, or the stromal proliferation and degree of fibrosis.

In a series of 94 biopsy cytosols from various types of breast carcinomas, 70 were positive (Table 5). Three of 5 lobular carcinomas were positive, indicating that MTGP is not restricted to cells of ductal differentiation. Numerous other histological types were inadequate, other than to note that a medullary carcinoma was also positive. Of all breast tumors studied to date, the 94 biopsies and 7 metastatic ductal carcinomas, 76.2% contained quantifiable amounts of MTGP.

**DISCUSSION**

The newly described glycoprotein, MTGP, may be a specific marker for breast carcinoma, since in the present study we have found this marker in most breast tumors studied to date but in no other tumor or benign tissue. It is present in most, though apparently not all, carcinomas of the breast. At the current level of assay sensitivity, MTGP was found in breast tumor of both ductal and lobular cell origin. As noted previously (12), MTGP was not demonstrated in normal or dysplastic mammary glands by sensitive immunohistochemical means nor in whole lactating breast milk or its glycoprotein fraction by quantitative elecroimmunodiffusion assay. In contrast to CEA-S in colonic tumors (3), the quantity of MTGP present in breast tumors did not correlate with the degree of differentiation, cytological features of anaplasia, or invasion. If MTGP were a normal differentiation product, one might anticipate higher concentrations in more highly differentiated tumors, whereas if it were an embryonic protein most characteristic of undifferentiated cells it should be present at higher concentrations in the least-differentiated tumors. Neither is suggested from the analyses in Table 4; rather MTGP is the frequent though minor product of the majority of these tumors.

It is not known whether MTGP is really absent from some breast tumors or whether the number of tumor cells present in some of the biopsies was too small to permit detection of MTGP. However, all 7 tumors with homogeneous tissue contained significant concentrations of MTGP. Considering the small quantities of this glycoprotein per cell in many tumors (Table 1 and Chart 2) and in other breast carcinoma cell lines, it is possible that MTGP might be present in the negative breast tumors. Statistical analysis suggests a normal Gaussian distribution of the concentration of MTGP and a significant possibility that MTGP concentrations in breast tumors may extend below the current detection threshold.

We suggest that the MTGP quantitated in this study was of cytoplasmic origin and was representative of the total MTGP content of the tumor. This is suggested from prior observations...
that only 1 to 9% of the total MTGP was recovered in the 100,000 × g sedimentable fraction of tumor homogenates (12). Immunofluorescent studies of fixed cells has indicated (7), we have not only shown the presence of this marker (11), as soluble rather than membrane-associated MTGP.4

Using a newly described breast carcinoma cell line, HS-578T (7), we have not only shown the presence of this marker (11), but have also estimated its concentration at about 80,000 molecules/cell. Although modest in number, this marker is present in sufficient concentrations to permit cellular studies of synthesis by sensitive techniques.

The presence of estrogen receptor has been used widely as a basic for prognosis and for therapeutic management of breast cancer patients (16). However, not only does the incidence and concentration of the estrogen receptor vary in breast carcinomas, but also this marker is present in benign breast tissues (10, 24), uterine muscle (19), and gastrointestinal neoplasms (15). We found no correlation between MTGP and estrogen receptor, i.e., MTGP occurred in both estrogen receptor-positive and -negative breast carcinomas. Thus, MTGP does not replace the estrogen receptor as a prognostic or therapeutic marker. Neither NCA nor CEA-S are tissue specific. We found carcinoembryonic antigen-related antigen in the majority (65%) of breast ductal carcinomas, as did Menendez-Botet, et al. (16), but no relationship to MTGP was apparent.

Since most tumors of both ductal and lobular cell origin contain MTGP, it may be of use to identify the tissue of origin of tumors by immunohistochemical techniques. In light of the relative tumor specificity of MTGP, it will be important to determine whether the synthesis of this molecule relates to the basic neoplastic change in the cell. Whether MTGP serves as a target antigen for the immune response of the tumor host also remains a highly provocative possibility. In this regard, the presence of humoral or cellular immune response to MTGP in breast cancer patients is under study.

ACKNOWLEDGMENTS

The authors are pleased to acknowledge the generous provision of breast carcinoma cell line HS-578T by Dr. Walter A. Nelson-Rees, Cell Culture Laboratory, School of Public Health, University of Calif., Berkeley. This study was conducted with the dedicated technical assistance of Nancy O’Rourke and William Garstka as well as the preparation of the manuscript by Mary Gortmaker and Sharon Garland.

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Frequency of Association of Mammary Tumor Glycoprotein Antigen and Other Markers with Human Breast Tumors

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