Cellular Specificity of NB70K, a Putative Human Ovarian Tumor Antigen

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ABSTRACT

We have studied the cellular expression of NB/70K, a glycoprotein proposed to be human ovarian tumor associated described previously. Analysis of cells bearing this antigen in malignant ascites shows expression in tumor cells of gynecological origin (ovarian, endometrial) and to a limited degree in breast cancer cells. Within such tumors, there is a weak inverse correlation between labeling index and antigen expression. Furthermore, evidence is presented to show that the cells bearing this glycoprotein are physically separable from those bearing carcinoembryonic antigen.

INTRODUCTION

A major therapeutic limitation in human ovarian carcinoma is imposed by the fact that the disease is frequently diagnosed at an advanced stage and that posttherapeutic recurrences are likewise frequently not recognized at a time early enough to allow the advantageous application of therapeutic procedures. In an attempt to improve therapy by earlier detection, several laboratories have utilized rabbit heteroantisera directed against human ovarian tumor-associated antigens to quantitate tumor bulk (1, 2, 5–12). Most of these antisera detect large tumor-associated glycoproteins and apparently do not cross-react with other common tumor-associated glycoproteins, such as CEA3 or a-fetoprotein (2, 6–11).

In this study, we have attempted to define the tissue and cellular specificity of an antisera directed against such ovarian-specific tumor-associated antigen (NB/70K) reported previously by Knauf and Urbach (11). This antisera has been shown to detect a polypeptide component (M 70,000) in the sera of patients with ovarian carcinoma as detected by analysis of immune precipitates. The tissue specificity has been supported by evidence of lack of cross-reactivity with normal serum and serum from patients with colon and lung carcinomas and with extracts of human colon carcinomas and normal lung, ovary, and serum (11).

Considerable heterogeneity is present within human tumor cell populations on the basis of cellular differentiation (4, 13). Cell subpopulations of differing degrees of differentiation can be fractionated through the use of equilibrium density gradient centrifugation (13). We have used this technology in the present report to analyze those cells expressing the antigen NB/70K in populations of ovarian tumor cells and to test the identity of such cells with those expressing CEA.

MATERIALS AND METHODS

Patients and Cells. Malignant effusions were obtained by paracentesis into heparinized (10 units/ml) containers from 46 patients with a variety of carcinomas (23 ovarian, 9 breast, 4 colon, one small-cell carcinoma of the lung, one melanoma, one jejunocecum, one bladder, one fallopian tube, 2 endometrial, 2 of unknown primary) undergoing therapy at Princess Margaret Hospital. Solid-tumor biopsies were obtained from 2 cases of adenocarcinoma of the colon and 2 cases of transitional cell carcinoma of the bladder. No patient had received therapy less than 1 month prior to study. Cells were harvested from effusions by centrifugation (600 × g, 5 min) and resuspended in McCoy’s Medium 5A containing 10% (v/v) fetal calf serum. Solid tumors were disaggregated mechanically by crossed scalpels and passage through needles of decreasing size to 25 gauge. Cellular differentials on cell populations were performed as described previously (3). Permanent cell lines of human origin serving as specificity controls were HL 60 (promyelocytic leukemia), 8226 (B-lymphoblastoid), MCF-7 (breast carcinoma), and MGU-1 (bladder carcinoma). All were grown in a minimal essential medium (14) (HL-60 and 8226 as suspensions; MCF-7 and MGU-1 as monolayers) containing 10% fetal calf serum and harvested once early enough to centrifugation (1000 × g, 10 min). The clear supernatant was stored at 4°C. At this point, the antiserum had no reactivity with mononuclear cells from normal peripheral blood (and therefore Staphylococcus A precipitation of undigested IgG, was deemed unnecessary).

Immunofluorescence Assay. Incubations were performed on aliquots of approximately 2 × 10^5 cells in PBS (pH 7.2) at 4°C in round-bottomed wells of titration plates (Cooke, Toronto, Ontario). Cell washes were performed by centrifugation (200 × g, 3 min) following resuspension in PBS before slide preparation by cytocentrifuge. Slides (mounted in glycerol buffer) were observed through a microscope equipped with a light source for excitation wavelength of 490 nm with appropriate filters. Positive fluorescence was characterized by bright punctate membrane ring staining. Negative cells were not illuminated (Fig. 1). At least 200 cells were counted in each case. Binding of anti-NB/70K to tumor...
cells was not dependent on cell preincubation with normal human serum, but the procedure was followed to avoid low-efficiency binding of second antibody to contaminating hemopoietic cells through Fc receptor.

Density Gradient Centrifugation. We have described the use of equilibrium density gradients to fractionate human ovarian tumor cell populations on the basis of cellular differentiation (13). Briefly, cells were fractionated by centrifugation to equilibrium in discontinuous bovine serum albumin density gradients. Cell layers were harvested consecutively by pipet, washed 4 times in PBS, and counted. Aliquots of cells from each density fraction were subjected to the immunofluorescent assay procedure for detection of NB/70K antigen and/or CEA.

RESULTS

Cellular Display of Antigen NB/70K. Fig. 1 shows tumor cells from a patient with serous ovarian carcinoma stained as described to detect NB/70K. Titration of antisera demonstrated a requirement for a 1/4 titer for adequate fluorescence. Positive cells displayed bright membrane fluorescence. Controls consisting of cells incubated with combinations of normal rabbit serum, fluorescent goat anti-rabbit IgG, and PBS instead of rabbit α-NB/70K were negative in all cases.

Tissue Specificity. Table 1 shows the extent of cellular positivity found in tumor samples of different histologies. Positivity in all cases was restricted to tumor cells; in those cases with infiltrating hemopoietic and mesothelial cells [assessed by cellular differentials (3)], designation of positive cells as tumor cells was made on the basis of cell size under phase microscopy. Additionally, the density distribution of positive cells (Chart 2) is inconsistent with positive fluorescence on mesothelial cells, since such cells have a density =1.04 g/ml. Positive cells were also enumerated in 2 cases of endometrial carcinoma and 3 of 9 breast carcinoma samples. No reactivity with the antisera was observed in 6 cases of adenocarcinoma of the colon, one case of carcinoma of the jejunum, one case of small-cell carcinoma of the lung, one case of fallopian tube carcinoma, one case of melanoma, 3 cases of transitional-cell carcinoma of the bladder, one case of carcinoma of the cervix, and 6 of 9 breast carcinoma cases. Two cases of malignant ascites with unknown primary demonstrated positive tumor cells. Extensive patient-to-patient variation was observed in the proportion of positive cells; the differences could not be attributed to histological subgrouping. Positive cells were also enumerated in 2 cases of endometrial carcinoma and 3 of 9 breast carcinoma samples. No reactivity with the antisera was observed in 6 cases of adenocarcinoma of the colon, one case of carcinoma of the jejunum, one case of small-cell carcinoma of the lung, one case of fallopian tube carcinoma, one case of melanoma, 3 cases of transitional-cell carcinoma of the bladder, one case of carcinoma of the cervix, and 6 of 9 breast carcinoma cases. Two cases of malignant ascites with unknown primary demonstrated positive tumor cells. The results where only one specimen of a tumor histology was studied cannot be regarded as significant. All cell lines tested (HL-60, 8226, MCF-7, and MGU-1) had no positive cells in either exponential-phase or stationary-phase cultures. Plastic adherent cell lines were also negative when harvested by scraping with a rubber policeman rather than trypsinization. As stated in "Materials and Methods," no reactivity was seen with peripheral blood mononuclear cells from 3 normal donors, with or without preincubation with normal human serum. Cells disaggregated from 2 normal human ovaries showed no (0 of 500) cells bearing NB/70K. Since the vast majority of such cells were not derived from the outer epithelial layers, it cannot be ruled out that a small fraction of epithelial cells in normal ovary do bear the antigen.

Tumor histology samples itive Median Mean Range

<table>
<thead>
<tr>
<th>Tumor histology</th>
<th>No. of samples</th>
<th>No. positive</th>
<th>Median</th>
<th>Mean</th>
<th>Range</th>
</tr>
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<tbody>
<tr>
<td>Ovarian</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serous</td>
<td>14</td>
<td>14</td>
<td>9.2</td>
<td>17.1</td>
<td>2.0–85.0</td>
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<tr>
<td>Endometrial</td>
<td>5</td>
<td>5</td>
<td>13.0</td>
<td>32.3</td>
<td>7.7–85.0</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>2</td>
<td>2</td>
<td>4.0</td>
<td>1.0</td>
<td>1.0–11.8</td>
</tr>
<tr>
<td>Clear cell</td>
<td>2</td>
<td>2</td>
<td>4.0</td>
<td>1.0</td>
<td>1.0–11.8</td>
</tr>
<tr>
<td>Breast (P)</td>
<td>9</td>
<td>3</td>
<td>0.0</td>
<td>1.2</td>
<td>0.0–4.3</td>
</tr>
<tr>
<td>Cervix</td>
<td>1</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Colon</td>
<td>6</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jejunum</td>
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<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bladder</td>
<td>3</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Lung (P)</td>
<td>1</td>
<td>0</td>
<td></td>
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<tr>
<td>Melanoma</td>
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<td>0</td>
<td></td>
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<td>0</td>
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<td>2</td>
<td>4.0</td>
<td>1.0</td>
<td>1.0–11.8</td>
</tr>
<tr>
<td>Unknown primary</td>
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<td>2</td>
<td>4.0</td>
<td>1.0</td>
<td>1.0–11.8</td>
</tr>
</tbody>
</table>

a P, pleural effusions; all others were ascites.
b Two of 6 samples were derived from solid tumors.
c Two of 3 samples were derived from solid tumors.

No attempt has been made to analyze the clinical implication of the variation in ovarian carcinoma ascites. However, in a number of instances, the proliferative status of the tumor cells was measured by a LI procedure (13). Chart 1 describes the relationship between LI and percentage of cells expressing NB/70K in samples from 14 ovarian cancer patients. An inverse relationship is apparent. Although this relationship is at the limit of statistical significance, the data suggest that the antigen does not express on cells in the proliferative compartment within the tumor. A similar analysis of the data from breast cancer effusions (3 of 9 positive) was possible since LI information is not available.

We further characterized the cells expressing NB/70K by analyzing cell populations separated from the total tumor population by equilibrium density centrifugation. Previous studies have shown considerable heterogeneity of cell function
among such fractionated populations and that ovarian tumor cells expressing differentiated functions have a higher cell density than those in the proliferative population (13). In this study, we have attempted to define the distribution of cells bearing NB/70K and to investigate their identity or lack of identity to those cells expressing CEA. The results of density gradient fractionation of the cells from 3 patients are shown in Chart 2. Cells from Patient 2 (Chart 2B) were analyzed only for expression of NB/70K, and those from Patients 1 and 3 (Chart 2, A and C) were analyzed for NB/70K and CEA using fluorescein- and rhodamine-labeled probes, respectively. The distribution of cells expressing NB/70K showed a peak at a density of approximately 1.06 g/ml in all 3 cases. In Patients 1 and 3 (Chart 2, A and C), the distribution of CEA expressing cells is shown to be distinct from the NB/70K peak, indicating that the cells bearing the 2 antigens are different and probably represent populations with different degrees of differentiation, CEA being expressed on more primitive cells than NB/70K. A small proportion (=1%) of cells in the overlapping areas of the density distribution could be seen to bear both antigens on the basis of displaying both fluorescein and rhodamine fluorescence.

**DISCUSSION**

The cellular expression of antigen NB/70K is largely restricted to tumor cells from gynecological cancers: ovarian carcinoma (23 of 23) and endometrial carcinoma (2 of 2). A small degree of cross-reactivity was seen in 3 of 9 samples of breast carcinoma. Considerable patient-to-patient variation was observed in the frequency of positive cells. The percentage of tumor cells expressing NB/70K ranged from 2 to 85% in the 23 ovarian carcinoma effusion samples. Although the data are not sufficient to draw statistically significant conclusions, there is no indication that the histology of the primary tumor is important in this regard. Our data are consistent with the proposal (11) that NB/70K is an antigen common to serous, mucinous, and endometroid histologies of ovarian carcinoma. The detection of antigen expression on 2 cases of unknown site of primary cancer points to a possible usefulness of this procedure for cases in which diagnosis is difficult, and treatment may depend on designation of the tumor as of gynecological origin. However, the small degree of cross-reactivity in breast carcinoma cells would render this use problematic.

As shown in Chart 1, expression of NB/70K is inversely related to LI, suggesting that the expression is linked to cellular differentiation. The patient-to-patient variation may therefore be an indication of the differentiation state of the individual tumors. This proposal is not testable on the basis of these clinical samples, since all but one of the serous tumors under study were classified as poorly differentiated at initial diagnosis.

We have further tested the idea that NB/70K may be expressed on cells undergoing differentiation by analyzing the distribution of positive cells with respect to density. We have shown previously that cell differentiation is related to cell density in ovarian tumor cell populations (13). The cells responsible for NB/70K expression are a subpopulation with density = 1.06 g/ml. The distribution of positive cells was similar in the 3 patients studied, despite large differences in the cell population distribution with respect to density. We have shown previously that proliferative ovarian tumor cells have a density of approximately 1.04 g/ml (13) and that loss of proliferative capacity and acquisition of differentiated features occur with an increase in cell density so that nonproliferative differentiated cells could be identified with a density = 1.07 g/ml. Cells expressing NB/70K therefore appear to be outwith the proliferative subpopulation (consistent with the inverse relationship with LI seen in Chart 1) and to represent a population intermediate in terms of cell differentiation.

In 2 patients, we analyzed the relative density distributions of cells expressing NB/70K and CEA. These antigens are representative of different but overlapping cell subpopulations, supporting the contention (11) that NB/70K and CEA are distinct antigens.

On the basis of this information, NB/70K is an antigen expressed by nonproliferating cells in tumors of gynecological origin. As such, the antisera directed against NB/70K may be of value in the study of cell differentiation in human ovarian carcinoma.

**REFERENCES**


Fig. 1. Human ovarian tumor cells from a malignant ascites viewed in phase contrast (A) and by fluorescence (wavelength, 490 nm) after staining with α-NB/70K and fluorescein-labeled goat anti-rabbit IgG (B).
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