ABSTRACT

Using a double-antibody panning procedure, we separated a unique cancer antigen cell line (BS-SHI-4M OVC-MU) expressing a mucinous ovarian cancer (OVC) antigen from a malignantly transformed Bloom's syndrome cell line. In order to gain information concerning a mucinous OVC antigen, we tested this unique cell line in the reaction to sera from patients with various OVCs, Krukenberg (KR) tumor, and signet ring cell cancer of the stomach under immunofluorescence and Western blotting protocols and determined the mucinous OVC antigen band at Mr 84,000. We also undertook an immune electron microscopic study to gain information concerning the antigen-antibody reaction [BS-SHI-4M OVC-MU cells-sera from patients with mucinous OVC and KR tumor] and concerning the antigenic determinant of the membrane using preembedding methods. Occasional protein A-gold particles were observed along the cell membrane of BS-SHI-4M OVC-MU cells, when treated with sera from mucinous OVC and KR tumor patients, but no labeling was observed in the cell membrane when treated with sera from normal patients and those with other cancers. Results of the immune electron microscopic study strongly support the data from the antigen-antibody reaction obtained by immunofluorescence and Western blotting analyses. The BS-SHI-4M OVC-MU cells separated here would be useful for serodiagnosis of mucinous OVC and KR tumors and for follow-up of patients after therapy.

INTRODUCTION

OVC is the most lethal of all gynecological cancers. Most ovarian tumors are of the epithelial type, of which the serous and mucinous cystadenocarcinomas predominate. The detection of OVCs is often delayed because of their location and lack of diagnostic methods for early detection; early diagnosis would greatly improve the prognosis. Our efforts during the past several years have been focused on finding tumor-distinctive markers that could be important tools for the serodiagnosis of OVCs using CA antigens originated from a malignantly transformed BS B-lymphoblastoid cell line (BS-SHI-4M). We have separated cells bearing OVC antigens from these mixed cell populations using the panning reaction with antibodies present in the sera of OVC patients. The cell lines obtained from the panning process appear specific to the kind of sera used and are valuable for diagnosis. Although BS-SHI-4M cells carried various OVC antigens (mucinous OVC, serous OVC, endometrial cancer, etc.) when BS-SHI-4M cells were established, the character of OVC antigen cells collected from the panning protocol remains unknown, particularly the specificity of the type of cancer (mucinous and serous OVCs). To clarify this point, we performed panning to collect OVC antigen-specific cells in the reaction with mucinous OVC serum alone, serous OVC serum alone, and the mixture of mucinous and serous OVC serum and separated a unique OVC antigen cell line which reacted specifically with mucinous OVC. In the present study, we tested this cell line in the reaction to sera from patients with various OVCs, KR tumor, and signet ring cell cancer of the stomach under IF and WB protocols for the establishment of serodiagnosis, since KR tumor is known to metastasize from gastric cancer (signet ring cell cancer) to the ovary (4–6). Signet ring cell cancers produce mucin, and their cell nuclei are often eccentric, making the cells signet ring in shape. However, the antigenic nature of KR tumors remains unclear, especially in connection with OVC originating from the ovary. We undertook an immunohistopathological study of KR tumor tissue in the reaction to sera of OVC patients and an immune electron microscopic study to gain further information concerning the ultrastructural features of the antigen-antibody reaction on the antigenic determinant of the membrane (BS-derived OVC antigen cells) in the reaction to sera from cancer patients (OVC and KR tumor) and protein A colloidal gold.

MATERIALS AND METHODS

Cell Line and Membrane Immunofluorescence. Malignantly transformed BS B-lymphoblastoid cells (BS-SHI-4M) were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum at 37°C in a CO2 incubator. Membrane IF for the detection of OVC antigen was performed as previously described (3), except that sera from both normal patients and those with cancer were treated with heat (56°C, 30 min) before the heat treatment was effective in excluding artificial reactions.

Separation of Cells with OVC Antigen by Panning Procedure. Since BS-SHI-4M cells reacted with the sera of mucinous, serous OVCs, endometrial cancer, etc. (1, 3), panning was undertaken to separate cell lines with mucinous and serous OVC antigens in the reaction to the antibodies of these patients. The panning procedures were basically the same as those described previously (3, 8), except that approximately 2 × 10^6 BS-SHI-4M cells, previously treated with 5% goat serum (blocking), were treated with antibodies (3000 µl; diluted 1:30 in PBS) of mucinous and serous OVC sera (heat treated, 56°C for 30 min.) separately, and a mixture of them, and then allowed to bind to dishes coated with anti-human IgG. The adhering antigen-positive cells were detached from the dishes, cultured for an appropriate period, and tested for IF and studied by immune electron microscopy.

Solubilization of Cell Membrane Antigen Proteins. Approximately 7 × 10^7 cells with OVC CAs, collected from panning using mucinous OVC and serous OVC sera (separately and combined sera) were dissolved in 10 ml of Tris-Trition buffer (10 mM Tris-HCl, pH 7.4-150 mM NaCl-0.5% Triton X-100-0.2 mM phenylmethylsulfonyl fluoride), centrifuged at 10,000 rpm (6700 × g) for 5 min to remove nuclei, and concentrated three times using a Millipore (Bedford, MA) membrane filter.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis and WB. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and WB were performed by the method described previously (3, 9). Each sample (20 µl; the exact antigen protein amount, 5 µg) was loaded into a sample chamber of 4–20% gradient acrylamide gel (Daiichi Chemical, Tokyo, Japan), and the gel was run for 3 h. Transferred nitrocellulose membranes (mucinous OVC antigens) were treated with 300 µl of sera from OVC patients (heat treated, 56°C for 30 min; mucinous and serous OVC, KR tumor, and normal sera; diluted at 1:10 in PBS) in each column as the first antigen-antibody reaction and were then processed with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA), followed by the diaminobenzidine (Sigma, St. Louis, MO) staining reaction (3, 10, 11).

Serum Samples. Sera from controls were obtained from 145 healthy individuals (45 males and 100 females, 40–81 years of age), who had no remarkable past clinical symptoms. Serum samples were obtained from 90 ovarian
cancer patients (45 mucinous OVCs, 30 serous OVCs, 10 endometrial cancers, and 5 KR tumors) and 25 STC patients including 15 with signet ring cell cancer. These cancer patients were diagnosed by pathological or other clinical examinations. The samples were collected at the time of each clinical assessment and were retrospectively selected to correlate with the onset of the first clinical change. Serum samples were obtained from nonheparinized blood and stored at −40°C until used. Levels of CA19-9 (gynecological cancer markers) in the sera from patient were determined with commercial assay kits manufactured by Toray Fiji Bionics, Inc. (Tokyo, Japan).

**Immunohistochemistry.** Sections of formalin-fixed and paraffin-mounted KR tumor tissues were processed for immunoperoxidase reactions as follows. Sections with paraffin removed were treated with 0.3% hydrogen peroxide in absolute methanol for 20 min in order to block endogenous peroxide activity. After the nonspecific binding of immunoglobulin was blocked by incubation in 5% goat serum at 37°C for 1 h, the sections were further incubated in sera from normal subjects and from patients with mucinous OVC and KR tumor at a 1:30 dilution in PBS for 1 h at room temperature. The slides were washed with running water for 1 h and incubated with biotinylated anti-human IgG for 1 h at room temperature according to the instructions of the ABC kit (Vectastain kit) (10, 11). After the slides were washed in running water (1 h) and PBS, they were incubated with horseradish peroxidase H-avidin D (Vectastain kit) for 1 h at room temperature. The slides were washed 3 times for 5 min with PBS, and peroxidase activity was detected with a substrate mixture of 0.12% 3-aminobenzidine (Sigma) (dissolved in dimethyl sulfoxide and 0.05% acetic buffer) and 0.05% H2O2 in PBS, resulting in cytoplasmic deposition of a brown-red pigment. Finally, slides were stained with hematoxylin eosin in order to recognize the histopathological features of signet ring cells.

**Immune Electron Microscopy.** The ultrastructural features of the antigenic determinant on the cell membrane of BS-derived CA cells recognized by serum antibody were studied using the preembedding method. BS-SHI-4M OVC-MU cells, which were equally processed for IF, were allowed to react with protein A colloidal gold (E.Y. Labs., Inc.; diluted 1:10 in PBS) in place of anti-human IgG, fixed with 2.5% glutaraldehyde (TAAB Laboratories, Ltd., Berkshire, England) in 0.1 M phosphate buffer (pH 7.4) at 4°C for 1 h, postfixed in 1% osmium tetroxide (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (pH 7.4) at 4°C for 2 h, dehydrated through a graded series of ethanol, and embedded in Spurr resin (TAAB). Ultrathin sections were stained with 4% uranyl acetate and 0.3% lead citrate and examined under a Hitachi H300 transmission electron microscope.

**RESULTS**

**Panning and OVC Antigen Cells.** When the BS-SHI-4M cells were treated with sera from 145 normal subjects and 45 mucinous OVC and 30 serous OVC patients (before the panning) by the indirect IF protocol, the positive frequencies were low and varied from 7–13% in the sera from cancer patients, although 6 of 45 mucinous OVC and 5 of 30 serous OVC sera were negative. As we indicated previously (2, 3), in general, low levels of IF reaction were found in the sera of healthy blood donors; the level of 5% IF positivity (mean ± 2 SD of normal subjects) was used to estimate the upper limit of normal sera (Fig. 1). Therefore, using this arbitrary cutoff level, sera which showed >5% IF positivity were considered to be abnormal. Three antigen cell populations for OVC were obtained from panning protocols using three combinations of sera: (a) mucinous OVC serum alone (BS-SHI-4M OVC-MU), (b) serous OVC serum alone (BS-SHI-4M OVC-Ser), and (c) the mixture of mucinous and serous OVC sera (BS-SHI-4M OVC-MU + Ser). The reactivity of these three OVC antigen cells and STC antigen cells (BS-SHI-4M STC, 3) to various OVC and STC sera as well as to normal sera was examined under the IF protocol (Fig. 1). Positive CA cell populations, obtained from the double-antibody panning procedure, exhibited 30–60% positivities in the reaction to the same type (antibody) of sera by IF (Figs. 1 and 2), although sera that showed negativity before the panning remained negative. In the BS-SHI-4M OVC-MU cells obtained from the panning, 3 of 145 normal individuals had increased IF positivity in the nontreated serum condition (Fig. 1), whereas these 3 sera became negative (<5%) following heat treatment at 56°C for 30 min. Since heat treatment itself did not have any effect on the cancer antibody activity, IF tests were done using heat-treated sera (O, nontreated; •, heat-treated sera). The mean IF-positive percentages in BS-SHI-4M OVC-MU cells were 50.1 ± 4.19 in 45 non-heat-treated mucinous OVC sera (○), 48.2 ± 3.81 in 45 heat-treated mucinous OVC sera (●), and 34.2 ± 2.21 in 5 heat-treated KR sera, as compared to 2.96 ± 0.41 (P < 0.001) in 145 non-heat-treated sera from healthy persons (O), 2.14 ± 0.26 (P < 0.001) in 145 heat-treated sera from healthy persons (●), and 3.98 ± 0.54 (P < 0.001) in 30 heat-treated serous OVC sera (●), and 2.87 ± 0.38 (P < 0.001) in 10 heat-treated sera from stomach cancer patients (●, Fig. 1). The mean IF-positive percentage of 15 heat-treated sera from signet ring cell cancer of stomach (●) was 6.71 ± 0.55 and significantly higher (P < 0.01) than those of healthy persons, serous OVC, and stomach cancer (heat treated). As shown in this figure, BS-SHI-4M OVC-MU cells obtained from panning with mucinous OVC serum were most specific to mucinous OVC sera, although IF-positive percentages were variable among different mucinous OVC cases following the reaction with sera.

![Fig. 1. Levels of mucinous OVC antigen-positive cells among BS-SHI-4M OVC-MU cells in the reaction with sera from patients (normal, ovarian cancers, and stomach cancer).](image-url)
MUCINOUS OVARIAN CANCER ANTIGEN

Fig. 2. Indirect IF micrographs showing antigen-antibody reaction between BS-SHI-4M OVC-MU cells and mucinous OVC serum. IF labeling was performed after panning as described in "Materials and Methods." A', phase contrast images of A.

Since it was uncertain how the cancer antibody activity of sera was related to the clinical symptoms of the patients, correlations between IF-positive percentages and clinical staging of OVC (International Federation of Gynecology and Obstetrics (FIGO), 1974) including KR tumor cases were studied (Fig. 3). As shown in Fig. 3, early stage (stage I) showed a higher IF percentage compared to the more advanced stages; two cases of stage III and four cases of stage IV showed negative levels. This differs from the pattern of serum values of CA19-9 (OVC markers, Ref. 12), which measures serum cancer-associated antigens in the reaction with monoclonal antibody and shows low values in stage I. In Fig. 3, the values of CA19-9 in 23 cases of mucinous OVCs and 5 cases of KR tumors are shown; an inverse correlation was noted between CA19-9 and IF positive percentages. Normal CA19-9 values, <37 units/ml.

Immunohistochemical Study. Microscopic examination of the ovaries from patients with KR tumor revealed infiltrating, poorly differentiated, metastatic adenocarcinoma of a mucin-secreting type, identical with that demonstrated in microscopic sections of the distal stomach. The ovarian tumor consisted of carcinoma cells which formed a solid cancer nest embedded in a string-like ovarian stroma. Some cells (so-called signet ring cells) had eccentric nuclei and single large intracytoplasmic vacuoles and were scattered among the dense fibroblastic stroma. The histological diagnosis was a poorly differentiated signet ring cell adenocarcinoma (Fig. 4). The cellular cytoplasm of cancer nests was immunostained positively with ovarian cancer serum antibody (Fig. 4, A-D). Mitoses, which contained positive immunostaining in the cytoplasm, were frequently seen (Fig. 4D). These various types of KR tumor cells revealed specific positive immunoreaction to mucinous OVC sera as well as to KR sera, but no significant positive staining was observed using normal sera. Positive immunostaining was not noted in the KR tumor tissue slides in the OVC serum (Fig. 4, E-H), which was previously absorbed with BS-SHI-4M OVC-MU cells. Serum absorptions were performed as described previously (2). These findings strongly support the contention that KR tumor cells carry antigen associated with mucinous OVC.

Immunohistochemistry. To analyze in further detail the ultrastructural features of cancer antigen on the cell membrane, ultrathin sections of BS-SHI-4M OVC-MU cells, previously treated with sera from cancer patients and protein A colloidal gold, were examined. Occasional gold particles were observed at the cell mem-

![Fig. 3. Comparison of the values of CA19-9 (top) in sera from 23 of 45 cases of mucinous OVC (OVC-MU) and 5 cases of KR tumor (KR TU) with the IF-positive percentages (bottom) in the clinically different stages (stages I-IV, FIGO, 1974) of mucinous OVC patients in the reaction with BS-SHI-4M OVC-MU cells. Inverse correlation was noted between CA19-9 values and IF positive percentages. Normal CA19-9 values, <37 units/ml.](attachment:image1.png)
brane of BS-SHI-4M OVC-MU cells when treated with sera from mucinous OVC and KR tumor patients, although early OVC patients tended to exhibit somewhat increased numbers of gold particles (Fig. 5). There was no labeling in the cytoplasm or in the occasional vacuoles observed in these cells (Fig. 5). No labeling was noted in the cell membrane or cytoplasm when treated either with sera from normal subjects, serous OVC patients, or other cancer patients or with sera which had been absorbed with BS-SHI-4M OVC-MU cells (2). Fig. 5, A and B, shows the ultrastructure of protein A-gold-labeled BS-SHI-4M OVC-MU cells in the reaction with mucinous OVC (stage I) sera; C and D show that in the reaction with KR tumor sera. As shown in Fig. 5, the localization of gold particles seemed parallel to that of the fluorescent parts of immunofluorescence in Fig. 2, although no correlation between a particular structure of the cell membrane and labeling loci was noted. Ultrathin sections in Fig. 5 exhibited irregular inclusion bodies and developed endoplasmic reticulum (Fig. 5A), which strongly supports malignant transformation from lymphoblastoid cells. The ultrathin sections of CA-positive BS-SHI-4M OVC-MU cells exhibited an irregular shape, with frequent vacuoles (mucin granules) and free ribosomes present (Fig. 5B). These findings also indicate that BS-SHI-4M OVC-MU cells carry mucinous OVC antigen on the cell membrane, supporting the association of mucinous OVC and KR tumor antigen.

**Western Blotting Analysis.** Antigen proteins of mucinous OVC were obtained from cell membranes of BS-SHI-4M OVC-MU cells by the panning protocol. In the WB analysis, a characteristic antigen band appeared at the $M_r$ 84,000 band. In the lectin reaction staining, the mucinous OVC antigen band reacted with wheat germ agglutinin, although this antigen band did not react with other lectins (concanavalin A, DBA, LCA, PHA-E4, PNA, RCA120, UEA-1) (16). The addition of wheat germ agglutinin lectin to serum antibody strongly inhibited the antigen-antibody reaction in the WB. The antigenic activity was less strongly inhibited by other lectins. This biochemical characteristic indicates that the antigenic epitope recognized by serum antibody exists on a high molecular weight glycoprotein. Fig. 6 shows WB analyses in the reaction with 2 mucinous OVC (Fig. 6, lanes 1 and 2), 2 KR tumors (Fig. 6, lanes 3 and 4), 2 serous OVC sera (Fig. 6, lanes 5 and 6), and one normal serum (Fig. 6, lane 7). As shown, there was a characteristic antigen band at $M_r$ 84,000 for mucinous OVC and KR tumor, although the band ($M_r$ 80,000) below $M_r$ 84,000 mucinous OVC antigen reacted with normal sera and serous OVC and was not usable for the diagnostic analysis.

**DISCUSSION**

With the use of the double-antibody panning procedure (3, 8), we separated characteristic BS-derived malignant cells (BS-SHI-4M OVC-MU) expressing strong mucinous OVC antigen on the cell membrane. The BS-SHI-4M OVC-MU cells separated here would be useful for serodiagnosis of mucinous OVC and KR tumors, since there was a significant difference in the percentage of IF-positive cells in the reaction to serum antibody between control individuals and patients with mucinous OVC. With the IF protocol using BS-SHI-4M OVC-MU cells, we were able to find an appropriate cutoff level (5% IF positivity) to make a distinction between mucinous and other types of OVC. There was a tendency for early OVC patients to exhibit rather high frequencies in IF and low values of CA19-9, whereas the advanced mucinous OVC groups including KR tumors exhibited low frequencies in IF or negativity (6 of 13) and high values of CA19-9. IF positivity disappeared in all of the OVC cases examined so far, when complete remission was induced by operation and chemotherapy, although the period until the disappearance of IF positivity was variable (usually about 3 months after remission). In this respect, serodiagnosis using BS-SHI-4M OVC-MU antigen cells seems to be useful for the early diagnosis and follow-up of mucinous OVC patients after therapy. However, with regard to negative OVC cases, it is postulated that the inability to detect cancer antibody to the CA (cell surface antigen and Western blot bands) might be due to the presence
of circulating antigen (or antibody) and formation of immune complexes, especially in patients with advanced cancer (13–15). We also assume that the inability to detect positivity of CA19–9 in stage II might be correlated with circulating immune complexes, although serum levels of CA19–9 were low in stage I, increased with OVC staging, and reached high values in stage IV. In this respect, our previous findings (3, 15) that early STC sera exhibited strong positivity to BS CAs, whereas most of advanced STC sera exhibited negative or weak positivity, may support the present results of OVC. The WB analysis using 4–20% gradient gels demonstrated that the antigen which characterized mucinous OVC was a band at $M_r$ 84,000, although there was a band ($M_r$ 80,000) below the MU-OVC antigen band, reacting to normal sera. The present WB analysis showed high positivity at the band ($M_r$ 84,000) in 19 of 21 mucinous OVC cases, supporting its validity in the early diagnosis of mucinous OVC. It is notable that the BS-SHI-4M OVC-MU membrane antigen protein did not cross-react with sera from normal, serous OVC, stomach, or lung cancers at $M_r$ 84,000, whereas membrane proteins from negative cells (nonmalignant original cells) did not exhibit any specific bands in this band region. In a previous report, monoclonal antibody OC133 was found to recognize a determinant on a molecule with an $M_r$ 80,000, which reacts only to serous OVC antigens (17). Therefore, the finding that mucinous and serous OVC antigens appear on different bands but close to each other seems of special interest considering the character of OVC antigen, although the antigen band ($M_r$ 80,000) reacted with serous OVC in the previous report (17) and with serous OVC and normal sera in the present study. In this respect, there seems to be a certain association between mucinous and serous OVC, although the exact difference at the antigen protein level remains unknown. Further studies are necessary to clarify the difference in these antigens under two-dimension electrophoresis and amino acid sequencing, thereby providing purified antigen useful for qualitative assay for serodiagnosis combined with radioimmunoassay. In the present study, we
further developed immunofluorescence protocols to immune electron microscopic study in order to gain information regarding the ultrastructural features of the antigenic determinant of the antigen-antibody reaction and detected grains of colloidal gold on the cell membrane of this unique cell line following the reaction with sera from mucinous OVC patients. This strongly supports our previous studies of BS-derived cancer antigen-antibody (sera from cancer patients) reaction with IF and WB protocols. To our knowledge, this is the first report clarifying the cellular morphological (or immunological) aspects of antigen-antibody reaction on the cell surface of cancer antigen cells with immune electron microscopy (preembedding methods), although labeling loci are not associated with a specific structure of the membrane. Other cancer antigen cells (BS-derived malignant lymphoma antigen, stomach CA cell lines, etc.) can now be studied by this approach, thereby providing useful data concerning the ultrastructural features of antigen-antibody reactions, as well as helping us to understand the observed ultrastructural differences among them.

Another interesting finding is that the mucinous OVC antigen cells (BS-SHI-4M OVC-MU) separated in the present study showed positivity to sera from all 5 KR patients examined and to approximately 20% sera of the signet ring cell type of STC, the tumor cells of which are known to secrete mucin. Among 5 KR tumor patients examined, three cases carried gastric cancers and ovarian tumors simultaneously (involving one of the ovaries) and their sera reacted to both BS-SHI-4M OVC-MU and BS-SHI-4M STC (3) with IF, whereas two cases, in which the stomachs and their cancers were already removed and KR ovarian tumors appeared after 6 months, showed positivity to only BS-SHI-4M OVC-MU. In these two KR tumors, which appeared 6 months after removal of the whole stomach including the cancer tissues, STC antibody of the sera from patients possibly disappeared because STC and its antigen were already removed, whereas OVC type antigen and its antibody in the serum would have been established with the appearance and proliferation of the ovarian tumor which had metastasized from the gastric cancer. Therefore, the expression of CA would be determined by the organ in which the tumor was established (even after metastasis from other organs). This seems of special interest considering the association of the nature of KR tumor antigen and signet ring cell cancer antigen of stomach, although the exact mechanisms of metastasis remain unknown. From the aspect of the CA, tumor antigen expressed on KR tumor in the ovary can be considered to correspond to that of mucinous OVC. This is supported by the immune electron microscopic and WB data in the reaction of BS-SHI-4M OVC-MU cell line and its derived antigen protein to the serum antibody which showed no difference in the reaction between mucinous OVC and KR tumor sera. The finding that KR tumor paraffin section slides reacted with mucinous OVC sera under the immunoperoxidase staining seems to parallel the positive reaction of KR tumor serum and BS-derived mucinous OVC antigen cells. In this respect, we have developed a unique diagnostic procedure for mucinous OVC (under immune electron microscope and WB) using diluted sera in the reaction with BS-derived mucinous OVC antigen.

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Characteristic Mucinous Ovarian Cancer Antigen Is Expressed in Malignantly Transformed Bloom's Syndrome Cells

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