Anti-tumor and Anti-ovarian Autoantibodies in Women with Ovarian Cancer

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Introduction

Despite progress in cancer therapy, mortality due to ovarian cancer has remained virtually unchanged over the past two decades.1 Ovarian cancer is one of the leading causes of cancer-related deaths among women.2 As there are no specific symptoms associated with ovarian cancer, and as there is a lack of early detection methods, the majority of women with ovarian cancer are diagnosed when they have advanced disease. The proportion of women with advanced disease who survive more than 5 years is around 30%. In contrast, the 5-year survival for women diagnosed with the disease confined to the ovaries is about 90%.3 Thus, there is an obvious need for better approaches to early detection of ovarian cancer.

Problem

There is a lack of validated marker(s) for the diagnosis of early-stage ovarian cancer (OVCA). The objective was to determine if women with OVCA had antibodies, to assess their potential as markers of ovarian cancer. The secondary objective was to compare the prevalence of antibodies to proteins from normal ovary and ovarian tumors to determine if antibodies primarily recognize tumor antigens, as many antigens are common to tumor and normal ovary.

Method of Study

Serum samples from patients with OVCA, borderline or benign ovarian tumors, endometrial cancer and healthy women were examined for anti-ovarian and anti-tumor antibodies by immunoassay. Immunoreactive proteins were characterized by one- and two-dimensional Western blot.

Results

Ovarian (81%, \( P \leq 0.001 \)) and anti-tumor (69%, \( P \leq 0.001 \)) autoantibodies in OVCA were significantly different from those of control sera. A majority of OVCA serum samples reacted with proteins at about 50 kDa from normal ovary or ovarian tumors in one-dimensional Western blot. While there were similar reactions in two-dimensional Western blots, there are differences between reactions to normal and tumor antigens and between reactions to autologous and allogeneic tumors.

Conclusion

Serum autoantibodies are significantly associated with OVCA. Anti-tumor antibodies may provide a useful marker for the detection of ovarian cancer.
ovarian cancer and identification of appropriate molecular markers.

Currently, none of the identified serum markers, including CA125, is effective for early detection of ovarian cancer.4 One consideration in the search for protein markers is that the first appearance of a protein may not be at a level detectable using existing technology. In addition, proteins are subject to degradation and metabolism, which reduces the utility of some proteins as early indicators of cancer.

Circulating antibodies are significant indicators of immune responses to inflammation and disease. Autoantibodies are standard serum markers for autoimmune diseases because of their specificity and high affinity for antigen as well as their stability.5 Similar to autoimmune diseases, cancers are complex and heterogeneous diseases that affect different target organs. There is increasing evidence for an immune response to cancer in humans, demonstrated in part by the identification of serum autoantibodies to tumor antigens,6–8 and by antigen-specific T-cell responses to tumors.1,9 The identification of a panel of tumor antigens that elicit a humoral response may have utility in cancer screening, diagnosis, and prognosis.

Individual antigens expressed in tumors have been identified using antibodies in serum samples.10 The prevalence of antibodies to individual tumor antigens ranges from 5% to 30% with rare exceptions.8 Studies with individual antigens support the concept that only cancer patients have anti-tumor antibodies to antigens expressed in tumors.11–13 Furthermore, these antibodies frequently recognize antigens that are also found in normal tissue.14 Anti-tumor antibodies have also been detected in ovarian cancer.8 However, relatively few antigens associated with anti-tumor antibodies in ovarian cancer have been identified compared with those reported for other cancers.8 In order to test for antibodies relevant to ovarian cancer there is a need to identify additional antigens. The first step is to identify positive sera for antigen identification. Many of the identified antigens are also expressed in the normal ovary. This is not surprising because tumors express antigens found in normal tissue but in altered amounts. Moreover, tumor tissues may include adjacent areas of normal tissue which may become involved in the inflammatory reactions. Therefore, the primary objective was to determine if a majority of women with ovarian cancer had anti-tumor antibodies. The secondary objective was to determine if these antibodies primarily recognize tumor antigens. In addition, for selected sera with anti-tumor antibodies, the specific reactions were compared by Western blot as a basis for identification of specific tumor antigens in subsequent studies.

Materials and methods

Study group

Blood samples from patients 35–85 years old (n = 80) were obtained at entry into the clinic for diagnostic evaluation of pelvic mass at the Rush University Medical Center. The study was approved by the Rush Institutional Review Board. Patients had no prior treatment or surgery for gynecologic cancer. The study group included patients with ovarian cancer (17 serous, four endometrioid, one mucinous, one clear cell, and three mixed type), benign ovarian tumors (including ovarian cysts, fibroids, etc.), borderline ovarian tumors or endometrial cancer (three serous, five endometrioid, one mixed) based on the pathology report. Sera from healthy women (age 20–60 years, n = 24) were obtained from ProMedDX (Norton, MA, USA) and used as experimental controls. Assay control sera were obtained from healthy volunteers without diagnosed autoimmune disease or a history of cancer (age 20–55 years, n = 10).

Serum and tissue preparation

Serum was separated, centrifuged (1000 × g, 20 min) and stored at −80°C until use. Tumor tissue was obtained at surgery. Snap-frozen normal human ovaries collected at surgery for benign conditions (e.g. hysterectomy) were obtained from the National Disease Research Interchange (NDRI). Tissues were stored at −80°C until use. Tissues were pulverized in a dry ice-acetone bath and homogenized with Polytron (Brinkman Instruments, Westbury, NY, USA) in ice-cold Tris-sucrose buffer (40 mM Tris, 4 mM MgCl2, 0.25 M sucrose, 1 μL/mL protease inhibitor cocktail; pH 7.4; Sigma, St Louis, MO, USA). The homogenate was centrifuged (1000 × g, 10 min) and the supernatant was collected. A portion of each homogenate was incubated with protein-G/magnetic bead complexes (MagnaBind; Pierce Biotechnology, Inc., Philadelphia, PA, USA; 0.5 mL/500 mg tissue protein, 30 min, 20°C) to reduce trapped immunoglobulin. The bound immunoglobulin complexes were removed with a magnet and the remaining supernatant used for immunoassay or one-dimensional gel
electrophoresis. The protein content of the extract was measured (Bradford protein assay kit; BioRad, Hercules, CA, USA) with bovine serum albumin (BSA) as the standard (Sigma). Extracts were stored at −80°C.

Immunoassay

Autoantibodies to ovarian antigens were detected by immunoassay as described previously.15 Tissues were homogenized and three extracts from normal ovary or ovarian tumors (malignant) at equivalent protein concentrations were pooled. Immunoassay plates (MaxiSorp, Nalge Nunc International, Rochester, NY, USA) were coated with ovarian or tumor extracts dissolved in a phosphate-based buffer (0.2 M NaCl, 1 M MgCl₂, pH 7.4; 0.05% Triton X-100) containing 1% BSA (Sigma) and tested in duplicate. The bound autoantibodies were detected with goat anti-human IgG-alkaline phosphatase conjugate (Fab-specific; Sigma) and washed to remove unbound conjugates. The bound alkaline phosphatase was allowed to react with p-nitrophenylphosphate substrate (Sigma) and plates were read at 405 nm in a plate reader (Thermomax; Molecular Devices, Sunnyvale, CA, USA).

Samples were assessed against the same negative control sera in every assay (n = 10, age 25–50 years). A positive assay control identified in immunoassays using normal ovarian antigens was used in every assay to monitor assay performance. Values considered positive for antibody had an optical density greater than three standard deviations (P < 0.05) above the mean optical density value for negative control sera.15 The proportion of sera positive for ovarian or tumor antibodies was determined for each group and results were compared using a chi-squared test, with P < 0.05 considered significant.

In addition, the mean optical density values were compared using a two-tailed Student’s t-test for unequal variance, with P < 0.05 considered significant. The correlation of optical density values for normal ovarian antigens and tumor antigens was compared using Spearman’s rho two-tailed test, with P < 0.05 considered significant.

One-dimensional Western blot

Tissue homogenates were mixed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample lysis buffer (2% SDS, 25% glycerol, 62.5 mM Tris-HCl, pH 6.8) to a final concentration of 300 µg protein/mL. Proteins were resolved in discontinuous 10% Tris-HCl SDS-PAGE preparative well precast gel (BioRad). Molecular size was determined by comparison with a chemiluminescent protein standard (MagicMarker Mix, broad range; Invitrogen, Carlsbad, CA, USA). Proteins were transferred to a nitrocellulose membrane (0.45 µm; BioRad). The blot was blocked overnight in Starting Blocker (Pierce, Rockford, IL, USA) containing 0.05% Tween-20 and then transferred to a multiscreen apparatus (BioRad) according to the manufacturer’s instructions. Serum samples were applied to each well (1:100, 1 h, 20°C) except for control wells in which serum was replaced with buffer or control serum. After incubation with sera, the blot was removed from the multiscreen and washed three times with Tris-buffered saline containing 0.05% Tween 20 (TBS-T). The blot was incubated with horseradish peroxidase-conjugated F(ab)₂ fragment goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA, USA) (1:10 000, 1 h, 20°C). Immunoreactions were visualized as a chemiluminescence product (Super Dura West substrate; Pierce) and the image was captured using a Chemidoc XRS (BioRad).

Two-dimensional Western blot

Two-dimensional gel electrophoresis was performed as reported previously,17 with minor modifications. Extracts (200 µg protein/strip) were diluted in rehydration buffer [Bio-Rad ReadyPrep 2-D rehydration buffer containing 7 M urea, 2 M thiourea, 1% (w/v) ASB-14, 40 mM Tris, 0.001% bromophenol blue] containing 1% (v/v) TBP-reducing agent (Bio-Rad), 0.5% (v/v) carrier ampholyte (Bio-Lyte 3–10; Bio-Rad) and 1.2% (v/v) destreaking agent (DeStreak Reagent; Amersham Biosciences, Upsala, Sweden). Samples were incubated with immobilized pH gradient (IPG) strips (Bio-Rad) according to the manufacturer’s instructions (16 hr, 20°C). Following rehydration of the strip, proteins were separated by isoelectric focusing in a PROTEAN IEF apparatus (Bio-Rad), in three steps according to the manufacturer’s instructions (step 1: 250 V, 15 min; step 2: 8000 V, 2.5 h; step 3: 8000 V, 35 000 V hr). After focusing, strips were equilibrated in two steps (20 min each, 20°C) using equilibration buffer 1 [6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol and 2.5% (w/v)
dithiothreitol] and buffer 2 [6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% SDS, 20% glycerol and 2.5% (w/v) iodoacetamide]. The second-dimension SDS-PAGE and Western blot were performed as described for one-dimensional Western blots.

Results

Prevalence of serum autoantibodies by immunoassay

Overall, ovarian cancer was associated with greater optical density values for both anti-tumor and anti-ovarian autoantibodies (Fig. 1). Only the mean optical density values of ovarian cancer sera were significantly different from those for controls (HOV, P < 0.001; TOV, P < 0.001). In addition, the optical density values for anti-ovarian and anti-tumor antibodies were significantly correlated (ovarian cancer, correlation coefficient = 0.69, P = 0.001; benign ovarian tumors, correlation coefficient = 0.45, P = 0.004; experimental controls, correlation coefficient = 0.85, P = 0.0001) in all categories except endometrial cancer (correlation coefficient = 0.56, P = 0.12). The correlation coefficient was not calculated for borderline tumors because of the small sample size.

The proportion of sera positive for ovarian autoantibodies (81%, P ≤ 0.001) or anti-tumor autoantibodies (69%, P ≤ 0.001) in ovarian cancer was significantly different from control sera (Table I). Autoantibodies in sera from patients with borderline or benign tumors did not differ significantly from those of controls. In ovarian cancer, there was no significant difference in autoantibody detection using tumor antigens or antigens from normal ovaries. In addition, there was no difference in antibody prevalence by tumor stage.

Comparison of autoantibodies detected by Western blot

The immunoreactions of selected ovarian cancer sera (n = 9) that were positive in immunoassay were compared by one-dimensional Western blot using normal ovarian antigens and tumor antigens. A majority of ovarian cancer sera had a common band of approximately 50 kDa in one-dimensional Western blot, in addition to bands of different sizes that were not evident in controls. Six sera with representative immunoreactions were selected for two-dimensional Western blot. Because of the heterogeneity of protein expression among tumors, sera from individual patients were tested against their own tumor antigens and tumor antigens from other patients’ tumors (Fig. 2). Characteristically, some immunoreactive spots had similar molecular weight and isoelectric points and some differed between the three antigen sources. In one example (Fig. 2, upper panel), immunoreactive spots at 50 kDa were observed against normal ovary and tumor ovarian proteins, along with distinctly different reactions at 20–30 kDa (normal ovary) and 30 kDa (tumor from same patient) and different isoelectric points. Both lower-molecular-weight reactions were absent against tumor antigens from a different patient. In another example, the reactions between normal ovary and tumor antigens were distinctly different (Fig. 2, lower panel).

Discussion

This is the first report of the prevalence of polyclonal autoantibodies associated with ovarian cancer. Specifically, the proportion of autoantibodies in sera of ovarian cancer patients was significantly greater than in sera of healthy control, borderline and benign tumors, and endometrial cancer by immunoassay.
Furthermore, in this study we found a similar prevalence of antibodies using antigens from normal ovary and ovarian tumors. This is consistent with previous reports that cancer patients produce antibodies to both normal and tumor antigens. While there was some apparent overlap in antigen recognition in Western blot analysis, there are obvious differences between reactions to normal and tumor antigens and between reactions to autologous and allogeneic (different) tumors.

Several methods are currently available for the identification of tumor antigens. In addition to proteomics, serological expression cloning (SEREX) and antigen and antibody microarrays have been used to identify antigens. SEREX does not provide a direct measure of the prevalence of anti-tumor antibodies and limits the detection of antigens to those expressed in the tumor from which the library was derived. Antigen reactions associated with altered post-translational modifications such as glycosylation are not likely to be detected by SEREX. Likewise, arrays are limited to known proteins. An alternate approach is to use a broad screen to identify common reactions and target those for further analysis. This approach also has the potential to identify antigens unique to ovarian cancer. In this study, we used a survey approach to examine the prevalence of a humoral immune response in ovarian cancer patients as a basis for a proteomic analysis of antibody reactions that would potentially distinguish ovarian cancer from benign tumors.

Many of the antigen reactions seen in Western blots were common to both normal and tumor antigens. These findings are consistent with two observations. First, the majority of tumor antigens identified to date are self antigens and many are unaltered. For example, the functional genome of melanoma cells is similar to normal proliferating melanocytes. Secondly, only cancer patients tend to produce autoantibodies to antigens otherwise found in normal tissue. For example, over 50% of cancer patients with tumors expressing NYESO-1 have antibodies to NYESO-1. Similarly, patients with mesothelin-expressing tumors have antibodies to mesothelin. Thus, the results of this study are consistent with previous observations that antibodies are a response to tumors.

Furthermore, assessment of reactions to nine antigens identified by SEREX screening shows a polyclonal but diverse pattern of reactivity in ovarian cancer patients, but not controls, that reflects the heterogeneous nature of tumors. As seen in this study, there are common reactions with ovarian proteins and tumor proteins, as well as divergent reactions, among sera from ovarian cancer patients. Thus, the detection of antibodies by immunoassay and Western blot represents multiple antigen reactions.

Table I Prevalence of Serum Antibodies Against Normal Ovary (HOV) and Ovarian Tumors (TOV)

<table>
<thead>
<tr>
<th>Patient category</th>
<th>n</th>
<th>% positive against HOV (n)</th>
<th>P-value</th>
<th>% positive against TOV (n)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovarian cancer</td>
<td>26</td>
<td>81 (21)</td>
<td>≤0.001</td>
<td>69 (18)</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Borderline</td>
<td>4</td>
<td>25 (1)</td>
<td>NS</td>
<td>25 (1)</td>
<td>NS</td>
</tr>
<tr>
<td>Benign</td>
<td>39</td>
<td>36 (14)</td>
<td>NS</td>
<td>29 (11)</td>
<td>NS</td>
</tr>
<tr>
<td>Endometrial cancer</td>
<td>9</td>
<td>33 (3)</td>
<td>NS</td>
<td>44 (4)</td>
<td>NS</td>
</tr>
<tr>
<td>Controls</td>
<td>24</td>
<td>25 (6)</td>
<td>ref</td>
<td>21 (5)</td>
<td>ref</td>
</tr>
</tbody>
</table>

Positive values were determined from the data shown in Fig. 1 by comparison to assay controls from women without diagnosed autoimmune disease or ovarian abnormality as described in Materials and methods. Significance was determined by chi-squared analysis and is indicated relative to experimental controls. NS, not significant.
One of the limitations of the present study is the use of immunoassay with a tissue extract as antigen. Clearly, some false-positives may be obtained and although the immunoassay is not directly useful for diagnostic purposes, the results showed the feasibility of anti-tumor antibodies as a marker for ovarian cancer. The immunoassay results will be useful to select sera for a proteomics-based approach to developing a specific antibody test for ovarian cancer.

The experimental control group also showed some positive reactions. This is not surprising because the group was an unselected sample from a healthy population. The group may contain women with ovarian autoimmune disease. The group may also include women who have ovarian cancer in the early stages.

In summary, the result of the present study showed that majority of the serum of malignant ovarian cancer patients contained anti-ovarian and anti-tumor antibodies. Thus, autoantibodies are a potential diagnostic marker for ovarian cancer.

Acknowledgments

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References

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