Humoral Immune Responses to Cathepsin D and Glucose-regulated Protein 78 in Ovarian Cancer Patients

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ABSTRACT

Many cancer patients develop tumor-reactive immune responses against antigens that are either expressed on the surface of tumor cells or released from them into the peripheral circulation. In this study, tumor-reactive immunoglobulins, present in the sera of ovarian cancer patients, were used to identify commonly recognized tumor-associated antigens on ovarian tumor cells. Western immunoblot analysis of cellular proteins, obtained from UL-1 ovarian tumor cell line, demonstrated several commonly recognized immunoreactive proteins. Two of these proteins (Mr 32,000 and 71,000) were selected for further investigation. Cellular proteins isolated from normal human ovarian epithelia, in a similar fashion, failed to exhibit corresponding immunoreactivity to these proteins. As an additional control, sera from normal (nontumor-bearing) individuals failed to identify these proteins on Western immunoblots. Furthermore, the absorption of the ovarian cancer patients' sera with normal ovarian epithelial tissue did not remove the reactivity of these two proteins. The Mr 32,000 and 71,000 proteins were subsequently purified by reverse-phase high-performance liquid chromatography, separated by SDS-PAGE, transferred to the polyvinylidene difluoride membrane, and digested with trypsin. These resulting tryptic fragments were separated by microbore reverse-phase high-performance liquid chromatography, and selected fragments were sequenced by mass spectrometry. This sequence analysis identified the Mr 32,000 protein as cathepsin D and the Mr 71,000 as glucose-regulated protein 78 (member of the heat shock protein family). The identities of cathepsin D and glucose-regulated protein 78 were confirmed by Western blot analysis. Additionally, the presence of cathepsin D was demonstrated in association with immune complexes in vivo. Currently, the common antigenic epitopes of these proteins are being defined.

INTRODUCTION

The inaccessibility of the ovaries and the lack of early symptoms make the diagnosis of ovarian cancer difficult until regional and distant metastases have occurred (1). To improve detection methods, research has focused on the identification of tumor-associated or tumor-specific antigens, which may result from transformation-linked expression of new or altered cell products and cell-surface components. Several classes of tumor-associated components have been described: those shared by all tumors of given histopathological type, those that are tissue specific, and those that represent embryonic antigens (for review, see Refs. 2–4). The phenomenon of both shared and distinct tumor-associated antigens has been observed in experimental and human cancers. In an effort to identify the antigens responsible for eliciting these responses, antisera to tumor cells or extracts of tumor cells have been prepared by inoculating other species. These antisera tend to be developed against strongly immunogenic epitopes on antigens and have tended to not be tumor specific.

The presence of tumor-reactive immunoglobulins has been observed in melanoma (5–7), breast (8), head and neck (9), and ovarian cancers (10, 11). These immune responses in cancer patients appear to be elicited by antigens that have been altered, overexpressed, or inappropriately (temporally or developmentally) expressed (4, 7, 12, 13). Autologous humoral immune responses represent a patient's antitumor immune response against immunogenic epitope(s) of physiologically relevant tumor-associated antigens (2, 9).

Some tumor-reactive immunoglobulins derived from the autologous response may recognize tumor-associated alterations in specific epitopes of commonly expressed antigens (14). Several antigens have been identified in melanoma by using the “autologous typing” technique; however, the molecular identification of many of these antigens remains elusive. This is in part due to poor antibody titer, a lack of sufficient quantities of antibodies, and/or formation of immune complexes with circulating antigens, as well as from the characteristics of the antigen, such as poor immunogenicity, loss of antigen expression, and antigen masking.

Recently, several tumor-associated antigens and viral and cellular oncopgenes or deregulated tumor suppressor gene products have been shown to elicit autologous antibody responses (12). These immune responses can result from aberrant glycosylation (mucins; Ref. 15), point mutations (in ras, Ref. 16; and in p53, Ref. 17), or overexpression (of c-erbB-2/HER2/neu, Refs. 8, 18; and of p53 protein, Ref. 13). Although these autologous humoral responses appear to be ineffective in the prevention or...
eradication of the tumor, the minor differences responsible for the immune response may serve as targets for immunotherapy.

Using autologous CTLs, antigens carrying minor alterations have been identified in melanoma. In the present study, autologous antitumor humoral responses of ovarian cancer patients are shown to be generated against specific tumor-associated antigens. The isolation and sequencing of two of these proteins by mass spectrometry are also presented. This study is the first study that defines the antigens, by using amino acid sequences, identified by a patient’s antibody response.

**MATERIALS AND METHODS**

**Cell Lines and Patient Materials.** The ovarian tumor cell line, UL-1, was developed from ascites fluid obtained from a patient 64 years of age with stage III ovarian cancer (19). These cells were grown in DMEM supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 200 mM L-glutamine, 100 mg/ml streptomycin, and 100 IU/ml penicillin in a humidified 5% CO₂ atmosphere. Cell viability was evaluated by trypan blue exclusion. All cultures used for this study were >95% viable.

Samples of normal ovary were obtained from the Gynecology Service from individuals undergoing hysterectomy and oophorectomy unrelated to ovarian cancer disease. The ovarian epithelium was aseptically dissected from the fresh ovary and was incubated in trypsin as described by Testa et al. (20). This procedure selectively removes surface epithelial cells. Serum samples were obtained from ovarian cancer patients undergoing treatment at the Division of Gynecological Oncology at the University of Louisville, and normal control sera were obtained from noncancer-bearing volunteers. Table 1 indicates the clinical data on the representative 9 (of 25) ovarian cancer patients whose Western immunoblot results are presented.

**Isolation and Characterization of Cellular Membranes.**

Monolayers of UL-1 cells, grown to confluence, were washed with 10 mM sodium phosphate-buffered saline (PBS), pH 7.2, and harvested from the dishes by scraping. Cells were pelleted by centrifugation at 1000 × g for 10 min and were resuspended in a hypotonic buffer consisting of 10 mM Tris (pH 7.4), 10 mM NaCl, 1 mM EDTA, and 1 mM PMSF at 4°C for 15 min. The cells were homogenized in a Dounce homogenizer for 15–20 strokes. The resulting cell homogenate was centrifuged at 1000 × g for 15 min to separate nuclei and unbroken cells (pellet). The supernatant was centrifuged at 100,000 × g for 60 min to isolate crude membranes. Crude membranes were resuspended in 8.5% sucrose and applied to a discontinuous sucrose density gradient of 8.5, 30, 35, and 50% sucrose prepared in 10 mM Tris (pH 7.4) and 1 mM PMSF solution and then were centrifuged at 100,000 × g for 1 h as described previously. The pellets were suspended in a buffer (10 mM Tris, pH 7.4; 100 mM NaCl; 10% glycerol; 1 mM DTT; and 1 mM PMSF), and protein concentrations of these fractions were determined by the Bio-Rad protein assay.

To evaluate the enrichment of plasma membranes, the level of 5’ nucleotidase was determined for each fraction. 5’ nucleotidase was assayed by measuring the released inorganic phosphate from AMP at 37°C for 30 min in a reaction mixture containing 5.6 mM sodium-AMP; 56 mM glycine-sodium hydroxide, pH 9.1; and 11.1 mM MgCl₂ (21). The sucrose gradient-isolated membrane fraction (8.5–30% interface) used in these studies exhibited a greater than 6-fold enrichment of 5’ nucleotidase.

**Immunoblot Analysis of Tumor-derived Immunoreactive Proteins.** Fractions I and II (40 μg) were separated by SDS-PAGE under reducing conditions using the method demonstrated by Laemmli (22), with 3% stacking gel and 12% separating gel. Molecular weight estimates were obtained by simultaneous electrophoresis of prestained molecular weight standards (Bio-Rad, Hercules, CA). Following the electrophoretic separation, the proteins were electrophoretically transferred to the nitrocellulose membrane. The nitrocellulose membrane was blocked by incubation with 5% nonfat dry milk in 30 mM Tris (pH 7.4), 150 mM NaCl, and 0.5% Tween 20 (wash buffer). The blots were incubated with patients’ sera diluted to 1:20 with PBS for 1 h. Blots were then washed three times with wash buffer for 15 min each and were incubated with peroxidase-labeled rabbit anti-human polyclonal immunoglobulins (Sigma Chemical Co., St. Louis, MO) for 1 h. Blots were then washed three times with wash buffer for 15 min each. The reactive bands were visualized by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). The resulting immunoreactive bands were compared to the molecular weight standards to determine the molecular weight.

For conformational studies, commercial antibodies against each of these proteins were obtained. The commercial antibodies used in this study were monoclonal anticalcineurin D (Calbiochem-Novabiochem International, San Diego, CA) and goat polyclonal anti-GRP78 (Santa Cruz Biotechnology, Santa Cruz, CA). Western blot analysis was performed with these as described above.

**Reverse-Phase HPLC Protein Separation.** Proteins from fractions I and II were solubilized and separated by

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1 The abbreviations used are: PMSF, phenylmethylsulfonyl fluoride; GRP, glucose-regulated protein; HPLC, high-performance liquid chromatography; PVDF, polyvinylidene difluoride.
reverse-phase HPLC using a Microsorb-MV C-18 column with a 300A pore size (4.6 × 250 mm; Rainin Instruments, Woburn, MA). Proteins were eluted from the column for 50 min, with a linear gradient of 99.9% H₂O with 0.1% trifluoroacetic acid to 99.92% acetonitrile with 0.08% trifluoroacetic acid. The elution was monitored by absorbance at 214 nm, and 1 min fractions were collected. Peak fractions were analyzed on 10% SDS gel followed by immunoblotting with patients’ sera as described above. The immunoreactive fractions from reverse-phase HPLC separation were transferred to the PVDF membrane, and the PVDF membrane was stained with 0.1% Ponceau S red dye in 1% acetic acid.

Trypsin Digestion and Mass Spectrometry of Peptides. The protein band of interest was cut from the membrane and rinsed with both 200 μM NaOH and water to remove the stain. Unoccupied sites on the membrane were saturated with polyvinylpyrrolidone (PVP-40) by soaking the band for 30 min at 37°C with a solution containing 0.5% PVP-40 in 0.6% acetic acid (23). Excess PVP was removed by washing the band extensively with water. After rinsing the membrane once with the buffer used in the enzyme digestion, 100 mM ammonium acetate (pH 8), the band was chopped into small pieces and overlaid with the same buffer containing between 0.1 and 0.5 μg of trypsin. Digestion with trypsin was allowed to proceed overnight at 37°C. The solution of tryptic peptides was removed from the pieces of membrane, which were then washed twice with 100 μl aliquots of ammonium acetate buffer. All solutions were combined into one, acidified with acetic acid to give it a final acid concentration of 1%, and reduced to a volume of 40 μl in a speed vac.

Aliquots of the trypsin digest were fractionated by HPLC on a fused-silica microcapillary column (75 × 200 μm, Polymicro Technologies, Inc., Phoenix, AZ) packed with Poros R2/H support (Perceptive Biosystems, Cambridge, MA). Peptides were eluted directly into the electrospray ion source of a triple quadrupole mass spectrometer (TSQ 70, Finnigan MAT, San Jose, CA) using a linear gradient of 0–80% acetonitrile in 0.1 M acetic acid (140 B solvent delivery system, Applied Biosystems, Foster City, CA; Refs. 24, 25). Mass spectra were acquired at 1.5-s intervals over a 300-1500 mass unit range. Peptides were detected down to the 500 fmol level. Additional aliquots of sample were used to sequence the detected peptides down to the 5–10 fmol level by collision activated dissociation (26). The BlastP program was used to search protein databases for identified sequences through the National Center for Biotechnology Information, NIH (Bethesda, MD; Ref. 27). Isolation of Circulating Immune Complexes. Serum samples (0.5 ml) from cancer patients and normal volunteers were initially incubated with Affi-Gel Blue 50–150 μm (Bio-Rad, Hercules, CA) for 1 h at room temperature with continuous gentle mixing to remove albumin, and then these samples were precleared by incubation with antimouse immunoglobulin coupled to Sepharose. The sera supernatants were applied to 1-ml columns of Protein G-Sepharose (Bio-Rad, Hercules, CA) that were equilibrated previously with PBS, pH 7.4. After the sera were applied, the columns were washed with 25 ml of 20 mM phosphate buffer (pH 7.4), and the absorbance at 280 nm was monitored to insure the complete removal of nonspecifically bound material. Bound immunoglobulins (free and immune complexes) were eluted with 10 mM glycine-HCl buffer, pH 3. The elution was monitored by absorbance at 280 nm, and the eluted fractions were immediately neutralized by the addition of 1 M Tris, pH 9. The eluted fractions were dialyzed and concentrated by a speed vac. These immunoglobulin fractions were separated by SDS-PAGE, and the association of specific antigens were assessed by Western immunoblotting.

RESULTS

Presence of Ovarian Tumor-derived Components Reactive with Patients’ Humoral Response. Membrane-enriched fractions were prepared as described in the “Materials and Methods” section. These associated-protein components were separated by SDS-PAGE, and the presence of components reactive with the humoral immune response was assessed by Western immunoblotting. This Western blot identified several bands

Fig. 1 Western blot analysis of UL1-derived proteins using ovarian cancer patients' sera as the primary antibody. Immunoreactive bands were visualized after incubation with peroxidase-conjugated goat antihuman immunoglobulins by enhanced chemiluminescence. Molecular weight determinations were based on prestained standards run simultaneously. In A, Lane 1 corresponds to patient 1 (Table 1), Lane 2 to patient 2, Lane 3 to patient 3, Lane 4 to patient 4, Lane 5 to patient 5, and Lane 6 to patient 6. In B, Lane 7 corresponds to patient 7 and Lane 7α to the serum of patient 7 after absorption with normal ovarian epithelium.
Table 2 | Sequence data for tryptic fragments derived from the Mr 71,000 and 32,000 proteins of UL-1 ovarian tumor cells and their identities based on sequence comparisons with known proteins by the BlastP program

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<th>Protein Name</th>
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<th>Mr 32,000 Protein</th>
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<td>Glucose-regulated protein (member of heat shock family)</td>
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<td>FDGILGMAYPR</td>
<td>VSTLPAITLK</td>
</tr>
<tr>
<td>Mature cathepsin D</td>
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![Fig. 2](Western blot analysis of UL-1-derived proteins using sera from normal (nontumor bearing) patient controls as the primary antibody. Immunoreactive bands were visualized after incubation with peroxidase-conjugated goat antihuman immunoglobulins by enhanced chemiluminescence. Molecular weight determinations were based on prestained standards run simultaneously.)

![Fig. 3](Representative Western blot analysis of cellular proteins from ovarian epithelial tissue (Lane 1) and UL-1 cells (Lane 2) using serum from ovarian cancer patient 8.)

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As controls for tumor specificity, the reactivity of normal sera was tested with UL-1 proteins. In addition, the reactivity of the cancer patients’ sera was tested against proteins obtained from normal ovarian epithelium. Sera from normal (nontumor-bearing) controls failed to recognize most of the UL-1-derived proteins except a Mr 42,000 band (Fig. 2A). Preabsorption of the normal sera with normal ovarian epithelium removed this reactivity (Fig. 2B). When the patients’ sera were adsorbed overnight with proteins derived from normal human ovarian epithelium and subsequently used in Western blot analysis against UL-1 derived proteins, many of the reactive bands were removed or diminished (Fig. 1B). The Mr 71,000 and 32,000 proteins were still recognized. In addition, the sera from ovarian cancer patients failed to identify the Mr 71,000 and 32,000 proteins on crude membranes prepared from normal ovarian tissue (Fig. 3).

Identification of the Mr 71,000 and 32,000 Immunoreactive Proteins. Although sera from ovarian cancer patients reacted with multiple UL-1-derived proteins following the absorption with proteins from normal ovary, the Mr 71,000 and 32,000 proteins were the most intensely recognized. The Mr 71,000 and 32,000 proteins were partially purified from the membrane-enriched fractions of UL-1 cells by reverse-phase HPLC on a C18 column. Immunoblot analysis of the resulting
fractions with patients' sera identified those fractions containing the immunoreactive M, 71,000 and 32,000 proteins. These fractions were further separated by SDS-PAGE and transferred to the PVDF membrane. Each molecular weight fraction was carefully cut from the PVDF membrane following Ponceau S red staining and was digested with modified trypsin. Multiple fragments for each protein were selected for sequencing by triple quadrupole mass spectrometry. The sequencing technique produced data from one peptide for the M, 71,000 protein and five peptides from the M, 32,000 protein (Table 2). Comparisons of these peptide sequences by the BlastP program identified them as GRP78 (a member of shock protein 70 family) and cathepsin D, respectively.

**Confirmation of the Identities of Cathepsin D and GRP78.** Because mass spectrometric sequencing data identified the M, 32,000 protein as cathepsin D and M, 71,000 protein as GRP78, the identities of these two proteins were confirmed by comparing patient immunoreactivity with commercial antibodies (Fig. 4). In Fig. 4A, the cathepsin D bands (mature and pro-forms) from UL-1 recognized by the commercial anticathepsin D antibody were found to be analogous to the bands recognized by ovarian cancer patients. Similar results were obtained by comparing commercial anti-GRP78 with patient reactivity (Fig. 4B).

**Presence of Circulating Cathepsin D and GRP78 in Vivo.** Because a humoral response to cathepsin D and GRP78 could be demonstrated in vivo, the presence of these specific antigens in the peripheral circulation was analyzed by Western immunoblotting. The Western immunoblot analysis of the cathepsin D antigen in patients' sera demonstrated the presence of reactive bands corresponding to the M, 52,000 procathepsin D and a M, 29,000 form (Fig. 6A). After the removal of the immune complexes, unbound cathepsin D was detected; however, only the M, 52,000 procathepsin D was observed (Fig. 6B).

**DISCUSSION**

In this study, we report the isolation and sequencing of two tumor-derived proteins that were commonly recognized by the humoral response of ovarian cancer patients. By using normal controls and preabsorption of ovarian cancer patients' sera with normal ovarian epithelium, we have demonstrated that immunoglobulins from ovarian cancer patients can be used to identify antigens associated with ovarian cancer. Previously, using established ovarian tumor cell lines SKOV-3 and OVCAR-3, as well as the UL-1, we demonstrated the ability of patients' sera to react with specific proteins (28).

In this study, the reactivity of the humoral response to antigens derived from UL-1 ovarian tumor cells was demonstrated in ovarian cancer patients (Fig. 1). For all ovarian cancer patients, these Western immunoblots identified multiple bands ranging in molecular weight from M, 32,000 to 140,000 (Fig. 1). Although the sera of all ovarian cancer patients recognized some antigens associated with the UL-1 tumor, the number and intensity of the immune interaction varied among patients. The varying intensities of the signal on the immunoblot may be due to the stage of disease, immunological status of the patients, or previous and current treatment, as well as other genetic factors. Although the specific proteins that were recognized varied among patients, some of these bands were recognized by most patients. Two such proteins expressed by the UL-1 ovarian tumor cells had approximate molecular weights of M, 32,000.
We have demonstrated previously the presence of tumor-reactive immunoglobulins in ovarian cancer patients and their correlation with stage of disease (10, 11, 29). In retrospective studies, the presence and levels of these tumor-reactive immunoglobulins have been shown to be prognostic (10). Although the characterization of the antigenic determinants was initiated (11), it has been technically difficult until recently to sequence the small quantities of immunoreactive materials. Two of the commonly recognized proteins \( (M_r = 32,000 \text{ and } 71,000) \) were purified by a combination of HPLC techniques and SDS-PAGE. Using mass spectrometric sequencing, these proteins were identified as cathepsin D \( (M_r = 32,000) \) and GRP78 \( (M_r = 71,000) \).

Cathepsin D, a lysosomal aspartic protease produced in variety of tumors and some normal cells, has been implicated in tumor invasion and metastasis (30). Cathepsin D in cells produced a \( M_r = 52,000 \) procathepsin D, and upon maturation, this proform is processed as a single-chain \( M_r = 48,000 \) active procathepsin D and subsequently, to a two-chain mature cathepsin D with \( M_r = 34,000 \) and 14,000 polypeptides. Altered processing, overexpression, and secretion with increased activity of cathepsin D appear to be associated with tumors versus normal tissue (31). Cathepsin D in the B cell lymphoma cell line A20 (32) and rabbit macrophages (33) has been shown to exist as a free lysosomal enzyme or as an endosome-associated (membrane-bound) enzyme. Both studies demonstrate that a precursor form \( (M_r = 45,000) \) of enzyme was associated with the endosome fraction. Endosomes have been shown to copurify with plasma membranes during discontinuous sucrose density gradient centrifugation, so the presence of membrane-associated cathepsin D may be the result of this cofractionation. In this study, reactivity
was demonstrated toward the $M_r$ 52,000 and 32,000 cellular enzymes; however, in vivo, the $M_r$ 52,000 and a smaller $M_r$ 29,000 form are recognized in immune complexes (Fig. 6). The presence of cathepsin D in these immune complexes further confirms the development of cathepsin D reactive antibodies in these patients. Previously, the presence of $M_r$ 52,000 and 27,000 forms of cathepsin D have been described in the sera of breast cancer patients (34). The significance of this lower molecular weight form is currently under investigation.

GRPs play a role as the prototype of genes that are regulated by signal transduction pathways originating in the ER and traveling to the nucleus. GRPs as molecular chaperones protect cells against adverse physiological conditions. GRPs can be induced by glucose deprivation, chronic anoxia, and acidic pH (35, 36). These stressors exist in many poorly vascularized or necrotic tumors. In many tumors, resistance to chemical and immunological cytotoxicity appears to be mediated by GRP induction (37). GRP78 is a $M_r$ 78,000 resident protein of the endoplasmic reticulum that is constitutively expressed in mammalian cells. Because GRP78 binds to mutated and malfolded proteins, it has been proposed that GRP78 functions to retain these abnormal proteins in the endoplasmic reticulum and prevent their secretion. GRPs exhibit similar functions to other members of the heat shock protein family, which has been documented to be expressed on the cell surface in cancer (38–41). Members of the heat shock protein family have been implicated in the formation of autologous immune responses, perhaps due to overexpression (42), and may even represent targets for the response (43). The possible immunogenicity of GRP78 may be related to autoimmune reactivity or to the presence of an altered immunogenic epitope on a tumor-derived GRP78. Although GRP78 was not detectable in the immune complexes, it was demonstrable in UL-1 cells. It is unclear whether GRP78 is not present or is present at levels undetectable by our assay.

The reactivity of the patients’ humoral responses with cathepsin D and GRP78 were initially confirmed by Western blot analysis using commercially available antibodies raised against each of these proteins (Fig. 4, A and B). The results of this study identify cellular antigens recognized by the tumor-reactive humoral response. Although the overexpression of normal proteins can elicit autoantibodies, and cathepsin D and GRP78 are associated with normal cells, the absence of cross-reactivity between the tumor-derived proteins and their normal counterparts suggests the presence of altered sites (mutated sequences or newly exposed epitopes). In the case of cathepsin D, the form isolated from circulating immune complexes appears to exhibit a lower molecular weight ($M_r$ 29,000) than the cellular protein ($M_r$ 32,000; Fig. 5). Current studies are directed at the identification of the immunogenic epitopes on these proteins.

Although immune effector functions have been demonstrated to be impaired in ovarian cancer, the immune recognition pathway appears to be functional. Thus, the immediate implication of the presence of a humoral response against specific antigens appears to be diagnostic. Other tumor types, such as breast, endometrial, colon, and thyroid have been demonstrated to overexpress and secrete cathepsin D. Thus, it is likely that our findings in ovarian cancer can be extended to other cancers expressing cathepsin D. If these antigens are found to contain unique epitopes, this finding will lead to investigations into the regulation and timing of their expression. Ultimately, the presence of altered epitopes could provide targets for eliciting effective cellular immune responses.

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S R Chinni, R Falchetto, C Gercel-Taylor, et al.