Characterization of the Antigen (CAK1) Recognized by Monoclonal Antibody K1 Present on Ovarian Cancers and Normal Mesothelium

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ABSTRACT

K1 is a monoclonal antibody that reacts with a cell surface antigen (CAK1) found in human mesothelia and nonmucinous ovarian tumors. In this article, the characteristics of the CAK1 antigen have been examined in detail. Using immunofluorescence microscopy, we have found that the CAK1 signal is removed from the cell surface by treatment with proteases or by phosphatidylinositol-phospholipase C, but not by neuraminidase and β-galactosidase. The phosphatidylinositol-phospholipase C-released material was found to contain the CAK1 antigen which was detected by a competition radioimmunoassay. The phosphatidylinositol-phospholipase C-released CAK1 antigen was examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting and found to be a ~40 kDa protein. The CAK1-K1 antibody complex remains on the cell surface and is poorly internalized, as shown by an acid wash immunofluorescence internalization assay. An immunotoxin composed of K1 and Lys-PE-40, a mutant form of Pseudomonas exotoxin lacking the cell binding domain, was not cytotoxic, supporting the conclusion that the CAK1-K1 antibody complex is not internalized. However, an immunotoxin composed of K1 and native Pseudomonas exotoxin was selectively cytotoxic to cells expressing the CAK1 antigen. This cytotoxicity is due to the fact that domain I of Pseudomonas exotoxin promotes internalization of antigens which are not internalized or bound to antibody alone. Our results suggest that CAK1 is a polypeptide that is expressed on mesothelial cells and many ovarian cancers, and that K1 may be useful as a targeting agent for the immunotherapy of human ovarian cancer.

INTRODUCTION

With the advent of monoclonal antibody technology, it was hoped that the isolation, identification and characterization of human tumor antigens would be greatly facilitated for the purposes of specific diagnosis, accurate monitoring, and more importantly, targeted therapy of human cancers. Since the first antitumor monoclonal antibody was generated (1), hundreds of Mabs to tumor-associated antigens have been developed based on the premise that the specificity inherent in these monoclonal antibodies would permit the elucidation and exploitation of novel tumor-specific antigens. Even though potentially useful antibodies have been produced, careful analysis on fresh frozen tissues have shown that specificity is still one of the major problems with monoclonal antibodies against human tumor antigens (2). In addition, several of the more specific monoclonal antibodies were found to react with the shed form of tumor antigens present in the blood of patients. While useful for diagnosis, this raises potential problems for immunotherapy.

There have been numerous studies on monoclonal antibodies that recognize antigenic determinants restricted to human ovarian epithelial carcinomas (3–17). To date, Mab OC125 (3) appears to be the best, based on its wide range of ovarian tumor reactivity and restricted normal tissue binding. In another report from this laboratory, we have described the isolation and preliminary characterization of Mab K1 that reacts with most human nonmucinous ovarian carcinomas (18). This Mab, similar to OC125, also reacts with cells of the mesothelium and the epithelia of trachea, but does not react with other normal human tissues. Immuno Peroxidase histochemical analysis has shown that the antigen identified by K1 may be highly conserved, since normal tissue reactivity is similar in human and cynomolgus monkey (18). Although Mab K1 reacts with many of the same types of tumors as OC125, several differences in reactivity have been noted which clearly indicate that it reacts with antigen different from CA125 (18). One difference is that the antigen recognized by K1, defined as CAK1, is not shed into the culture supernatant of K1-positive cell lines, and is not found in the blood of patients with ovarian carcinoma (18). Thus, K1 may be useful for the immunotherapy of ovarian carcinoma. In the present study, we have examined the biochemical characteristics of the CAK1 antigen and evaluated its potential ability to act as an immunotoxin target.

MATERIALS AND METHODS

Monoclonal Antibodies and Cell Lines. Monoclonal antibody K1 was generated and purified as described previously (18), and 125I-labeling of K1 was performed according to the method of Bolton and Hunter (19); OC125 and 125I-labeled OC125 were purchased from Centecor (Malvern, PA) and Amersham (Arlington Heights, IL); IgG1x murine myeloma protein MOPC-21 was obtained from Sigma (St. Louis, MO). The human tumor cell lines OVCAR-3, AGS, and HeLa were obtained from the American Type Tissue Collection (Rockville, MD). Cell lines were cultured in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY), supplemented with l-glutamine (2 mM), penicillin (50 μg/ml), streptomycin (50 units/ml), and 5–10% fetal bovine serum (Hazelton, Denver, PA).

Exoglycosidase and Protease Digestion. AGS, HeLa, and OVCAR-3 cells, plated in 75-cm² flasks, were incubated with either: (a) neuraminidase, type X (Sigma), 0.2 unit/ml in 0.5 volume of PBS+ and 0.5 volume of PBS/A at pH 4.5; or (b) 0.2 unit/ml neuraminidase with 0.5 unit/ml of β-galactosidase in PBS/A buffer; or (c) with 10 mg/ml of porcine pancreatic trypsin, type IX (Sigma); or (d) 0.2 mg/ml of proteinase K in 0.1 M Tris-HCl, 50 mM CaCl₂ at pH 7.8 for 2 h at 37°C. All these enzymatically treated cells were then removed from the flask by shaking or scraping, and sedimented in PBS+ onto 35-mm dishes precoated with poly-L-lysine (Sigma) for about 30 min until most of the cells attached to the bottom of the dishes. The treated cells on the dishes were then assayed for antibody binding by using indirect or double-labeling cell surface immunofluorescence staining (18).

Phosphatidylinositol-Phospholipase C Digestion. AGS, HeLa, and OVCAR-3 cells, grown to confluency in 35-mm dishes were treated with 10 units/ml of PI-PLC (from Bacillus cereus; Boehringer Mannheim Biochemicals) for 1 h at 37°C, followed by washing in PBS three times. The treated cells were then plated onto 35-mm dishes precoated with poly-L-lysine and processed for immunofluorescence labeling when they had attached to the bottom of the dish. To collect the PI-PLC-cleaved supernatant, three 162-cm² flasks each containing about 15 × 10⁶ cells were incubated with 10 units/ml of PI-PLC for 2 to 4 h at 23°C. The supernatant was collected and concentrated through CentriCon 30, so that the lower molecular weight molecules, including

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2 The abbreviations used are: Mab, monoclonal antibody; PBS, phosphate-buffered saline; PBS+, PBS with Ca²⁺ and Mg²⁺; PBS/A, PBS with 100 mM sodium acetate; PI-PLC, phosphatidylinositol-phospholipase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS/BSA, PBS with 0.2 mg/ml bovine serum albumin; PBS/T, PBS with 0.05% Tween 20; PE, Pseudomonas exotoxin.
most of the PI-PLC, were removed in the filtrate. This preparation, designated as PI-PLC concentrated supernatant, was used in the antigen competition studies. For SDS-PAGE and immunoblotting procedures, this material was further concentrated by lyophilization.

K1 Antibody Internalization Assay. The internalization capacity of the antibody was evaluated by an immunofluorescence cytochemical assay designed to allow visualization of the internalized antibody without interference from surface-bound antibody. OVCAR-3 cells, plated 1 day before the assay on 35-mm dishes, were washed in cold PBS/BSA for 5 to 10 min, followed by incubation with 10 μg/ml of K1 or HB21 at 4°C for 1 h. The dishes were then warmed to 37°C for 15 to 20 min to allow the surface-bound antibody to internalize into the cells. After washing in PBS/BSA, the dishes were incubated with an acid buffer (pH 3) containing 0.5 M NaCl and 0.2 M acetic acid at 23°C for 15–20 min, then fixed in 3.7% formaldehyde in PBS for 10 min, washed in PBS/BSA three times, and incubated with PBS containing 4 mg/ml normal goat globulin and 0.1% saponin for 10 min. After a subsequent 1-h incubation of the cells with rhodamine-labeled goat anti-mouse IgG (H + L) (25 μg/ml) in 4 mg/ml normal goat globulin, 0.1% saponin-PBS, the dishes were washed and fixed again with 3.7% formaldehyde for 10 min, followed by PBS washes. The controls for this assay included dishes that were not warmed to 37°C, and/or dishes that were not acid washed.

Indirect and Double-labeling Immunofluorescence Analysis. The cells that had been treated with exoglycosidases, proteases, or PI-PLC were plated onto poly-L-lysine-coated 35-mm dishes and subjected to live cell immunofluorescence labeling by using methods previously described (18, 20). Direct conjugates of K1 and OC125 with rhodamine (K1) and fluorescein isothiocyanate (OC125) were prepared as previously described (18). These conjugates were mixed together and the cells were simultaneously labeled at 4°C.

Western Blotting for PI-PLC Supernatant of HeLa and OVCAR-3 Cells. The PI-PLC supernatants containing 50 to 100 μg total protein/lane from approximately 10⁶ HeLa or OVCAR-3 cells were separated by using SDS-PAGE with a 12.5% gel. The separated protein bands were then transferred to nitrocellulose paper at 150 mA overnight with the use of a solution containing 20% methanol, 25 mm Tris, and 200 mm glycine, pH 8.5, with a Bio-Rad blotter. The nitrocellulose was then blocked with 3% low-fat milk powder in PBS for 1 h at 23°C. After washing with PBS/T five times, each nitrocellulose sheet was incubated with 5 μg/ml of K1, OC125, or MOPC-21 in 3% milk/PBS for 6 to 16 h, followed by extensive washing with PBS/T for at least five times for 60 min total. Following an overnight incubation of the paper at 4°C with 10 μl of affinity-purified horseradish peroxidase-conjugated goat anti-mouse IgG (H + L) (Jackson ImmunoResearch) in 3% milk/PBS, and a 1-h wash with PBS/T, the reactive bands were visualized by reaction with 0.4 mg/ml diaminobenzidine (Sigma), 0.01% H₂O₂ in PBS for about 10 min, and the reaction was terminated by rinsing the paper in distilled water.

Antigen Competition Live Cell Radioimmunoassay. Fifty to 100 μl duplicate or triplicate serial dilutions of PI-PLC supernatants of OVCAR-3 cells were incubated with 50 to 100 μl (10,000 cpm) of 125I-labeled K1 or 131I-labeled OC125 at 23°C for 2 h. While this incubation was proceeding, OVCAR-3 cells were plated at 7 x 10⁶ cells/well in the wells of a 96-well microtiter plate precoated with 1:100 diluted Cell-Tak (Collaborative Research, Waltham, MA), and incubated with 250 μl/well of blocking buffer (3–5% BSA in PBS or RPMI 1640) at 37°C for 1 h, followed by brief washes with PBS/BSA. The antigen-absorbed iodinated K1 and OC125 were then added to the wells and incubated at 23°C for 2 h. The unbound radioactive antibodies were aspirated off and the wells were extensively washed with PBS/BSA buffer. The individual wells were separated from the plates, and their content of radioactive material was measured in a gamma counter (Beckman, Gamma 5500B).

K1-PE Cytotoxicity Experiments. Mab K1 was coupled to PE or LysPE40 and purified as previously described (21, 22). To measure the effects of the immunotoxin, a protein synthesis inhibition assay (22) was used to document cell killing. MOPC-21 (Sigma), a murine myeloma antibody (IgG1) without known reactivity with mouse or human tissues, was used as a negative control in our cytotoxicity experiments.

<table>
<thead>
<tr>
<th>Enzyme Treatment</th>
<th>Concentration</th>
<th>OVCAR-3</th>
<th>AGS</th>
<th>HeLa</th>
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<tbody>
<tr>
<td>Exoglycosidase</td>
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<tr>
<td>Neuraminidase</td>
<td>0.2 unit/ml</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>Neuraminidase + β-galactosidase</td>
<td>0.2-0.5 unit/ml</td>
<td>+++</td>
<td>++</td>
<td>+++</td>
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<tr>
<td>Protease</td>
<td></td>
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<tr>
<td>Tryptsin</td>
<td>10 mg/ml</td>
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<tr>
<td>Protease K</td>
<td>0.2 mg/ml</td>
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</tr>
<tr>
<td>Pl-phospholipase C</td>
<td>10 units/ml</td>
<td>-</td>
<td>±</td>
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</table>

* Live cell immunofluorescence was performed as described in "Materials and Methods." The effectiveness of the treatment of OVCAR-3 cells with the exoglycosidases was compared with a control antibody, OVB1. This mouse Mab recognizes a cell surface antigen which is destroyed by the exoglycosidases (Ref. 17). +++, strong fluorescence signal; ++, moderate signal; ±, very weak signal; and -, no detectable signal.

RESULTS

Analysis of the K1 Antigen by Enzymatic Digestion. To determine the nature of the K1 antigen, three K1-positive tumor cell lines were incubated with the enzymes, and immediately stained by using live cell immunofluorescence labeling (Table 1). The reactivity of Mab K1 on HeLa, AGS, and OVCAR-3 cells was completely abolished after digestion with either trypsin or protease K, suggesting that the antigen is associated with a polypeptide or is a polypeptide itself. In contrast, when these cells were treated with neuraminidase alone or neuraminidase and β-galactosidase together, K1 reactivity was retained. In addition, K1 was examined for its reactivity with a panel of 34 neoglycoproteins containing a wide variety of carbohydrate residues (23) and no reactivity was detected. Altogether, these data strongly indicate that Mab K1 recognizes a polypeptide epitope.

Since some membrane proteins are attached by a covalent linkage to glycosyl-phosphatidylidylinositol (24), we treated cells with PI-PLC and found that this abolished the ability of K1 to bind to HeLa or OVCAR-3 cells as shown in Fig. 1. It also substantially decreased the binding of K1 to other K1-positive cells, such as AGS and A1847 cells (data not shown). Whereas the CA125 antigen, which is also present on OVCAR-3 and HeLa cells, was not removed by treatment with PI-PLC, as demonstrated in a double immunofluorescence analysis. As shown in Fig. 1E', fluorescein-labeled OC125 was able to bind to these PI-PLC treated OVCAR-3 cells, whereas rhodamine-labeled K1 was not (Fig. 1E).

The failure to detect reactivity with Mab K1 could also have been due to internalization of CAK1 by endocytosis as a consequence of PI-PLC treatment. This did not appear to be the case, because cells permeabilized with saponin after fixation, which allows antibodies to react with internal antigens (20), did not show a fluorescence pattern consistent with endocytic vesicles after cells had been incubated with PI-PLC at 37°C (data not shown). To determine whether CAK1 release was a consequence of or resulted in cell death, PI-PLC-treated cells were monitored for their viability by staining with 0.4% trypan blue. Over 99% of the PI-PLC-treated cells were viable. In addition, the PI-PLC-treated cells could be recultured successfully, and reexpression of normal amounts of CAK1 on the surface was detected by using immunofluorescence after culture for 16 h (data not shown).

3 J. Magnani, personal communication.
Evidence That CAK1 Is Released by PI-PLC Treatment. Proteins that are released by PI-PLC treatment can usually be recovered intact. Therefore, we attempted to measure the presence of CAK1 by a radioimmunoassay. To do this, wells containing OVCAR-3 cells were exposed to $^{125}$I-K1 in the presence of increasing amounts of supernatant from PI-PLC-treated OVCAR-3 cells. As shown in Fig. 2, competition for binding of $^{125}$I-K1 was clearly evident. No competition occurred when supernatants from A431 cells (CAK1-negative cells) were used, or when supernatant from OVCAR-3 cells not treated with PI-PLC was used. A similar assay was set up in which $^{125}$I-OC125 binding to OVCAR-3 cells was studied to further verify if PI-PLC treatment also released CA125. As also shown in Fig. 2, supernatants from both untreated and PI-PLC-treated OVCAR-3 cells displaced the binding of $^{125}$I-OC125. PI-PLC-treated supernatant appeared to be less active, probably due to the smaller amount of CA125 antigen shed from the cells during the 2-h incubation with PI-PLC, compared to the amount contained in a harvest from a 2-day culture in regular medium. This experiment again showed, in agreement with previous data (25), that CA125 is a shed antigen.

The molecular mass of the CAK1 antigen on OVCAR-3 and HeLa cells released into the supernatant after PI-PLC treatment was revealed in Western blots using Mab K1, following 12.5% SDS-PAGE. As shown in Fig. 3, a band with an $M_r$ of about 40 kDa was detected from both cell lines. This band was specific for Mab K1 and did not react with Mab MOPC-21. Mab OC125 did not react with the 40 kDa band but, as expected, reacted with a band with a very high molecular weight.

Fig. 1. K1 cell surface immunofluorescence labeling. Immunofluorescence surface labeling was performed before and after PI-PLC treatment as described in "Material and Methods." A and B show the appearances of K1 (A) and OC125 (B) by using indirect immunofluorescence without PI-PLC treatment of OVCAR-3 cells. C, D, E, and $E'$ show the appearance of such cells after PI-PLC treatment by using antibody K1 (C, E) or OC125 (D, $E'$), using either indirect immunofluorescence (C, D) or double-label (E, $E'$) immunofluorescence. $A'$, $B'$, $C'$, $D'$ and $E'$ show phase contrast images of A-E. x 630; bar, 6 $\mu$m.

Fig. 2. Quantitative antigen competition radioimmunoassays. Aliquots of serially diluted supernatants from phosphatidylinositol phospholipase C (M)-treated OVCAR-3 cells were assayed for CAK1 and CA125 content by a live cell competition radioimmunoassay as described in "Materials and Methods." Supernatants from a regular culture of OVCAR-3 (C) and A431 (G) were also used as a positive and negative controls, respectively.
CHARACTERIZATION OF CAK1 TUMOR ANTIGEN

Immunotoxins Composed of Mab K1 and PE. To determine if K1 could be effectively used to deliver toxins to cancer cells bearing the CAK1 antigen, Mab K1 was coupled to native Pseudomonas exotoxin and LysPE40, a mutant form of PE that does not bind to the PE receptor which is present on most types of cells (22, 27). As shown in Table 2 and Fig. 5, K1-PE was very toxic to A1847, AGS, and SCC-4 cells with 50% inhibitory dose of about 0.1 ng/ml, and moderately toxic to OVCAR-3, KB, and HBT103 cells. The cytotoxic effect on these K1-positive cells was specific, since it was blocked by the presence of excess Mab K1 at 50 µg/ml (Fig. 5). Whereas MOPC21-PE, an immunotoxin made of murine IgG1 that does not react with human cells, had minimal cytotoxic activity to the tested cells (5% inhibitory dose > 400 ng/ml). Although no detectable signals have been observed in SCC25 and A431 cells by using indirect immunofluorescence labeling with Mab K1, both cell lines showed low level of sensitivity to K1-PE. This is very likely due to the limited numbers of CAK1 sites on the surface of these cells that was below the detection limit of immunofluorescence (usually <1000 sites/cell). In contrast, K1-LysPE40 was not toxic to these cells (Fig. 5). The explanation for the finding that K1-PE is cytotoxic and K1-LysPE40 is not, lies in the fact that when native PE is present in an immunotoxin, binding of domain I of PE promotes internalization of the immunotoxin antigen complex. However, when domain I is absent, as in LysPE40, the immunotoxin is internalized to the same degree as unconjugated antibody (22, 27).

DISCUSSION

In this article we have shown that CAK1 is a protease-sensitive, glycosidase-insensitive antigen that is present on the surface of OVCAR-3 cells and several other cultured cells; the CAK1 antigen is not shed in significant amounts into the culture medium. We have also shown that PI-phospholipase C treatment of such cells removes the CAK1 antigen from the cell surface, and that this antigen can be recovered in the supernatant after PI-PLC treatment, concentrated, and detected on SDS-PAGE immunoblots as a ~40 kDa band.

We have previously shown that CAK1 is expressed only in a limited number of normal tissue sites, most prominently in the mesothelia of the peritoneum, pleura, and pericardium (18). K1 reacts with this antigen in both human and monkey tissues, suggesting that this antigen may be a conserved molecule. In addition, CAK1 has been found in a placental membrane that is probably the amnion. These distributions suggest that the expression of CAK1 is under very precise regulation. As a result, the presence of this antigen in ovarian tumors and in some squamous tumors of esophagus and cervix may provide a unique target for directed immunotherapy.

The tissue distribution of CAK1 and CA125 (3) is remarkably similar, although there are notable differences as have been previously shown (18). Both CAK1 and CA125 are protease-sensitive antigens on the cell surface of OVCAR-3 cells. As shown previously (18), however, there are many cultured cell lines which express these two antigens independently, and tumors show many differences in their expression of these two antigens. The CA125 antigen is shed into the supernatant of cultured cells and can be found in the blood of patients with ovarian cancer, a property that CAK1 does not show (18, 25). Further, as shown in this paper, CAK1 requires PI-phospholipase C treatment for its release from the cell surface, whereas CA125 does not. When examined by SDS-PAGE and immunoblotting, CAK1 is found to be a ~40 kDa polypeptide,
whereas the CA125 antigen is found in a high molecular weight complex >200,000.

When K1 antibody is complexed on the cell surface with CAK1, the complex is not well internalized by the cell and remains on the cell surface. However, immunoconjugates made from K1 and whole PE are selectively toxic to target cells in culture, because domain I of PE (missing in LysPE40) promotes the internalization of such conjugates. These results indicate that Mab K1 may be useful as an antitumor agent when coupled to native PE. On the other hand, the lack of spontaneous endocytosis of the K1-CAK1 complex implies that Mab K1 may be useful as a therapeutic agent in those settings in which internalization might not be required for activity, such as with

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<th>Table 2</th>
<th>Cytotoxic effects of K1-PE on Several Human Cancer Cell Lines</th>
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<td>Cell lines</td>
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<tr>
<td>A431</td>
<td>Epidermoid</td>
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*ID₅₀, concentration that reduces the protein synthesis of treated cells to 50% compared to that of untreated control cells.

*Negative with Mab K1 as tested using immunofluorescence.
radiotherapeutic agents or enzymes that convert produgs into toxic agents (28), or for antibody-directed cell killing. In addition, Mab K1 may be useful for radioimaging of human tumors, or immunohistopathological diagnosis and differentiation of tumors, using immunohistochemical methods.

REFERENCES