Reactivity of Monoclonal Antibody DF3 with a High Molecular Weight Antigen Expressed in Human Ovarian Carcinomas

Eliot L. Friedman, Daniel F. Hayes, and Donald W. Kufe

ABSTRACT

We have previously described the monoclonal antibody (MAb) DF3, prepared against a human breast carcinoma. MAb DF3 reacts with a high molecular weight glycoprotein detectable in human breast carcinomas and in human milk. Previous studies have demonstrated that DF3 antigen levels are elevated in the plasma of patients with breast and ovarian cancer. The present study has further examined the reactivity of MAb DF3 with human ovarian carcinomas. Immunoperoxidase staining demonstrated reactivity of MAB DF3 with 95% of benign, borderline, and malignant tumors (serous, mucinous, and endometroid) of the ovary. Furthermore, malignant tumors contained cytoplasmic DF3 antigen while benign tumors expressed the antigen only on apical surfaces. Western blot analyses demonstrated that the MAb DF3 reactive ovarian antigen (DF3-O) was a glycoprotein with a heterogeneous molecular weight ranging between 300,000 and 450,000. This antigen was detectable by immunofluorescence on the cell surface of five of six cultured human ovarian carcinoma cell lines. The extent of cell surface reactivity with MAb DF3 was equivalent to or greater than that obtained with MAb OC125, an antibody generated against coelomic epithelium and developmental antigen. Furthermore, uptake of 125I-labeled MAb DF3 by human ovarian carcinoma xenografts in athymic mice was 5.4- and 6.2-fold higher than the respective uptake noted in liver and control tumor (P = 0.031). These findings suggest that DF3-O antigen is similar if not identical to the antigen detected in human breast carcinomas by MAb DF3. Thus, MAb DF3 may be a useful reagent in immunodiagnostic evaluation of patients with ovarian cancer.

INTRODUCTION

We have previously described MAb-designated DF3, which was prepared against a membrane-enriched fraction of human breast carcinoma (1). The breast cancer-associated antigen with which MAb DF3 reacts has been designated DF3 antigen. DF3 antigen is a component of the human milk fat globule membrane and has a heterogeneous molecular weight of 300,000–450,000 (2). Detailed biochemical analysis has demonstrated that the DF3 antigen is a glycoprotein with the MAb DF3 binding site involving the glycosidic linkage between carbohydrate and protein moieties. Immunoperoxidase staining has demonstrated the presence of DF3 antigen in apical borders of secretory mammary epithelial cells and in the cytosol of less well-differentiated malignant cells (1). DF3 antigen expression has been shown to correlate with clinicopathological parameters related to degree of breast tumor differentiation and estrogen receptor status (3). These findings suggest that MAb DF3 reacts with a differentiation antigen present in both human milk and breast carcinoma cells (4).

A double-determinant EIA has been developed in our laboratory and used to monitor plasma levels of DF3 antigen (5). We detected elevated levels of circulating DF3 antigen in 42 of 58 patients with breast cancer and in only 6 of 111 age-matched controls without cancer (5). In contrast, elevated circulating DF3 EIA levels were not observed in patients with esophageal, gastric, or colorectal cancer. However, 20 of 43 patients with ovarian cancer had elevations of an antigen reactive with MAb DF3 (5). A subsequent study demonstrated that the circulating ovarian cancer-associated antigen DF3-O is also heterogeneous with molecular weights ranging between 300,000 and 450,000 (6).

In the present study, we have demonstrated by immunoperoxidase staining the presence of DF3-O antigen in ovarian carcinomas. Analysis by Western blot and fluorescent flow cytometry has further characterized the molecular weight, heterogeneity, and cell surface expression of the DF3-O antigen. Finally, the ability to specifically localize 125I-MAb DF3 in ovarian carcinoma xenografts in nude mice suggests that MAb DF3 may be a useful reagent for radioimmunodiagnosis of patients with epithelial ovarian carcinomas.

MATERIALS AND METHODS

Preparation of MAb DF3. MAb DF3 was produced by the immunization of BALB/c mice with a partially purified membrane-enriched fraction of human breast carcinoma metastatic to liver (1). The antibody was purified from ascites by Protein-A-Sepharose-CL-4B chromatography (Pharmacia Fine Chemicals, Piscataway, NJ) (5).

Immunoperoxidase Studies. Two-μm sections of frozen tissue were stained by a modification (7, 8) of the avidin-biotin-peroxidase complex (9) (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA). Histopathological grading of all biopsy specimens was performed and specimens were classified as either benign or malignant. All malignancies were further graded as borderline (Grade 1), well-differentiated adenocarcinoma (Grade 2), or poorly differentiated adenocarcinoma (Grade 3).

Cell Lines. Six human ovarian cell lines were grown in media comprising of Dulbecco’s modified Eagle’s medium (GIBCO Laboratories, Chagrin Falls, OH) with 10% fetal bovine serum, 1% penicillin/streptomycin, 1% penicillin/streptomycin, 1% nonessential amino acids, and 1% sodium pyruvate. Each line exhibited a distinctive epithelial morphology, karyotype, and pattern of growth in vivo (10). Breast carcinoma cell line ZR-75-1 (11) was grown in RPMI 1640 medium (GIBCO) with 10% fetal bovine serum, 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 1% penicillin/streptomycin. The cell lines were maintained in logarithmic growth phase by monolayer cultures and disrupted with trypsin and 0.02% EDTA (4).

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using a 3–15% gradient gel in a modified Laemmli buffer system (12). Ovarian and breast carcinoma tissue culture cell lines were harvested and resuspended in protease inhibitory solution composed of phosphate-buffered saline (pH 7.1), phenylmethylsulfonyl fluoride (0.2 mm), and aprotinin (0.015 trypsin inhibitor units/ml). Cell suspensions were sonicated and 5 μg protein was applied to the gradient gel and electrophoresed at 160 V for 15 h. Additionally, tissue culture supernatant from each cell line was analyzed simultaneously on the gel. Protein measurement of each sample was determined using the Bio-Rad protein assay. (Bio-Rad Laboratories, Richmond, CA).
Monoclonal Antibody DF3 in Ovarian Cancer

Immunoblotting. Transfer of the DF3-O and DF3 antigens to nitrocellulose paper was performed by the method of Towbin et al. (13). Nonspecific binding sites on the nitrocellulose paper were blocked by incubation with 5% bovine serum albumin in phosphate-buffered saline for 1 h at room temperature. The nitrocellulose paper was then incubated with MAb DF3 (0.25 μg/ml) for 2 h, rabbit anti-mouse IgG at a dilution of 1:500 for 1 h, and then 125I-Protein A at 5 × 105 cpm/ml for 2 h. The blots were washed 5 times, dried, and exposed to Kodak XAR-5 film.

Indirect Immunofluorescent Cell Sorter Analysis of Cell Lines with Monoclonal Antibodies. Cultured cells (1 × 10⁶) were washed extensively and incubated with 0.1 ml of a 1:20 dilution (final concentration, 50 μg/ml) of MAb DF3, MAb OC125 (14), or MAb 287 (an isotype-identical nonreactive control antibody). Cells were incubated at 4°C for 60 min and then washed twice. Cells were then reacted with 0.1 ml of a 1:100 dilution of goat anti-mouse fluorescein isothiocyanate (Tago, Inc., Burlingame, CA), incubated at 4°C for 30 min, washed 3 times, and analyzed on a dual-beam fluorescence-activated cell sorter (Coulter, Hialeah, FL). Intensity of fluorescence was determined for 10,000 cells in each population and was compared with the fluorescence of the nonreactive immunoglobulin of the same isotype.

Growth of Human Tumor Xenografts in Athymic Mice. Athymic (Swiss nude) mice (aged 8–10 weeks) were implanted simultaneously with 2–3 × 10⁶ OVCAR-3 human ovarian carcinoma cells in one flank and 0.3 ml of 1–2 mm³ pieces of HPB-ALL (a human T-cell lymphoma cell line that does not react with MAb DF3) in the other flank. Tumors grew for approximately 3 weeks, achieving mean sizes of 0.5–1.0 cm³ for OVCAR-3 and 1–2 cm³ for HPB-ALL.

Iodination of MAb DF3. MAb DF3 was iodinated by the iodogen technique as described previously (15). MAb DF3 (200 μg) was reacted with 1.4 μCi of iodine-125. Free iodine-125 was separated by filtration using a G-25 Sephadex column (Pharmacia). Final specific activity of 0.5 μCi/μg of protein was obtained.

Tumor Distribution Studies. The athymic mice bearing the two human tumor xenografts were injected with 10 μg of 125I-MAb DF3. Mice were sacrificed and dissected at 24, 72, and 96 h. Radioactivity of each tissue was determined using a Minigam counter (LKB/Wallac, Turku, Finland). The amount of tissue (cpm/g) was calculated for each organ.

Statistical Determinations. The difference between cpm/g of OVCAR-3 and cpm/g of other organs was analyzed using the Wilcoxon signed-rank test for paired data (16).

RESULTS

Immunoperoxidase Staining of Ovarian Neoplasms and Benign Ovarian Tissue. MAb DF3 reactivity with benign tissue specimens, borderline ovarian tumors, and malignant tumors was evaluated by immunoperoxidase staining of frozen tissues. Of the 43 patients studied, all but 2 had positive staining. MAb DF3 reacted with all histologies, regardless of grade, including serous, mucinous, endometrioid, clear cell, and undifferentiated adenocarcinomas. Additionally, 2 samples of normal ovary reacted with MAb DF3. Furthermore, 9 of 11 (82%) benign borderline tumors demonstrated positive immunoperoxidase staining. Of these, one benign mucinous tumor and one borderline mucinous tumor failed to stain with MAb DF3. All 31 true epithelial ovarian malignancies (Grades II and III) demonstrated reactivity with MAb DF3.

Of the 7 patients with mucinous tumors, 3 (43%) demonstrated apical staining alone and 2 (28%) demonstrated both apical and cytoplasmic staining (Fig. 3C). In contrast, only 2 of 17 (12%) malignant tumors demonstrated apical staining alone and 15 (88%) displayed cytoplasmic staining with or without apical staining (Fig. 3D). Of these 15 tumors, 4 (27%) had a considerable number of cells that demonstrated cytoplasmic reactivity (Fig. 3D).

Of the 7 patients with mucinous tumors, 3 (43%) demonstrated apical staining alone and 2 (28%) demonstrated both apical and cytoplasmic staining (Fig. 4A). Of the 5 patients with an endometrioid histology, 2 (40%) demonstrated apical staining only and 3 (60%) displayed cytoplasmic reactivity with MAb DF3 with or without apical staining (Fig. 4B). Likewise, MAb DF3 reacted with other histological subclasses of epithelial ovarian malignancies, including clear cell, mixed clear cell, and endometrioid, mixed serous, and mucinous and undifferentiated adenocarcinoma. However, no clear pattern of staining predominated (Fig. 4C). All 6 tumors demonstrated apical staining with 4 of the 6 (67%) additionally demonstrating cytoplasmic staining. Neither the intensity nor pattern of the staining correlated with the histological type of malignancy or

Fig. 1. Reactivity of MAb DF3 with frozen tissue sections from normal ovary and malignant tumors. A, × 40; B, × 60.
grade of malignancy. The patterns of staining are summarized in Table 1.

Tissue obtained from multiple sites from each of 11 patients was stained with MAb DF3 to examine tumor heterogeneity. Five of the 11 patients (45%) demonstrated identical staining patterns in the primary ovarian tumor and either the contralateral ovarian tumor or distant metastases. The other 6 patients (55%) demonstrated distinct staining of primary tumors and metastases or contralateral ovarian tumors (Fig. 5).

In addition, specimens of normal gynecological tissue and other gynecological malignancies were examined for reactivity with MAb DF3. Reactivity was demonstrated in tissue from one uterus and 2 endocervices but was not present in any of the 3 exocervices. Reactivity, when noted, was restricted to the glandular epithelium. Two specimens of cancer in situ of the cervix exhibited cytoplasmic staining patterns with MAB DF3. While all 7 specimens of adenocarcinoma of the uterus demonstrated apical reactivity with MAB DF3, 5 (71%) exhibited a cytoplasmic staining pattern.

Western Blot Analysis. Cell extracts of 5 human ovarian cancer cell lines and one human breast cancer cell line (ZR-75-1), as well as the tissue culture supernatants of each cell line, were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and monitored for reactivity with MAB DF3 (Fig. 6). Western blot analysis of a ZR-75-1 cell extract (Lane 1) demonstrated 2 antigens with distinct molecular weights of approximately 300,000 and 450,000. Both antigens were shed into the tissue culture supernatant as illustrated in the second lane (s/n ZR-75-1). Expression of the MAB DF3 cross-reactive antigen in 5 ovarian cell line extracts (OV-A, OV-D, OV-M, OV-CAR-3, and OV-S) was heterogeneous. The relative quantity of DF3-O antigen expressed per 5 μg extract was different for each cell line, as reflected by the relative intensity of the bands on the autoradiograph (Fig. 6). Two of the ovarian carcinoma cell lines expressed little or no DF3-O (OV-M, OV-S). In those cell lines that did contain DF3-O, 2 distinct molecular weight antigenic species were demonstrated. These 2 molecular weight moieties correlated with those observed in the ZR-75-1 breast cancer cell line.

Table 1 Pattern of immunoperoxidase reactivity of human ovarian tumors

<table>
<thead>
<tr>
<th>Histology</th>
<th>Number of specimens</th>
<th>Number reactive with MAB DF3</th>
<th>Number with apical staining pattern</th>
<th>Number with cytoplasmic staining pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal ovary</td>
<td>2</td>
<td>2 (100)</td>
<td>2 (100)</td>
<td>0</td>
</tr>
<tr>
<td>Ovarian tumor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign</td>
<td>2</td>
<td>1 (50)</td>
<td>1 (50)</td>
<td>0</td>
</tr>
<tr>
<td>Borderline</td>
<td>8</td>
<td>7 (88)</td>
<td>7 (88)</td>
<td>3 (43)</td>
</tr>
<tr>
<td>Malignant</td>
<td>31</td>
<td>31 (100)</td>
<td>29 (94)</td>
<td>22 (71)</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, percentage.
cells. However, 2 of the ovarian carcinoma cell lines (OV-A, OVCAR-3) expressed only the lower molecular weight antigen of approximately 300,000, while a third cell line (OV-D) expressed only the species with a higher molecular weight of 450,000.

Shedding of the DF3-O antigen into the tissue culture supernatant was also heterogeneous (Fig. 6, lanes s/n). The presence of DF3-O in tissue culture supernatant was demonstrated by Western blotting for cell line OV-D. However, DF3-O was not found in the tissue culture supernatants of either OV-A or OVCAR-3, despite detection of the antigen in the cell extracts.

Cell Membrane Reactivities of MAb DF3. Antigens MAb DF3, MAb OC125, and MAb 287 were analyzed for surface binding to a panel of human breast and ovarian carcinoma cell lines. The fluorescence histogram patterns are illustrated in Fig. 7. Whereas MAb DF3 bound strongly to the human breast carcinoma cell line ZR-75-1, binding of MAb OC125 was identical to that of the control (Fig. 7A). The cell surface expression of the DF3-O antigen was heterogenous in these 6 ovarian cell lines, consistent with the findings obtained by Western blot analysis. However, the binding of MAb DF3 to 5 of the ovarian cell lines was equivalent or greater than MAb OC125 (Fig. 7A). None of the 3 monoclonal antibodies demonstrated significant reactivity with a sixth ovarian carcinoma cell line (Fig. 7G). Trypsinization of the monolayer cells did not affect their cell surface reactivity with MAb DF3.

Tumor Distribution Studies. Athymic mice bearing OVCAR-3 and HPB-ALL cell lines were given injections of 10 /µg of DF3 IgG labeled with iodine-125 to a specific activity of 0.5 µCi/µg. The amount of radioactivity per g in the OVCAR was compared to that of various tissues and control tumor over a 4-day period (Fig. 8). The cpm/g value for OVCAR-3 increased from 5.3 x 10⁴ at 24 h to 8.8 x 10⁵ at 72 h and decreased to 7.3 x 10⁵ at 96 h (Table 2). Conversely, the mean cpm/g value of liver decreased from 3.9 x 10⁵ at 24 h to 1.6 x 10⁵ at 72 h and to 1.4 x 10⁵ at 96 h. A similar trend was noted in the HPB-ALL control tumor. The blood pool remained stable at approximately 5.0 x 10⁴ cpm/g of tissue throughout the experimental period (Fig. 8). The difference between cpm/g of OVCAR-3 and cpm/g of liver and control tumor at 72 and 96 h was significant (P = 0.031).

At 24 h, the ratio of cpm/g of OVCAR-3 to cpm/g of liver and control tumor was 1.4 and 2.3, respectively (Table 2). At 72 h the ratio of cpm/g in OVCAR-3 to liver and control tumor was 5.4 and 6.2, respectively (Table 2). At 96 h this ratio was 5.2 with respect to both liver and control tumor. The ratio of cpm/g of OVCAR-3 to blood pool at 72 and 96 h was 1.3 and 1.4, respectively.

DISCUSSION

Several monoclonal antibodies have demonstrated varying patterns of immunological reactivity with gynecological malignancies. MAb OC125 reacts with both serous and endometrioid adenocarcinomas but lacks reactivity with mucinous adenocarcinomas (17). Conversely, MAb OCA and OCAA react with mucinous adenocarcinomas but lack reactivity with serous and endometrioid adenocarcinomas (18). In contrast, monoclonal antibody F36/22, generated against a human breast carcinoma, cross-reacts with 100% of human ovarian carcinoma, regardless of histology (19).
In the present study, immunoperoxidase staining has demonstrated reactivity of MAb DF3 with an antigen present in serous, endometrioid, mucinous, clear cell, and undifferentiated epithelial ovarian carcinomas. A pattern of reactivity was noted in the tumors that reacted with MAb DF3: Although only a few samples were examined, benign tumors and specimens of normal ovaries demonstrated no cytoplasmic staining. However, 72% of malignant tumors demonstrated cytoplasmic staining. Tumors with borderline histology had a smaller percentage of cytoplasmic staining (50%) commensurate with their lower histological grade. A similar distinction between benign and malignant breast tumors has been described (1). While apical staining is present in 100% of 13 benign breast lesions, cytoplasmic staining is present in only one of 13 (8%). In contrast, 3 of 20 patients (15%) with nonovarian gynecological malignancies, and only 4 of 59 control women (7%) have elevated circulating DF3-O antigen (6). In the present study, only the MAb DF3-reactive antigen of molecular weight 450,000 was detectable in the tissue culture supernatant from a single cell line. In contrast, our prior study has shown that serum from patients with epithelial ovarian carcinoma may contain MAb DF3-reactive antigens of molecular weight ranging 300,000–450,000 (6). Thus, the finding of DF3-O antigens in plasma could result from cell surface shedding or from lysis of tumor cells. Regardless of the mechanism, these antigens circulate at higher levels in patients with ovarian cancer as compared to normal women.

In the present study, we monitored ovarian carcinoma cell lines for cell surface expression of DF3-O antigen. Five of the 6 ovarian carcinoma cell lines demonstrated cell surface reactivity with MAb DF3. The intensity of immunofluorescence staining paralleled the amount of DF3-O antigen detected in these cells by transblot analysis. More importantly, in each case reactivity with MAb DF3 was equivalent to or more intense than that obtained with MAb OC125. These findings would suggest that DF3-O antigen is expressed to an equal or greater extent than CA-125 on the surface of ovarian carcinoma cells. Furthermore, expression of the two antigens by ovarian carcinoma cells may be complementary.

Radioimaging studies of human ovarian carcinoma have been performed with limited success using other monoclonal antibodies, including those generated against human milk fat globule membranes (20, 21), placental alkaline phosphatase (22, 23), and osteogenic sarcomas (24). The intense cell surface expression of DF3-O antigen on the surface of ovarian carcinoma cells suggested that MAb DF3 might be useful for radioimaging. Distribution studies were thus performed with 125I-labeled MAb DF3 in nude mice bearing the OVCAR-3 ovarian carcinoma xenograft. Radiolabeled MAb DF3 preferentially accumulated in the OVCAR-3 tumor as compared to liver, blood pool, and a control tumor. Thus, the demonstration of cell surface DF3-O antigen expression and selective uptake of radiolabeled MAb DF3 in ovarian carcinoma xenografts in nude mice indicates that MAb DF3 may also be useful in the radioimmunodetection of this disease.

Finally, we have recently demonstrated that the DF3 antigen detectable in human breast carcinomas is a high molecular weight glycoprotein (2). Further studies will be necessary to determine whether the DF3-O antigen is identical or simply related to the DF3 antigen in breast carcinoma. Preliminary characterization has demonstrated that the DF3-O antigen is
periodic acid-Schiff positive, thus confirming the presence of a carbohydrate moiety.

Other high molecular weight glycoproteins have been described as tumor-associated antigens. The DF3-O antigen is distinct from CA-125 as reflected in differences in their molecular weights, as well as the complementary nature of reactivity with sera from patients with ovarian cancer (6). An antigen defined by MAB MOV2 appears to be a high molecular weight glycoprotein present in secreted mucus of a variety of malignancies (25, 26). MAB MOV2 exhibits a different pattern of cross reactivity than MAB DF3. MAB HMFG1 recognizes a high molecular weight component of human milk and breast carcinoma cell lines which may be similar to DF3-O (27). However, the reactivity of MAB HMFG1 with colon carcinoma (20) clearly distinguishes this MAB from MAB DF3 (1).

Recent studies have suggested that several MAB-defined, high molecular weight, mucin-like glycoproteins may represent a family of tumor-associated antigens.4 For example, the cell membrane determinants defined by MAB Ca1, designated the CA antigen, are two major glycoproteins with estimated molecular weights of approximately 350,000 and 390,000 (28, 29). These antigens are structurally similar to the DF3 antigen in that most of the carbohydrate is O-glycosidically linked to a polypeptide. However, the CA antigen has been detected in a wider range of human tumors (28, 29) than the DF3 antigen (1). Furthermore, the MABs CA1 and DF3 clearly react with different epitopes on these antigens as determined by solid-phase inhibition assays.5 The reactive determinants defined by MABs F36/22 (19) and 115-D8 (30) also reside on the same high molecular weight glycoproteins detectable in human breast carcinomas and human milk, as demonstrated by double-determinant radioimmunoassays and immunoblot analysis. However, these MABs are also distinguished from MAB DF3 by reactivity patterns in fixed tissues and solid-phase inhibition assays. The purification and further identification of each of these components will be necessary to determine the extent of their biochemical and immunological similarities.

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4 M. Abe and D. W. Kufe. Identification of a distinct family of high molecular weight tumor-associated glycoproteins, submitted for publication.

5 MABs CA1 and DF3 clearly react with different epitopes on these antigens as determined by solid-phase inhibition assays.
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