Coexistence of Autologous Antibodies and Decay-accelerating Factor, an Inhibitor of Complement, on Human Renal Tumor Cells

Toshiro Terachi, Gabriel Stanescu, J. Edson Pontes, M. Edward Medof, and Michael J. Caulfield

Section of Immunology, Research Institute of the Cleveland Clinic [T. T., M. J. C.] and the Department of Urology, Cleveland Clinic Foundation [T. T., J. E. P.] Cleveland, Ohio 44195; and the Institute of Pathology, Case Western Reserve University [M. E. M.], Cleveland, Ohio 44106

ABSTRACT

Autologous immunoglobulin was detected on the cell surface of tumor cells freshly isolated from cancerous kidneys of patients with renal cell carcinoma by flow cytometry after staining with murine anti-human IgG monoclonal antibodies. Cells isolated in parallel from macroscopically normal regions of the tumorous kidneys were not specifically stained with the anti-human IgG reagents. In further studies, tumor cells were stained with an antibody to decay-accelerating factor (DAF), a known inhibitor of complement. Flow cytometry of these cells revealed that nearly all tumor cells expressed DAF, and that the intensity of staining with the anti-DAF monoclonal antibody correlated with the staining of cells with anti-IgG. The results suggest that tumor cells coated with autologous antibody may be resistant to complement-mediated cytotoxicity in vivo through the expression of high levels of DAF.

INTRODUCTION

There is a body of evidence suggesting that cancer patients make an antibody response against their own tumors (1-6). Absorption studies reveal that the sera from such patients may contain antibodies that react specifically with autologous tumor cells, although antibodies that cross-react with normal tissues are more commonly detected (4-5). Rather than mediating immunity to cancer, however, antibodies have been associated with enhancement of tumor growth (7, 8). Several mechanisms have been proposed to account for this phenomenon, the simplest being that antibodies facilitate tumor growth by masking neoantigens thereby protecting the tumor cells from cell-mediated attack (9). A second possibility is a blockade of the effector arm of the immune response in which antigen/antibody complexes dissociated from tumor cells saturate Fc receptors on natural killer cells and activated macrophages thereby preventing them from attacking tumor cells (10). These studies provide possible mechanisms for the escape of tumor cells from cell-mediated immunity; however, they do not explain why anti-tumor antibodies do not effect complement-mediated cytotoxicity of cancer cells in the first place. DAF is a plasmanylinositol-linked membrane glycoprotein (11, 12) that prevents assembly of C3 and C5 convertases on cell surfaces, thereby protecting host cells from autologous complement attack (13-15). Recently, Cheung et al. (16) showed that DAF expressed on certain cultured human tumor cell lines protects the cells (following in vitro sensitization with a specific monoclonal antibody) from complement-mediated cytotoxicity. The present study was undertaken to examine tumor cells for the presence of autologous IgG bound to their surfaces in vivo, and if present, to determine whether the binding of such antibodies is correlated with the expression of DAF.

MATERIALS AND METHODS

Preparation of Cell Suspensions. Tumorous kidneys removed during surgery were the source of tumor and normal kidney cells. Macroscopically normal tissue adjacent to the primary tumor was used as the source of normal kidney cells. Fresh tissues were minced into small pieces and digested at room temperature overnight in 20 ml of Dulbecco's modified Eagle medium (GIBCO, Grand Island, NY) containing 0.1 unit/ml of collagenase type III (Worthington Biochemicals, Freehold, NJ), 1 ng/ml DNase (Sigma Chemical Co., St. Louis, MO), and 0.1% antibiotic-antimycotic solution (GIBCO). The cell suspension was filtered through a wire mesh, washed twice with Tris-ammonium chloride buffer to lyse erythrocytes (17), and then washed with staining medium [phosphate-buffered saline with 5% bovine serum albumin (HyClone, Logan, UT) and 0.1% sodium azide].

mAbs. A murine IgG1 mAb specific for human IgG was obtained from AMAC, Inc. (Westbrook, ME). The anti-DAF mAb (IIIH6) is a murine IgG1 mAb prepared as described elsewhere (18). AB1-2 and 19 were murine IgG1 mAbs used as an isotype-matched control mAb.

Immunostaining and Flow Cytometry. For staining, 0.5-1.0 million cells were pelleted in 12 x 75-mm tubes and then resuspended in 25 µl of staining medium containing the specific antibody (anti-human IgG or anti-DAF) (80 µg/ml), or with an isotype-matched (IgGl) control murine mAb. After 30-min staining on ice, the cells were washed 3 times and then incubated with 25 µl of 5 µg/ml solution of biotin-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL). The cells were washed 4 times and then incubated with phycoerythrin/Texas Red-conjugated streptavidin (Duochrome, Becton Dickinson, Mountain View, CA). All staining was performed for 30 min at 4°C in foil-wrapped tubes. After staining, the cells were washed 5 times and then fixed with 0.5% paraformaldehyde. Flow cytometry was performed on a FACSscan instrument (Becton Dickinson). An argon-ion laser was used for excitation at 488 nm and a 650 nm long-pass filter was used for detection of fluorescence emitted by Texas Red. Lymphocytes were gated out from tumor cells by both low forward scatter (smaller size) and low 90-degree side scatter (low granularity) (20).

RESULTS

Single cell suspensions were prepared from fresh tumors or the corresponding normal kidney from 13 patients with renal cell carcinoma. For flow cytometry analysis, the cells were stained with murine monoclonal antibodies followed by staining with biotin-labeled goat anti-mouse IgG and then with fluorochrome-labeled streptavidin. Because the tumor cells often had high autofluorescence at the fluorescein isothiocyanate (530 nm peak) and phycoerythrin (585 nm peak) emission wavelengths, the tumor cells were analyzed for specific staining by using emission by the Texas Red fluorochrome (618 nm peak). Fig. 1 contains three examples of the presence of autologous IgG on tumor cells. As shown in this figure, freshly isolated tumor cells exhibited specific fluorescence when stained with the mAb directed against human IgG. Cells stained with an isotype-matched control mAb had a broad peak of weak fluorescence that was indistinguishable from that of unstained cells. The
presence of membrane-bound IgG on the tumor cells suggests that the patients were producing antibodies reactive with their own tumor, and that these antibodies remained bound to the tumor cells during the digestion with collagenase and DNase. Although IgG was detected on tumor cells from each patient, two peaks were detected indicating that IgG was not bound to all tumor cells. This finding suggests that the renal cell carcinoma tumors may be heterogeneous or that tumor-associated antigens may be shed from the cell surface as in other cancers (21).

Figure 2 contains examples from three additional renal cell carcinoma cases in which freshly isolated cells from macroscopically normal kidney sections adjacent to the primary tumor were used as controls for staining and flow cytometry analysis. As shown in Fig. 2 (Column b) the normal kidney cells were not stained with the anti-human immunoglobulin reagent, whereas the tumor cells (Column a) exhibited strong staining as in Fig. 1. With the recent observation (16) that the expression of DAF on certain tumor cell lines mediates resistance to complement-mediated cytotoxicity in vitro, we decided to look for the coexistence of DAF and autologous IgG on freshly isolated tumor cells. As shown in Fig. 2 (Column c), DAF was expressed uniformly on tumor cells from each patient. Consistent with previous immunochemical studies of normal kidney (22), DAF was also detected on some normal kidney cells, although the fluorescence index was lower than that of tumor cells stained with the anti-DAF mAb.

To determine whether there was a correlation between the level of DAF expression on tumor cells and the level of endogenous IgG binding, the intensity of anti-DAF staining (fluorescence index) was plotted against that of anti-IgG staining of tumor cells. As shown in Fig. 3, there was a strong correlation ($r = 0.95$) between these parameters, suggesting that DAF may protect cancer cells from complement-mediated cytotoxicity in vivo.

In one tumor specimen with particularly low autofluorescence, we were able to compare the intensity of staining of DAF on tumor cells versus that of tumor-infiltrating lymphocytes. The tumor cells could readily be separated from tumor-infiltrating lymphocytes by size (forward scatter) and granularity (side scatter). A flow cytometry histogram of un gated cells from this experiment is shown in Fig. 4. It is evident from this analysis that anti-DAF antibodies stained two populations of cells present within the tumor bed. This histogram shows two peaks of fluorescence following staining with the anti-DAF mAb (Fig. 4a). Gating on cells with low forward and side scatter (Fig. 4b) revealed that the resulting population, which consists primarily of lymphocytes, comprised the first peak in the un gated histogram. Conversely, gating on cells with higher forward and side scatter (Fig. 4c) selected for tumor cells which were present in the second peak. These results suggest that tumor cells may possess a higher concentration of DAF molecules on their surface than do lymphocytes. This finding may have physiological relevance since lymphocytes have been shown to express relatively high concentrations of DAF (18).

**DISCUSSION**

The results from this study indicate that tumor cells freshly isolated from primary renal cell carcinomas are coated with autologous IgG antibodies. (Proteolytic enzymes were not used in the digestion of the tumor and normal tissues in order to preserve the bound antibodies.) It should be emphasized that the cells were not further treated with autologous or heterologous serum following their isolation and that the autologous antibodies remained bound to the cells throughout the isolation procedure. Thus, the staining with the anti-human IgG mAb detected antibodies that were bound to the tumors in vivo. Examination of stained cells by UV microscopy confirmed that the fluorescence was due to specific staining of the cell membrane, whereas background staining resulted from fluorescence of intracellular bodies probably containing lipids.

Whether the autologous antibodies detected on the surface of tumors cells are binding to tumor-associated antigens remains to be determined; however, it is clear that cells isolated from normal tissue taken from the same kidneys as the primary tumors were not stained for the presence of human IgG. This suggests that the autologous antibodies are in some way specific for the tumor cells; however, an alternative possibility is that the autologous antibodies could be bound to the tumor through Fc receptors. We think this is unlikely for the following reasons: (a) there was no membrane fluorescence when tumor cells were stained for total IgG (Columns a and b) as in Fig. 1. Columns c and d are histograms of the staining of tumor and normal kidney cells with a mouse IgG anti-DAF mAb (thick lines) overlayed onto histograms of cells treated with the isotype control (thin lines).

![Fig. 1. Flow cytometry analysis of three cases of freshly isolated renal tumor cells stained with an anti-IgG mAb. Histograms depicting the relative fluorescence intensity of specifically stained cells (thick lines) are overlayed on histograms of cells incubated with the isotype control antibody (thin lines).](image1)

**Fig. 1.** Flow cytometry analysis of three cases of freshly isolated renal tumor cells stained with an anti-IgG mAb. Histograms depicting the relative fluorescence intensity of specifically stained cells (thick lines) are overlayed on histograms of cells incubated with the isotype control antibody (thin lines).

![Fig. 2. IgG binding and DAF expression on cells from the primary tumor or normal kidney of 3 patients with renal cell carcinoma. Cells were isolated and stained for total IgG (Columns a and b) as in Fig. 1. Columns c and d are histograms of the staining of tumor and normal kidney cells with a mouse IgG anti-DAF mAb (thick lines) overlayed onto histograms of cells treated with the isotype control (thin lines).](image2)

**Fig. 2.** IgG binding and DAF expression on cells from the primary tumor or normal kidney of 3 patients with renal cell carcinoma. Cells were isolated and stained for total IgG (Columns a and b) as in Fig. 1. Columns c and d are histograms of the staining of tumor and normal kidney cells with a mouse IgG anti-DAF mAb (thick lines) overlayed onto histograms of cells treated with the isotype control (thin lines).

![Fig. 3. Correlation between binding of human IgG and expression of DAF on tumor cells. The channel number containing the peak (mode) of cells stained with anti-IgG or anti-DAF was divided by the modal channel number containing cells stained with the isotype control to obtain an index of fluorescence staining. The fluorescence index obtained for anti-IgG staining was plotted against that obtained for anti-DAF staining for the 12 cases of primary renal cell carcinoma (O). (•), data from a case of metastatic renal cell carcinoma. The correlation coefficient for these two parameters was 0.95.](image3)

**Fig. 3.** Correlation between binding of human IgG and expression of DAF on tumor cells. The channel number containing the peak (mode) of cells stained with anti-IgG or anti-DAF was divided by the modal channel number containing cells stained with the isotype control to obtain an index of fluorescence staining. The fluorescence index obtained for anti-IgG staining was plotted against that obtained for anti-DAF staining for the 12 cases of primary renal cell carcinoma (O). (•), data from a case of metastatic renal cell carcinoma. The correlation coefficient for these two parameters was 0.95.
stained with the isotype-matched control antibodies or with fluorescein-labeled goat anti-rabbit or goat anti-rat antibodies (data not shown). (b) Staining of tumor cells with mAbs specific for different human IgG subclasses revealed that IgG4 antibodies were the predominant antibodies bound to the tumors in certain cases (data not shown), and human IgG4 antibodies do not bind to known Fc receptors (23). (c) An established renal carcinoma cell line (24) does not bind human IgG4, although it does express high levels of DAF (data not shown). It should also be mentioned that lymphocytes were gated out of the population that was analyzed by flow cytometry, and that the cells analyzed were of the expected size of tumor cells.

Previously, it was shown (16) that DAF is expressed at low levels on certain tumor cell lines such as neuroblastoma lines, whereas it could be expressed at high levels on other tumors such as breast carcinoma lines. In some tumors such as melanoma, the expression of DAF was variable, being high on some melanoma lines and undetectable on others. Following sensitization with a specific monoclonal antibody, cells expressing low levels of DAF could be killed with the addition of complement, whereas cells expressing high levels of DAF were resistant to complement-mediated lysis (16). In a similar manner, we suggest that tumor cells sensitized in vivo with autologous antibodies could be protected from complement-mediated lysis by expressing high levels of DAF. Indeed, the association of high levels of DAF expression with high levels of autologous IgG binding to tumor cells observed in the present study supports this hypothesis.

The wide distribution of DAF on tumor cells may constitute an impediment to cancer therapy using monoclonal or chimeric antibodies. However, addition of anti-DAF antibodies to sensitized tumor cells expressing high levels of DAF was shown to render the cells sensitive to complement-mediated lysis (16). It will be of interest to determine whether inhibitors of DAF can improve therapy with monoclonal antibodies or enhance autologous immunity to cancer.
Coexistence of Autologous Antibodies and Decay-accelerating Factor, an Inhibitor of Complement, on Human Renal Tumor Cells

Toshiro Terachi, Gabriel Stanescu, J. Edson Pontes, et al.