Growth-associated Shedding of a Tumor Antigen (CE7) Detected by a Monoclonal Antibody

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ABSTRACT

A monoclonal antibody was developed against an antigen, termed CE7, which was highly expressed on the surface of rat fibrosarcoma KMT-17 cells (clone A3) cultured in low serum medium (A3-1% FCS). The CE7 antigen was not detectable on A3 cells cultured in ordinary high serum medium (A3-10% FCS), on in vivo passeed A3 cells, or on parental in vivo KMT-17 cell line. However, immunoelcctron microscopy and Western blot analyses indicated that CE7 antigen was produced by these tumor cells in all circumstances but was shed from their surfaces in vesicular form into the surrounding tissue culture medium or ascites, unless low serum concentration prevailed and disappeared from their cell surfaces. We have previously reported that the immunogenicity of A3 cells was increased when the serum concentration was lowered from 10% to 1% and the phenomenon paralleled the CE7 antigen expression on the A3 cells. These results suggest that the CE7 antigen could be a tumor-associated rejection antigen and that the expression of the CE7 antigen on A3-1% FCS cells (which is shed by high serum culture or in vivo transplantation and disappears from the cell surface) may play a role in immunological responses against the tumor cells.

INTRODUCTION

The surface phenotype of tumor cells varies according to the situation of tumor cells (1-5) or the growth environments (6-13). However, the mechanisms governing these changes are still unknown.

We previously reported (6) that the immunogenicity of a subclone (A3) of KMT-17 fibrosarcoma cell line was enhanced and the tumorigenicity was diminished in a reversible manner when the growth of the tumor cells was suppressed by serum deprivation. While the expression of original TAA, which was detected on KMT-17 cells, did not change in any growth conditions, a unique antigenic determinant(s) was/were detected on the surface of growth-suppressed A3 cells by an antiserum obtained from rat hyperimmunized with growth-suppressed A3 cells, and the enhanced immunogenicity correlated with the expression of the antigen on the growth-suppressed A3 cells. These observations suggest that the in vitro culture condition of tumor cells modifies the surface of tumor cells and that the modification of the antigenic expression influences both the tumorigenicity and the immunogenicity. In this report, we describe a monoclonal antibody against this antigen, termed CE7, and investigate the mechanisms which lead to a change in the expression of the antigen associated with tumor cell growth properties.

MATERIALS AND METHODS

Tumor. KMT-17 is a transplantable fibrosarcoma cell line induced by 3-methylcholanthrene in a WKA rat and maintained in ascites form (14, 15).

Received 2/1/89; revised 4/12/89; accepted 4/19/89.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by a Grant-in-Aid for Cancer Research from the Japanese Ministry of Education, Science and Culture.

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Cell Culture. A cultured KMT-17 cell line (c-KMT-17) was maintained in Eagle's minimal essential medium supplemented with 10% heat-inactivated FCS, sodium pyruvate (1 mm), L-glutamine (2 mm), glycine (0.1 mm), and L-serine (0.25 mm); the cells were incubated at 37 °C in a humidified atmosphere of 5% CO₂ and were directly subjected to cloning in vitro by limiting dilution method. Several clones were isolated from the c-KMT-17 cell line (1). Clone A3 was used in the experiments reported here because it demonstrated the highest level of tumorigenicity and the lowest level of immunogenicity. The A3 cells were found to be negative for Mycoplasma or viral contaminations (6).

Growth Conditions of A3 Cells. In order to suppress the growth of the A3 cells, the FCS concentration in the growth medium was decreased from 10% to 1%. The doubling time was prolonged from 14.9 h to 31.5 h (6). The cells are referred to A3-10% FCS and A3-1% FCS, respectively.

Monoclonal Antibodies. The A3-1% FCS cells were used for immunization to WKA rats. Rats were successively immunized i.d. with 10⁴, 10⁵, and 10⁶ viable cells every week followed by a final i.v. injection of 10⁵ cells 7 days later and 3 days before fusion. Fusion and selection were carried out using a standard technique (16). RPMI 1640 supplemented with 10% FCS and sodium pyruvate (1 mm) was used to grow hybridoma clones. Hybridomas were initially screened by enzyme-linked immunoassortment assay (17). As a result of these screenings, we decided to use anti-CE7 antibody (IgG) for following assays.

Quantitative Immunofluorescence Assay. A3-10% FCS and A3-1% FCS cells were harvested using 1 mm EDTA in PBS, washed 3 times with PBS, and incubated in the hybridoma culture medium containing anti-CE7 antibody. After 1 h, the culture medium was removed by washing twice with PBS. Fluorescein isocyanate conjugated goat antirat IgG (heavy and light chain specific; Cappel Laboratory) was then added to the cells. The mixture was incubated at room temperature for 1 h and washed with PBS. Finally, the cells were analyzed using FACScan (Becton-Dickinson FACScan Systems).

Immunoelectron Microscopic Studies. A3 cells were plated on glass slides and cultured for 1 day in medium containing 1% or 10% FCS, and after washing with PBS or without washing, the plates were fixed with peroxide-lysine-parafomaldehyde fixing solution (0.01 M sodium metaperiodate-0.075 M phosphate buffer-2% paraformaldehyde). Staining was carried out using a Vectastain ABC Kit (Vector Laboratories). The cells were then fixed with 2.5% glutaraldehyde in cacodylate buffer at pH 7.4 for 20 min, washed using cacodylate buffer 3 times, and postfixed with 2% osmium acid for 45 min. After serial dehydration with alcohol from 30 to 100%, the cells were embedded in Epon 812. Ultrathin sections were cut for examination by electron microscopy (HITACHI 800).

Extraction and Preparation of Glycoprotein from A3 Cells by n-Butyl Alcohol or NP-40 Treatment. n-Butyl alcohol extraction was performed using modification of the method of Sato and Kikuchi (18). The glycoprotein was extracted from 10⁷ A3 cells with 1.25% n-butyl alcohol for 15 min providing about 200 μg/ml of protein yield without cellular lysis (data not shown). NP-40 lysis buffer contained 1 mm EDTA, 1 mm phenylmethylsulfonyl fluoride, and 1% (v/v) NP-40 in PBS. A3 cells were washed with PBS and then extracted with 1 ml of lysis buffer per 10⁷ cells. Conditioned media and ascites were also centrifuged at 15,000 rpm and their precipitates were resuspended in the lysis buffer. After sonication, these samples were centrifuged at 100,000 x g for 1 h, and then the supernatants were harvested as samples.

The abbreviations used are: PC'S, fetal calf serum; TAA, tumor-associated antigen; PBS, phosphate-buffered saline; A3-1% FCS, A3 cells cultured in a medium containing 1% FCS; A3-10% FCS, A3 cells cultured in a medium containing 10% FCS; NP-40, Nonidet P-40.
Gel Electrophoresis and Protein Transfer (Western Blotting). SDS-PAGE was performed as described by Laemmli (19). After electrophoresis, the proteins (40 μg/lane) were transferred to nitrocellulose filter (0.22-μm pore size; Millipore Corporation, Bedford, MA) for 90 min at 160 mA in transfer buffer (25 mM Tris 192 mM glycine-20% methanol). The filter was incubated in PBS containing 2% bovine serum albumin overnight at 4°C and then washed 3 times with saline containing 0.025% Tween 20. Fifteen ml of hybridoma supernatant were added, and the mixture was incubated for 2 h at room temperature and washed with saline 3 times. Staining was carried out using a standard technique (20).

RESULTS

Flow Cytometry Analysis of CE7 Antigen Expression. The anti-CE7 monoclonal antibody was used for quantitation of the expression of CE7 antigen. Fig. 1 shows that changing the growth conditions from 10% to 1% serum is accompanied by a time-dependent increase in the expression of the CE7 antigen which becomes maximum at 14 days. Conversely, switching from low to high serum concentration leads to a time-dependent decrease in the expression of the CE7 antigen, with return to low base-line expression at 7 days.

Immunoelectron Microscopic Studies of CE7 Antigen Expression. To clarify the mechanisms of the change in the expression of CE7 antigen, we carried out immunoelectron microscopic analysis (Fig. 2). Anti-CE7 antibody strongly reacted with growth-suppressed A3 cells on the cell surface (Fig. 2a). When the growth of A3 cells was stimulated by high serum medium, large numbers of vesicles were detected around the cell surface which stained strongly with anti-CE7 antibody (Fig. 2b). In Fig. 2c, the growth-stimulated A3 cells were washed with PBS before fixation. The washing decreased the number of vesicles detectable around the cell surface and no anti-CE7 antibody reactivity was observed on the cell surface.

Western Blot Analysis. Using cell extracts and conditioned media of A3-1% FCS or A3-10% FCS cells, it was demonstrated that the vesicles around the growth-stimulated A3 cells contain the "shed" antigen (Fig. 3). Anti-CE7 antibody detected a protein with a molecular weight of about 42,000 in the extracts of growth-suppressed A3 cells (A3-1% FCS; Fig. 3, Lane 6), but did not react with the extracts of growth-stimulated A3 cells (A3-10% FCS; Fig. 3, Lane 7). In contrast, anti-CE7 antibody reacted strongly with the conditioned medium of growth-stimulated A3 cells whereas the conditioned medium of growth-suppressed A3 cells showed no detectable antigen at the same molecular weight (Lanes 8 and 9).

When A3 cells or KMT-17 parent cell line were transplanted i.p., no reactivities of anti-CE7 antibody with these cells were observed (Fig. 4a, Lanes 7 and 8), while anti-CE7 antibody reacted with proteins from ascites of both cells at molecular weights of 75,000, 58,000, 49,000, and 47,000 (Fig. 4b, Lanes...
DISCUSSION

In this report, we have demonstrated that the CE7 antigen is expressed only on the surface of the A3-1% FCS cells, while A3-10% FCS cells or in vivo-transplanted A3 cells produce the CE7 antigen but shed it from the cell surface to the culture medium or ascites in vesicular form associated with their growth stimulation. The change in the expression of the CE7 antigen is reversible with the cell growth condition in a time-dependent manner.

We previously described (6) that the immunogenicity of A3-1% FCS was enhanced relative to A3-10% FCS and we also detected a unique antigen on the cell surface by anti-A3-1% FCS antiserum. The original TAA, which is detected on KMT-17 cells, did not change in any growth conditions. The enhanced immunogenicity of A3-1% FCS was therefore correlated with the unique antigen expression. These phenomena are also associated with the expression of an antigen termed CE7 which was strongly expressed on A3-1% FCS cells. Thus, these observations suggest that the CE7 antigen is selectively shed (but original TAA is not shed) from the cells which are grown under ordinary 10% FCS culture condition or in vivo, but that the shedding of CE7 antigen from the surface of A3 cells grown in low serum media is inhibited leading to higher immunogenicity relative to A3-10% FCS cells.

It is well known that expression of certain tumor antigen varies according to the environment of tumor cells (1-5) and several explanations for these changes have been proposed. Although there are several reports relating changes in antigen expression with growth properties (6-13), the mechanisms governing these changes are still unknown. In this report, we have confirmed the identity of CE7 antigen as a tumor antigen and have demonstrated one mechanism of control of antigen expression in A3 cells. While the CE7 antigen expressed on A3-1% FCS cells may induce high immunogenicity, A3-10% FCS cells which shed CE7 antigen could not induce effective antitumor immunity, indeed the CE7 antigen which is shed from the A3 cells may suppress antitumor immunity and may help immunological escape of the tumor cells. They may thereby support tumor formation as a result.

A number of mechanisms about immunological escape of tumor cells have been described, including: (a) masking of tumor antigen (21); (b) antigenic modulation (22); (c) change in tumor antigen expression by immunological selective pressure; (d) loss of tumor antigen by rapid growing (23) or prolonged cultivation (24); and (e) elimination of tumor antigen by shedding.

The shed antigen or antigen-antibody complex, in particular, may block host immune response. Ladisch et al. (25) have suggested that gangliosides shed by tumor cells are extremely potent enhancers of tumor formation. Recently, Bernhard and Dippold (26) have indicated the possibility that shedding of ganglioside (GD3) may cause a local accumulation at the tumor site with immunosuppressive function. Thus control of tumor antigen shedding may be a potential route to achieving more effective antitumor immunity.

Although there are several reports demonstrating shedding
of tumor antigen (27–33), the mechanisms remain unknown. In situations such as these, however, our observations indicate that a reversal of this antigen shedding might lead to enhanced host immune response and that strategies to control this phenomenon might prove to be of therapeutic benefit.

ACKNOWLEDGMENTS

The authors wish to thank Drs. Jean Viallet and Richard Osborne for valuable discussions and comments on this manuscript.

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ACKNOWLEDGMENTS

The authors wish to thank Drs. Jean Viallet and Richard Osborne for valuable discussions and comments on this manuscript.

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