Inhibition of Tumor Cell Growth in Vitro by Murine Monoclonal Antibodies That Recognize a Proliferation-associated Cell Surface Antigen System in Rats and Humans

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ABSTRACT

The mouse monoclonal antibody (MoAb) B3 raised against a rat bladder cancer cell line and the MoAbs HBJ127 and HBJ98 raised against a human bladder cancer cell line recognize homologous antigens predominantly present on proliferating cells of the corresponding species. Examination of MoAb-defined antigen and epitopes revealed that both HBJ127 and HBJ98 MoAbs defined a human cell surface glycoprotein complex having an apparent molecular weight of 125,000–130,000 which was composed of a heavy subunit of a glycoprotein nature (M, 90,000–95,000) and a disulfide-linked light subunit of protein nature (M, 30,000–35,000), but the HBJ127 and HBJ98 MoAbs recognized a protein epitope and a sugar epitope on the heavy subunit, respectively. Likewise, the B3 MoAb recognized a protein epitope on the heavy subunit of a rat cellular glycoprotein complex of similar composition to the HBJ127/HBJ98-defined human antigen. Addition of the B3 MoAb to rat and the HBJ127 or HBJ98 MoAb to human tumor cells inhibited the nucleic acid synthesis or the proliferation of the tumor cells in vitro in a dose-dependent manner. The target tumor cells exposed to MoAb could regrow when they were freed from the antibody, indicating that the effect of these MoAbs on the tumor cells is cytostatic and reversible. These MoAbs did not cause down-regulation of the cell surface antigen and did not arrest the cell cycle in a certain phase. These observations indicate that the M, 125,000 glycoprotein cell surface component detected in both rat and human systems may play a requisite role for cell proliferation and that our MoAbs could inhibit the function by binding to the functionally proximal region of the component.

INTRODUCTION

In a previous report (1), we communicated that the murine MoAb B3 raised against the BC47 rat bladder cancer cell line recognized a rat cell surface antigen which was distributed in a variety of cultured cell lines, mitogen-activated lymphocytes, and proliferating portions of normal tissues, such as the basal layer of the skin and esophagus, and that the MoAb immunoprecipitated a glycoprotein complex having an apparent molecular weight of 125,000–130,000 and being composed of 2 subunits of molecular weights of 85,000 and 43,000. A similar antigen system (gp125) in humans has been detected with the 2 mouse MoAbs, HBJ127 and HBJ98, raised against the human bladder cancer cell line, T-24 (2). Since the B3 MoAb to rats and the HBJ127 and HBJ98 MoAbs to humans seem to recognize the antigen predominantly expressed on proliferating cells, we have been interested in the role of the MoAb-defined antigen in cell growth. In this light, we examined the effect of these MoAbs on tumor cell growth in vitro. In addition, we characterized antigenic determinants (epitopes) recognized by the HBJ127 and HBJ98 MoAbs.

This paper communicates that these MoAbs can inhibit tumor cell growth in vitro and that the HBJ127 MoAb, which defines a protein epitope on a heavy subunit of the gp125 antigen, is more inhibitory for tumor cell growth than is the HBJ98 MoAb which defines a sugar epitope on the antigen.

MATERIALS AND METHODS

Cell Lines

The BC47 rat bladder cancer cell line has been established from a nitrosamine-induced cancer (3). The Y3 rat myeloma cell line was donated by T. Watanabe, Saga Medical College, Saga, Japan. Human tumor cell lines were obtained from the following sources in Japan: the T-24 bladder cancer cell line from T. Suzuki, Niigata University; and the Molt-4 T-lymphoma, Daudi B-lymphoma, and HL-60 premyleocytoma cell line from T. Tachibana, Tohoku University, Sendai.

The bladder cancer cell lines and the lymphoid cell lines were maintained in Eagle's minimal essential medium and RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, respectively.

MoAb

The B3 (lgG1) and the B31 (lgG1) MoAbs were obtained from hybridoma clones established by a fusion between P3-x63Ag8.653 mouse myeloma cells and spleen cells of a mouse immunized against BC47 mouse bladder cancer cells (1). The B31 MoAb defined a glycoprotein complex (M, 62,000 and 30,000) widely distributed in rat cell surfaces. The HBJ127 (lgG1), HBJ98 (lgG2a), and HBJ27 MoAbs are products of hybridoma clones established by a fusion of spleen cells of a mouse immunized against T-24 human bladder cancer cells (2). The HBJ27 MoAb defined a glycoprotein (M, 85,000) widely distributed in human cell surfaces. All these MoAbs showed species specificity in their reactivity. All MoAbs used were purified from hybridoma culture supernatants by ammonium sulfate precipitation followed by DEAE-cellulose column chromatography.

Characterization of Antigenic Determinants Recognized by MoAbs

Cells bearing a MoAb-defined antigen were labeled externally with 125I as described previously (4) or internally with [3H]glucosamine (New
Assay for Binding of MoAb on Tumor Cell Surface

To examine the additive binding of the HBJ127 and HBJ98 MoAbs, Molt-4 lymphoma cells (1 x 10^6) were treated in triplicate at 4°C for 1 h with a saturating amount (about 1 µg) of the HBJ127 MoAb, HBJ98 MoAb, or their mixture. After washing, the cells were stained with FITC-conjugated F(ab')2 fragments of rabbit anti-mouse IgG antibody (Cappel, Cochranville, PA) and then assessed for their fluorescence intensity by means of flow cytometry with a FACS analyzer (Becton-Dickinson, Mountain View, CA). To examine the competitive binding of these MoAbs, Molt-4 cells (1 x 10^6) were treated at 4°C for 1 h with a mixture of biotinylated MoAb (1 µg) and unmodified MoAb (10 µg). After washing, the cells were stained with FITC-conjugated avidin DCS (Becton-Dickinson, CA) and analyzed as above.

Assays for Effect of MoAb on Tumor Cell Proliferation and Nucleic Acid Synthesis

Counting of Cell Number. Aliquots (2 ml) of target cell suspension containing 2 x 10^6 cells were plated in triplicate in Costar No. 3512 plastic plates and were cultured in the presence or absence of MoAb. Portions of the cell suspension were harvested daily for 4 days and assessed for viability by means of the trypan blue dye exclusion test.

Nucleic Acid Synthesis. To estimate the effect of MoAb on cellular DNA or RNA synthesis, aliquots (200 µl) of a test cell suspension containing 1 x 10^6 cells were cultured in triplicate for 2-3 days in a Falcon No. 3072 microtest plate with or without MoAb. Hydroxyurea, a potent inhibitor of cellular DNA synthesis (5), was used as a positive control which was added to the culture at 5 mM. The cells in each well were pulsed with [3H]thymidine (0.5 µCi/well) or [3H]uridine (1 µCi/well) for the last 4 h of the culture periods. The cells were collected with a FACScan analyzer and fixed in 70% ethanol. The fixed cells were washed twice, and then fixed in 70% ethanol. The fixed cells were washed twice, and then fixed in 70% ethanol.

Cell Cycle Analysis

Target cells were cultured in triplicate at 37°C with or without MoAb, washed twice, and then fixed in 70% ethanol. The fixed cells were examined for DNA content by staining the cells with propidium iodide (Sigma) in the presence of RNase (Sigma; 1 mg/ml), followed by measuring the red fluorescence intensity by using a FACS analyzer (6). The cell cycle distribution was analyzed by the Dean method (7).

RESULTS

Characters of MoAb-defined Cell Surface Antigen and Epitopes. Brief structures of the B3 MoAb-defined rat antigen and the HBJ127 MoAb- or HBJ98 MoAb-defined human antigen have been indicated in our previous reports (1, 2). We further determined the structure of these subunits and the position of MoAb-defined epitopes.

BC47 rat bladder cancer cells and T-24 human bladder cancer cells were metabolically labeled with [3H]glucosamine and subjected to immunoprecipitation followed by SDS-PAGE (Fig. 1). Under nonreducing conditions, 2 components of apparent molecular weights of 125,000 (gp125) and 90,000 were manifested from the T-24 cancer extract with either the HBJ127 or HBJ98 MoAb (Fig. 1A), while only one component of an apparent molecular weight of 90,000 (gp90) was detected under reducing conditions (Fig. 1B). A similar result was obtained by combination of the B3 MoAb with an extract from BC47 cancer cells. The M, 90,000 component detected under nonreducing conditions is suspected to be a cytoplasmic-free gp90 which could not be detected after surface labeling with 125I (2). These results indicate that both HBJ127 and HBJ98 are reactive with a similar glycoprotein, that the antigen is composed of a glycosylated subunit (M, 90,000) and an unglycosylated subunit (M, 35,000), and that the HBJ127/HBJ98-defined antigen has an apparently similar molecular structure with the B3 MoAb-defined rat cell surface antigen.

In order to confirm whether HBJ127 and HBJ98 MoAbs could recognize the same antigenic molecule, we performed sequential immunoprecipitation of the 125I-labeled T-24 cancer cell extract with the use of these 2 MoAbs (Fig. 2). Preclearance of the extract with HBJ98 MoAb-bound Sepharose diminished the amount of the antigen immunoprecipitated with the HBJ127
MoAb (see Fig. 2, Lane c), and preclearance with HBJ127 MoAb-bound Sepharose completely removed the antigenic components which could be immunoprecipitated with the HBJ98 MoAb (see Fig. 2, Lane d). Incomplete clearance of the HBJ127 MoAb-defined antigen with the HBJ98 MoAb may not be due to the insufficient amount of the added HBJ98 MoAb, since increase of the antibody amount did not alter the result (data not shown). This result may indicate that a portion of the gp125 antigen possesses the HBJ127 MoAb-defined epitope but lacks the HBJ98 MoAb-defined epitope.

To determine the localization of MoAb-defined epitopes, the extract of 125I-labeled T-24 cancer cells was treated with dithiothreitol prior to immunoprecipitation with either the HBJ127 or HBJ98 MoAb. Only a component of molecular weight of 90,000 was precipitated with either MoAb (Fig. 3), indicating that the epitopes recognized by either MoAb are present in the heavy subunit of the gp125 antigen. A similar result was also obtained as to the B3 MoAb with the extract from 125I-labeled BC47 bladder cancer cells (data not shown).

Antigenic Modulation of HBJ127 MoAb- or HBJ98 MoAb-defined Target Cell Surface Antigen with Corresponding MoAb. The following studies were performed by means of flow cytometric analysis. To confirm whether the HBJ127 and HBJ98 MoAbs recognize different epitopes on the same antigen molecule, we examined the additive and competitive binding of the HBJ127 and HBJ98 MoAbs (Fig. 4). In these experiments Molt-4 T-cell leukemia and HL-60 promyelocytoma cells were selected as the target, because both cell lines were found to express the HBJ127/HBJ98-defined antigenic determinants and because these cells are rapid growing and able to be obtained as a free cell suspension for flow cytometric analysis without protease treatment. The antigenic component immunoprecipitated with
the HBJ127 or HBJ98 MoAb from the extract of 125I-labeled Molt-4 cells was found to show the same molecular characteristics (gp125 composed of 2 subunits) to that observed with T-24 cells (data not shown). A mixture of the biotinylated HBJ127 MoAb and the HBJ98 MoAb stained Molt-4 cells more intensely than when either was used alone (Fig. 4A). Furthermore, binding of the biotinylated HBJ127 MoAb was not blocked by a 10-fold excess amount of the HBJ98 MoAb (Fig. 4B), and this was also the case in the reciprocal MoAb combination (Fig. 4C). These results substantiate that the HBJ127 MoAb and the HBJ98 MoAb recognize different epitopes.

We next examined the effect of MoAbs on the expression of the corresponding antigens by culturing target cells in the presence of the HBJ127 MoAb (50 µg/ml) or HBJ98 MoAb (200 µg/ml) for 48 h at 37°C and subsequently estimated the antigen expression on the cells (Fig. 5). Before the culture with the MoAbs, the epitope to the HBJ127 MoAb was more intensely expressed than that to the HBJ98 MoAb (Fig. 5, A-1), but after the culture with the MoAbs, expression of the HBJ98 MoAb-defined epitope was augmented as to be comparable to that of the HBJ127 MoAb-defined epitope (Fig. 5, A-2). This was more strikingly indicated when HL-60 cells were used as a target; cultures with the HBJ127 MoAb or especially with the HBJ98 MoAb resulted in markedly augmented expression of the corresponding epitopes (Fig. 5B). These results indicate that the regulation of the antigen expression with the corresponding MoAb is, in contrast to some cell surface molecules (8-11), not downward but rather upward (see Fig. 5, A-3, A-4, B-3, and B-4).

Inhibitory Effect of MoAbs on DNA Synthesis and Growth of Tumor Cells. To examine the effect of MoAbs on cell proliferation, MoAb was added to tumor cell cultures and examined for their inhibitory effect on DNA synthesis and growth of tumor cells. The B3 MoAb inhibited cellular DNA synthesis of BC47 rat bladder cancer and Y3 rat myeloma cells in a dose-dependent fashion, about 70% inhibition being observed with the MoAb at a concentration of 5 µg/ml (BC47) or 10 µg/ml (Y3), whereas the B31 MoAb, which could strongly bind with these target cells but was unreactive with the gp125 antigen, did not significantly inhibit the DNA synthesis even at a concentration of 200 µg/ml (Fig. 6). Like the B3 MoAb in the rat system, the HBJ127 and HBJ98 MoAbs could inhibit the DNA synthesis of human tumor cells such as T-24 bladder cancer and Molt-4 T-lymphoma cells (Fig. 7). When the inhibitory activities of these 2 antibodies were compared in terms of concentrations accounting for 50% inhibition, the HBJ127 MoAb showed about 5 times greater inhibitory activity for the cellular DNA synthesis than did the HBJ98 MoAb.

Molt-4 cells were cultured in the presence of MoAbs and examined for growth by counting the number of viable cells (Fig. 8). The HBJ127 MoAb inhibited the cell growth at concentrations...
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Fig. 8. Effect of monoclonal antibodies on the growth of Molt-4 cells. Molt-4 cells (2 x 10^5/well) were cultured with or without MoAb. Viable tumor cells were counted by a dye exclusion method. O, without MoAb; •, with MoAb; , 12.5 µg/ml; , 25 µg/ml; , 50 µg/ml; , 100 µg/ml.

Lower than 25 µg/ml, whereas the HBJ98 MoAb could show a significant but lower inhibitory effect on the cell growth at a concentration as high as 100 µg/ml and only after 3-4 days of culture. These results indicate that both HBJ127 and HBJ98 MoAbs were able to almost completely inhibit the cellular DNA synthesis, but the HBJ127 MoAb which recognizes a protein epitope on the gp125 antigen is much more inhibitory for cell growth than is HBJ98 MoAb which recognizes a sugar epitope on the antigen.

Characteristics of MoAb-mediated Tumor Cell Growth Inhibition. To further assess the characteristics of the MoAb-mediated cell growth inhibition, we examined the effect of the HBJ127 MoAb on cellular RNA synthesis, reversibility of the effect, and cell cycle changes of the target cells exposed to the MoAb. Hydroxyurea, a potent inhibitor for cellular DNA synthesis, and the HBJ27 MoAb, which was unreactive with the gp125 antigen, were used for a positive and a negative control, respectively.

Molt-4 cells were cultured in the presence or absence of the HBJ127 MoAb (50 µg/ml), and cellular DNA and RNA syntheses were periodically examined, respectively, by [3H]thymidine and [3H]uridine incorporation (Fig. 9). At a period of 12-h culture, the HBJ127 MoAb did not show a significant inhibitory effect on either DNA or RNA synthesis, whereas hydroxyurea already inhibited the DNA synthesis by 50% of control. However, additional 12-h culture with the HBJ127 MoAb resulted in about 80% inhibition of DNA and 50% inhibition of RNA synthesis, although HBJ27 MoAb did not show significant inhibitory effect. Further culture with HBJ127 MoAb did not increase the degree of inhibited DNA synthesis, and a substantial amount of [3H]thymidine (2000-2500 cpm) was incorporated, as opposed to the case of hydroxyurea (incorporation at 300-600 cpm). Decline of [3H]uridine incorporation was manifested more slowly than that of [3H]thymidine incorporation, and a substantial amount of [3H]uridine was incorporated over 36 h as was [3H]thymidine.

To examine the reversibility of the MoAb-mediated inhibition of nucleic acid synthesis, Molt-4 cells were precultured with HBJ127 MoAb for 24 h, washed, cultured in fresh medium, and estimated for cellular DNA synthesis. Culture of the cells for 24 h with the MoAb decreased the capacity of cellular DNA synthesis to 10% of the control (cultured without MoAb); however, when the cells were washed and then cultured in MoAb-free medium, the repressed DNA synthesis recovered to 60% of control within 12 h and 80% of control within 24 h (Fig. 10). When the cells were precultured up to 48 h in the presence of MoAb, the cells recovered the capacity of DNA synthesis after further culture for 48 h in MoAb-free medium (data not shown).

These findings indicate that the effect of the HBJ127 MoAb on cellular DNA synthesis is reversible and that the MoAb is not cytotoxic but cytostatic.

Effect of MoAb on Cell Cycle Distribution of Target Cells. The effect of the HBJ127 MoAb on the cell cycle distribution of Molt-4 cells was examined by means of flow cytometry (Fig. 11). Only a small accumulation of the cells in S phase was manifested by culture of the cells for 1-5 days in the presence of the MoAb. These observations suggest that the MoAb does not arrest the target cells in a certain phase of cell cycle even at a concentration by which nucleic acid synthesis of the target cells was almost perfectly inhibited.

Fig. 10. Effect of washing on the inhibited cell growth of Molt-4 cells by HBJ127 MoAb. After culturing Molt-4 cells (2 x 10^5/well) in the presence or absence of HBJ127 MoAb (50 µg/ml) for 24 h, the cells were washed and then cultured in fresh medium for an additional 12 to 48 h. The cells were harvested, and aliquots (1 x 10^6 cells) of the cell suspension were pulsed for 4 h with 0.5 µCi of [3H]thymidine (Tdr). Ordinate indicates [3H]thymidine incorporation (percentage of untreated control).

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the expression of the HBJ98 MoAb-defined epitope is probably due to the heterogeneity in the O-linked carbohydrate processing conducted by the posttranslational modification of the core polypeptide of the heavy subunit. The weaker growth-inhibitory effect of the HBJ98 MoAb might be attributable either to such heterogeneity in the HBJ98 MoAb-defined epitope or to that the HBJ98 MoAb-defined epitope is present in the less proximal region of the biologically functional region of the gp125 component than is the HBJ127 MoAb-defined epitope.

An additional polypeptide (M, 56,000) immunoprecipitated with the HBJ98 MoAb, but not with the HBJ127 MoAb, from the extracts of [3H]-labeled T-24 cells (Figs. 2 and 3) has not been observed in the experiment shown in Fig. 1 and the previous work (2), where the target cells that adhered on plastic were radiolabeled in situ and the antigenic components were extracted from the nontrypsinized cells. However, in the experiments in Figs. 2 and 3, a single cell suspension of T-24 cells was prepared by brief trypsinization, and then the cells were immediately radiolabeled. Therefore, the polypeptide appears to be a tryptic fragment of the gp125 molecule which retained the HBJ98 MoAb-defined epitope but lost the HBJ127 MoAb-defined epitope.

Recently, Haynes et al. (12) selected a mouse MoAb, 4F2, from MoAbs raised against human T-lymphoma cells and proved that it reacted with a variety of human cell lines, mitogen- or alloantigen-stimulated lymphocytes, and monocytes. 4F2 MoAb was also found to define a disulfide-linked glycoprotein complex of apparent molecular weight of 125,000, which was composed of a glycosylated heavy subunit (M, 85,000) and an unglycosylated light subunit (M, 41,000) (13). These findings strongly suggest that both 4F2 and HBJ127/HBJ98 MoAbs define the same antigen molecule, although the epitopes recognized by these MoAbs seem to be different from each other. 4F2 MoAb was reported to partially suppress the mitogen-induced DNA synthesis of human T- and B-cells (12) but inhibit neither allogenic mixed lymphocyte reactions nor the proliferative responses of the human T-cell line to IL 2 nor DNA synthesis of malignant cells (12, 14). By contrast, HBJ127 and HBJ98 MoAbs did block all these DNA synthesis or proliferative responses (data for lymphocyte responses will be published elsewhere). Therefore, the HBJ127 and HBJ98 MoAbs appear to recognize the more biologically functional regions of the gp125 antigen than does the 4F2 MoAb.

As did the HBJ127 and HBJ98 MoAbs, antibodies to a glycolipid component on human melanoma cells (15) and the receptors for growth factors such as transferrin (16), IL 2 (17), and epidermal growth factor (18) are reported to inhibit the cell growth in vitro. For example, the 42/6 MoAb against transferrin receptor blocks the transferrin binding to the receptor-bearing tumor cells, leading to the cell growth inhibition (16). The HBJ127 and HBJ98 MoAbs did not, but the 42/6 MoAb was found to, arrest the target cells in S phase of the cell cycle (16). Regulation of cellsurface components by exposure to the corresponding antibodies is a unique characteristic of the functional cell-surface components. Expression of the known growth factor receptors such as receptors for epidermal growth factor, insulin, transferrin, and IL 2 is found to receive a down-regulation by exposure to the corresponding antibody (8–11). By contrast, the gp125 antigen on either activated lymphocytes (data will be published elsewhere) or neoplastic cells could not be down-regulated by the
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antibody. Therefore, the gp125 antigen seems to regulate cell growth in a unique fashion quite different from the known growth factor receptors.

As described in the present report, the gp125 antigen in both human and rat systems, which is predominantly expressed on proliferating cells, seems to play some requisite role in cell growth, but its biological function is yet unknown. The putative function of the gp125 component may be as follows: (a) function as a cell surface receptor for some factor requisite for cell growth and (b) the component itself functions as a regulator for cell growth by mediating a cascade reaction requisite for the cell growth.

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