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ABSTRACT

In order to find lung cancer-specific markers, monoclonal antibody 15 (MAb15) was produced against a variant-type cell of small cell lung carcinoma. Its gp85/45 antigens were demonstrated in 70% of lung cancers, and particularly in the proliferating zone of cancer cell nests, but they are scarcely detected in noncancerous tissues. Immunoelectron microscopy revealed that gp85/45 antigens were expressed alternatively on the cell membrane of living cancer cells according to their biological states. MAb15 added to the culture medium inhibited the proliferation of lung cancer cells, depending on its concentration, but cell death rate did not increase. The growth inhibition by MAb15 was reevaluated by a colony-forming assay. On DNA histogram, MAb15 decreased the number of DNA-synthesizing cells in the S phase with an elevation of the G1 peak, indicating a G1-S boundary block in the cell cycle. gp85/45 detected by this lung cancer-associated monoclonal antibody could be a functional membrane unit, such as a growth factor receptor, which is related to the cell proliferation of lung cancer. The growth inhibition by MAb15 may be caused by the blocking of a growth factor receptor which is specific to lung cancer.

INTRODUCTION

As a result of advances in immunology, many kinds of monoclonal antibodies against cancers have been produced in order to find cancer-specific markers. Interestingly, some of them are related to cancer cell growth and influence cancer cell proliferation in vitro (1, 2). The antigens detected by these monoclonal antibodies may be functional molecules associated with cancer cell growth as well as cancer-specific markers, and they are very important in the analysis of the characteristics of cancer.

MAb15 is one of the monoclonal antibodies produced in our laboratory (3, 4). It was raised against a human lung cancer cell line TKB-2, which is classified as a variant type of small cell carcinoma according to its biochemical and morphological features (5, 6). Its isotype belongs to murine IgG1K. The antigens detected by MAb15 are M, 85,000 and M, 45,000 glycoproteins, designated gp85/45. A peptide portion of gp85/45 was recognized by MAb15 as a determinant. The gp85/45 antigens are common antigens shared by four major histological types of lung cancer cell lines and tissues. They are demonstrated immunohistochemically in about 70% of lung cancers, but rarely detected in noncancerous tissues or in cancer tissues other than lung cancer. The gp85/45 antigens were not detected serologically in the patients' blood (3, 4).

To discover the biological function of gp85/45 recognized by MAb15, we examined the manner of their expression and also studied the relation between expression of gp85/45 and cancer cell growth. We further analyzed the effect of MAb15 on cell proliferation of lung cancer in vitro.

MATERIALS AND METHODS

Lung Cancer Tissues. Surgically removed materials from human lung cancers, including all histological types, were fixed with periodate-lysine-paraformaldehyde solution (7), then frozen in Tissue-Tek O.C.T. compound (Miles, Naperville, IL), and stored at -80°C.

Human Lung Cancer Cells. Four major histological types of human lung cancer cell lines which had high immunological reactivity with MAb15 (3) were used for this experiment. They were all established in our laboratory: TKB-2 (MAb15 immunogen) from a small cell carcinoma; TKB-3 from a large cell carcinoma; TKB-4 from a squamous cell carcinoma; and TKB-6 from an adenocarcinoma. These cells were maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories, Chagrin Falls, OH) supplemented with 10% heat-inactivated fetal bovine serum (M. A. Bioproducts, Walkersville, MA).

Immunoperoxidase Staining and Immuno electron Microscopy. Lung cancer tissues were prepared as 5-µm-thick cryostat sections, treated with cold acetone (10 min, 4°C), and washed 3 times with PBS. After preincubation with 3% bovine serum albumin-PBS and 1% normal horse serum, tissue sections were incubated with MAb15 for 90 min at room temperature and stained using the avidin-biotin-conjugated peroxidase method (ABC kit; Vector Laboratories, Burlingame, CA). The histological distribution of antigen-positive cells in lung cancer tissues was studied in detail. Lung cancer cells which were cultured in chamber slides (Lab-Tek No. 4802; Naperville, IL) were fixed with cold acetone (10 min, 4°C) and stained by the same method as described above.

To examine the intracellular localization of the gp85/45 antigens, cryostat sections of TKB-2 cells fixed with periodate-lysine-paraformaldehyde solution were examined by preembedding immunoelectron microscopy, using the peroxidase-labeled antibody method (8). The variety of antigen expression on the cell surface of living cells was investigated by the following immunoelectron microscopic method. Thirty min after the addition of MAb15 to the culture medium, cancer cells were suspended by pipetting and washed 3 times in cold PBS supplemented with 0.1% sodium azide; the suspended cells were then made to react with the peroxidase-conjugated anti-mouse IgG (Cooper Biomedical No. 3311-81; Malvern, PA) and fixed with 1% glutaraldehyde for 10 min at 4°C; next the specimens were resuspended in 8% albumin-PBS and congealed into gelatinous blocks using 1% glutaraldehyde; thereafter the specimens were prepared as usual electron microscopy materials without counter staining and inspected with a 1200-EX electron microscope (JEOL, Tokyo, Japan).

Assay for Effect of MAb15 on Tumor Cell Proliferation and Cell Cycle in Vitro. To study the biological effects of MAb15 on the immunogen cell TKB-2, cell counting assays were performed. Cancer cells harvested in the exponential growth phase were plated in duplicate 35-mm dishes and cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated fetal bovine serum for 6 h. Varying concentrations of MAb15 (0.01 µg/ml, 0.2 µg/ml, 4 µg/ml) and indifferent murine IgG (4 µg/ml) as a control were then added to the culture medium. The culture media were changed every 3 days. Cell number and viability were assessed at certain intervals until the sixth day by using a dye (erythrosin B) exclusion test (9).

Cancer cells were also
cultured in chamber slides in the same manner, fixed by 95% alcohol, and stained by Papanicolaou's method to examine the morphological changes of cancer cells.

A colonogenic assay was done in order to evaluate the effect of MAb15 on four major types of lung cancer cells with which MAb15 reacts immunologically, and to reevaluate the results of the cell-counting assay. Single cell suspensions of each cancer cell (1 to 2 × 10^5) were plated in ten 60-mm dishes and cultured with or without MAb15 for 14 days. Aggregated colonies larger than 100 μm in diameter were scored, and their sizes were measured by a computed image analyzer (SPICCA; Nippon Avionics, Tokyo, Japan).

Assay for Effect of MAb15 on DNA Synthesis and DNA Histogram. Cancer cells which were cultured in chamber slides for 6 days in the same manner as in the cell-counting assay described above were incubated with 100 μM BrdUrd for 30 min, fixed by 70% alcohol, and stained immunocytochemically with anti-BrdUrd monoclonal antibody (No. 7580; Beckton-Dickinson, Mountain View, CA). The S-phase labeling index and mitotic index were evaluated microscopically. For the DNA histogram analysis, cancer cells were also cultured in chamber slides for 6 days with and without MAb15 and fixed by 70% alcohol. They were stained with Feulgen’s reaction, and DNA histograms of the cancer cells were examined by the microphotometric method using a color image analyzer specially modified for DNA analysis (NASCCA; Nippon Avionics, Tokyo, Japan).

RESULTS

Histological Distribution of gp85/45 Antigen-positive Cells. MAb15 reacted immunohistologically with 76% of squamous cell carcinoma (13 of 17), 71% of adenocarcinoma (12 of 17), 44% of small cell carcinoma (3 of 7), and 100% of large cell carcinoma (15 of 15). These data have been previously reported (3). The histological distribution of MAb15 antigen-positive cells was investigated in detail. Marginal zones of cancer cell nests or frontier portions of infiltrating cancer were strongly positive, particularly in squamous cell carcinomas (Fig. 1). In the other histological types, the antigen-positive cells showed no peculiar distribution except that necrotic cells tended to lose the staining activity in general. MAb15 did not react with most noncancerous tissues, though parts of the basal layer of esophageal squamous epithelium were stained slightly by MAb15.

Intracellular Localization of gp85/45 Antigen. The immunoelectron microscopic study using cryostat sections revealed that gp85/45 antigens were present on the cell membrane locally. The antigens were also expressed to a lesser extent in the endoplasmic reticulum (Fig. 2).

Intercellular Variety of gp85/45 Antigen Expression on the Surface of Living Cells. Immunocytochemical study using culture cells of four major histological types of lung cancer showed that the gp85/45 antigens were expressed in different degrees, even among the same lineage of cancer cells. Antigen-positive cells tended to form clusters, and the mitotic cells seemed to be stained more strongly in the large cell carcinoma cell line (TKB-3; Fig. 3) and the squamous cell carcinoma cell line (TKB-4).

The variety of gp85/45 expression was also demonstrated by an immunoelectron microscopic examination for membrane surface antigens of living cells, particularly in TKB-3 cells. Cancer cells with strong expression usually had a large nucleolus and electronically dense cytosol (Fig. 4).

Influence of MAb15 on Lung Cancer Cell Growth in Vitro. A cell-counting assay revealed that the cell proliferation of TKB-2 which was the immunogen cell line of MAb15 was inhibited by 4 μg/ml and 0.2 μg/ml of MAb15 (Fig. 5). The inhibitory effect was statistically significant after 4 days, compared with a control, and it depended on the concentration of MAb15 (P < 0.05). This experiment was repeated twice, and the result was almost the same. Microscopic examination showed that cancer cells did not undergo any morphological changes. Cell death rate did not increase throughout the experiment, suggesting that the inhibitory effect of MAb15 was not cytotoxic but cytostatic, and can be attributed to the prolongation of the doubling time.

A clonogenic assay was carried out to evaluate the effect of MAb15 on cell growth of four types of lung cancers. In TKB-2, 3, and 4, colony-forming efficiency was obviously less in the MAb15-added medium than in the control (Table 1). The sizes...
of colonies were also smaller in MAb15-added medium than in
the control. The effects were especially pronounced in TKB-2,
and this confirmed the result of the cell-counting assay. The
growth-inhibitory effect on TKB-3 was also statistically signif-
ificant. TKB-6 did not form any colonies in either the MAb15-
added medium or the control.

Assay for Effect of MAb15 on DNA Synthesis and DNA
Histogram. The percentages of DNA-synthesizing cells labeled
by BrdUrd (labeling index) and of mitotic cells (mitotic index)
were evaluated simultaneously (Fig. 6). The labeling index
decreased steadily in TKB-2 cells cultured in the MAb15-added
medium as compared with the control cells. The mitotic index
of the cells cultured with MAb15 was, however, constant as was
that of the control. That is, the total DNA synthesis decreased
even though the relative turnover of cell cycle did not change.

The DNA histograms showed that the proportion of the cells
in S phase decreased in the groups cultured with MAb15,
corresponding to the elevation of the G1 peak, though it was
variable in cancer cell lines. The decrease was marked in TKB-
3, but there was no difference in TKB-6 (Fig. 7). These findings
indicated that MAb15 suppressed the transition from G1 phase
to S phase and induced cell accumulation in the G1 phase. That
is, the G1-S boundary block mechanism may be present as a
cytostatic effect of MAb15, thus changing DNA histograms.

DISCUSSION

MAb15 is a lung cancer-associated monoclonal antibody
which has a broad binding capacity to various types of lung

Table 1 Colonogenic assay for late effects of MAb15 on cancer cell growth in vitro

<table>
<thead>
<tr>
<th></th>
<th>TKB-2</th>
<th>TKB-3</th>
<th>TKB-4</th>
<th>TKB-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAb15 (4 μg/ml)</td>
<td>57.0 (5.48)b</td>
<td>16.6 (7.19)</td>
<td>25.6 (5.83)</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>80.0 (7.80)</td>
<td>20.0 (8.59)</td>
<td>28.4 (7.00)</td>
<td>27.6 (7.02)</td>
</tr>
</tbody>
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a Number of colonies/10⁴ at the 14th day.
bP < 0.05.
cNo colonogenesis.
dNumbers in parentheses, average size of colonies (mm²).
cell surface indicated that the degree of expression varied even
more extensively than was expected particularly in the cells having high prolif-
erative activity. On the other hand, the observation of the living cancer cells cultured with MAb15 (4 µg/ml) (■) and without MAb15 (○) for 4 days.

Fig. 6. Labeling index by BrdUrd (L.I.) and mitotic index (M.I.) of TKB-2
cells cultured with MAb15 (4 µg/ml) (■) and without MAb15 (○).

Fig. 7. DNA histograms of lung cancer cells: a, TKB-2; b, TKB-3; c, TKB-4;
and d, TKB-6. Lung cancer cells were cultured with MAb15 (4 µg/ml) and without
MAb15 (control) for 4 days.

DNA CONTENTS

MAb15 AGAINST CELL GROWTH-RELATED ANTIGEN

cancer and reacts with about 70% of lung cancers immunohistochemically. The antigens detected by MAb15 are glycoproteins with molecular weights of 85,000 and 45,000 (gp85/45). MAb15 recognizes the peptide portion of gp85/45 as a determinant. The present immunoelectron microscopic study using frozen sections revealed that gp85/45 was locally distributed on the cell membrane. That is, gp85/45 is a glycoprotein included in the cell membrane.

Detailed observation of the immunohistochemical specimens revealed that the cancer cells expressing the gp85/45 were distributed in the margin of cancer cell nests, particularly in squamous cell carcinomas. Proliferating cells are abundant in marginal zones of cancer cell nests in squamous cell carcinoma in contrast to the rather uniform distribution in other histological types of carcinoma (10, 11). Expression of gp85/45, therefore, was expected particularly in the cells having high proliferative activity. On the other hand, the observation of the living cancer cell surface indicated that the degree of expression varied even within a lineage of cultured cells. It is interesting that cancer cells expressing gp85/45 tend to contain a large nucleolus and have electronically dense cytosol, probably according to the biological condition or the phases of the cell cycle. These morphological studies suggest that gp85/45 antigens have a biological significance connected with cell growth, and they could be diversely expressed on the membrane surface of each living cancer cell in proportion to its proliferative state.

The biological significance of gp85/45 expression in the lung cancer cells was clarified by several in vitro studies using the culture cells. A small amount of MAb15 added to the culture medium inhibited cell proliferation of TKB2 cancer cell in a dosage-dependent manner. The inhibitory effect was not cytotoxic but cytoplastic, for the cell death rate did not increase. MAb15 also inhibited the cell proliferation of other lung cancer cells in vitro, though its degree was variable. A colonogonic assay verified that MAb15 had a growth-inhibitory effect in vitro on various cancer cells. The inhibitory effect of MAb15 varies among the cancer cell lines, and this may depend on the difference of gp85/45 expression in quantity.

Analysis of DNA synthesis and DNA histograms showed that MAb15 decreased the number of DNA-synthesizing cells in the S phase, corresponding to the elevation of the G1 peak in the DNA histogram. MAb15 attached to the living cells' surface seems to suppress the transition from G1 phase to S phase, leading to the interpretation that the growth-inhibitory effect of MAb15 is a cytostatic G1-S boundary block of the cell cycle.

The most unique biological characteristic of cancer cells is autonomic growth distinguishing them from nonneoplastic cells. Some known growth factors have been reported to be involved in the proliferation of human cancers (12-14). Cancer cells as well as nonneoplastic cells have receptors on the cell surface against several growth factors. When the receptors that cancer cells possess are effective enough, the growth factors corresponding to these receptors influence the proliferation of cancer cells (15), but they are not always specific to cancer. Monoclonal antibodies raised against growth factor receptors also possibly influence cancer cell growth; indeed several examples of receptor blocking by monoclonal antibodies have recently been reported (16-19). On the other hand, many kinds of monoclonal antibodies against cancers have been produced in order to find cancer-specific markers. Some of the cancer-associated monoclonal antibodies are of interest in relation to cancer growth, and they influence cancer cell growth in vitro (1, 2). The antigens detected by these antibodies may be unique growth factor receptors as well as cancer-specific markers, and these monoclonal antibodies may block the receptors in vitro.

Concerning the mechanism of the growth-inhibitory effect of MAb15, the most attractive explanation is the blocking of a membrane receptor for growth factor. However, there may be other possible mechanisms. Cytolysis via the complement system is ruled out, since fetal bovine serum added to the medium inhibited cell proliferation of TKB2 cancer cell in a dosage-dependent manner. The inhibitory effect was not cytotoxic but cytoplastic, for the cell death rate did not increase. MAb15 also inhibited the cell proliferation of other lung cancer cells in vitro, though its degree was variable. A colonogonic assay verified that MAb15 had a growth-inhibitory effect in vitro on various cancer cells. The inhibitory effect of MAb15 varies among the cancer cell lines, and this may depend on the difference of gp85/45 expression in quantity.

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It is reported that transferrin receptors are expressed on the surface of proliferating cells (20) and that EGF receptors are demonstrated in the basal cells of squamous epithelium (21),
but the molecular weights of both receptors are different from gp85/45. Recently gastrin-releasing peptide and bombesin appear to be specific growth factors for small cell lung carcinoma (22), but they too are different from gp85/45. The MAb15 that recognizes gp85/45 reacted broadly with lung cancers, and rather more strongly with types of carcinoma other than small cell carcinoma. MAb15 antibody has been produced against a (22), but they too are different from gp85/45. The MAbIS that gp85/45. Recently gastrin-releasing peptide and bombesin appear to be specific growth factors for small cell lung carcinoma cell line named TKB-2; however, TKB-2 is classified not into the classic type, in which gastrin-releasing peptide works as an autocrine growth factor, but into the variant type by morphological and biochemical characteristics. It is quite possible that gp85/45 recognized by MAb15 is a new growth factor receptor of lung cancer.

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