Growth Inhibition of Human Tumor Cells in Athymic Mice by Anti-Epidermal Growth Factor Receptor Monoclonal Antibodies

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ABSTRACT

Monoclonal antibodies (MoAbs) were raised against epidermal growth factor (EGF) receptors on a human epidermoid carcinoma cell line, A431. Administration of anti-EGF receptor MoAbs inhibited tumor formation in athymic mice by A431 cells and by another epidermal carcinoma cell line, T222. When one of the same MoAbs was used in therapy against Li-7 (a human hepatoma) and HeLa cells (a cervical carcinoma), tumor growth was not affected. The number of EGF receptors on A431 cells was about 100-fold higher than on T222, Li-7, and HeLa cells, suggesting that the number of EGF receptors may not be an important determinant in suppressing tumor growth. Three anti-EGF receptor MoAbs were used in the present studies. MoAbs 528 (immunoglobulin G2a) and 225 (immunoglobulin G1) are capable of competing with EGF for receptor binding and inhibit proliferation of A431 cells in culture. The other MoAb, 455 (immunoglobulin G1), is incapable of blocking the binding of EGF to its receptors and has no effect on the proliferation of cultured A431 cells. All three MoAbs inhibited A431 tumor growth in athymic mice, indicating that the antibody isotype and the site of binding on the EGF receptor are not the determinants of antiproliferative activity in vivo. The observation that MoAb against the receptor for EGF is cytostatic rather than cytocidal in vitro against A431 cells, yet completely prevents tumor growth in vivo, suggests that some host animal responses also may be involved in the antitumor effect. MoAbs against growth factor receptors could provide useful immunotherapeutic agents.

INTRODUCTION

There have been many attempts at passive immunotherapy of malignant tumors, but there are only a few reports in which the administration of conventional antiserum inhibited tumor growth in experimental animals or in cancer patients (26). Thus, passive immunotherapy has not been established as a treatment for human cancer. The major reasons are the difficulties in identifying tumor-associated antigens and in preparing homogeneous antibody in large quantities by conventional methods.

Development of the hybridoma technique by Kohler and Milstein (14, 15) has overcome these difficulties, and experimental cancer immunotherapy has been revived in recent years using MoAbs. Several recent reports demonstrated that MoAbs inhibit the growth of human tumor xenografts in athymic mice (8, 9, 16, 27). Furthermore, the results of several clinical studies on the treatment of leukemia and lymphoma using MoAbs have been published, but in these cases, clinically significant results have not been obtained (17, 18, 20, 23–25), with one exception. This was a report on the treatment of a human B-cell lymphoma with anti-idiotype antibody, in which the patient has been free from disease for more than 1.5 years (19).

In most of these studies, MoAbs against tumor-associated antigens have been used for treatment. Another type of MoAb which might be used for cancer immunotherapy is an antibody against plasma membrane receptors for growth factors. It is well known that the proliferation of tumor cells in culture is controlled by various growth factors, and a similar control mechanism is postulated for the control of tumor cell growth in vivo (2). Therefore, MoAbs against growth factor receptors, which could block access of growth factors to their receptors, may provide useful therapeutic agents. Recently, Trowbridge and Domingo (29) reported that treatment with anti-transferrin receptor MoAb can inhibit tumor formation by a human melanoma cell line in athymic mice. A431 cells, a human epidermoid carcinoma cell line, express an unusually large number of EGF receptors on the cell surface membrane (1 to 3 x 10^6/cell) (4, 7), and addition of EGF to the culture medium inhibits the proliferation of these cells in culture (1, 6). We have developed MoAbs against the EGF receptor using partially purified EGF receptors from A431 cells as antigen (13). In this paper, we report that the administration of anti-EGF receptor MoAbs inhibits tumor formation in athymic mice by human epidermoid carcinomas, A431 and T222. However, growth of 2 other xenografts is not affected.

MATERIALS AND METHODS

Animals. BALB/c athymic mice 6 to 8 weeks of age were used in the present studies. These mice were bred and maintained at the Athymic Mouse Colony Facility at the University of California, San Diego. The procedures for breeding and maintenance have been published previously (22).

HUMAN TUMOR CELLS. A431, T222, Li-7, and HeLa cells were maintained in a mixture of DME and Ham's F-12 medium (Grand Island Biological Co.), containing 15 mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer and 5% newborn calf serum, at 37°C in 5% CO_2:95% air atmosphere.

The Li-7 human hepatoma tumor line was established originally at the Cancer Center Research Institute, Tokyo, Japan (10). This tumor was obtained after 50 passages and has been transplanted serially in this facility for 22 passages over the past 3 years. The human lung epidermoid carcinoma (T222) was established in our laboratory by inoculating fresh tumor tissue from surgery and was used after 47 to 50 passages in the

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6 The abbreviations used are: MoAb, monoclonal antibody; EGF, epidermal growth factor; DME, Dubecco's modified Eagle's medium; PBS, phosphate-buffered saline.
present studies. Cell lines were established from these tumors in our laboratory and were cultured as described above.

Anti-EGF Receptor MoAbs. In the present studies, we used 3 anti-EGF receptor MoAbs, referred to as Nos. 528, 225, and 455, raised against the EGF receptor on A431 cells, derived from a epidermoid carcinoma of the vulva. The development and characterization of these murine MoAbs have been published elsewhere (Footnote 7; Ref. 13). MoAb 528 has the isotype IgG2a and competes with EGF for binding to its receptors. When MoAb 528 is added to culture medium, proliferation of A431 cells is inhibited. MoAb 225 of the isotype IgG1 also competes with EGF binding to receptors and inhibits proliferation of cultured A431 cells. In contrast, MoAb 455, also of the isotype IgG1, is incapable of blocking EGF from binding to its receptors and does not affect growth of A431 cells in culture.

Hybridoma cells producing MoAbs were cultured in a 2:1:1 RPMI-1640:DME:Ham's F-12 mixture containing 15 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, insulin (10 μg/ml), 10 μM mercaptoethanol, and 10 μM ethanolamine. To raise large amounts of MoAbs, cells were harvested and injected i.p. into athymic mice (107 cells/animal). After 2 to 3 weeks, ascites fluid was collected, centrifuged to remove floating cells, and kept frozen until purification. MoAbs were purified by ammonium sulfate fractionation followed by DEAE-cellulose column chromatography. The purity of immunoglobulins in these preparations was more than 90%, as judged by sodium dodecyl sulfate-polyacylamide gel electrophoresis. Ascites fluid from one animal provided 3 to 5 mg of purified MoAb.

A murine-murine hybridoma (ARB229), which produces MoAb against a haptene (tyrosyl arsenate), obtained from Hybritech, Inc., was used as a control.

Assay of Tumor Growth in Athymic Mice. A431 cells, T222 cells, Li-7 cells, or HeLa cells from stock cultures were trypsinized and washed with serum-free DME:Ham's F-12 (1:1) medium by centrifugation. Tumor cells were injected s.c. into athymic mice at the specified doses in 0.2 ml of medium. Tumor size was measured twice weekly using the formula, \( \frac{1}{2} \times \pi \times \text{length} \times \text{width} \times \text{height} \) (cm). In some experiments with Li-7 hepatoma, T222 epidermoid carcinoma, and A431 epidermoid carcinoma, cells for injection into mice were obtained from tumor xenografts. In these experiments, tumors of 2- to 4-g weight were removed, minced finely with scissors, and washed with DME:Ham's F-12 medium, and a portion was injected s.c. into athymic mice through a 18-gauge needle. For treatment, varying quantities of MoAb were dissolved in 0.5 ml of PBS, pH 7.4, and injected i.p. into mice. Initially, injections were given 3 times per week, but identical results were obtained in later studies using a twice weekly schedule. Control animals received 0.5 ml of PBS. Each experimental group consisted of 5 or 6 athymic mice unless otherwise described.

125I-EGF Binding Assay. The number of EGF receptors on A431 cells, T222 cells, Li-7 cells, or HeLa cells from stock cultures were trypsinized and washed with serum-free DME:Ham's F-12 (1:1) medium by centrifugation. Tumor cells were injected s.c. into athymic mice at the specified doses in 0.2 ml of medium. Tumor size was measured twice weekly using the formula, \( \frac{1}{2} \times \pi \times \text{length} \times \text{width} \times \text{height} \) (cm). In some experiments with Li-7 hepatoma, T222 epidermoid carcinoma, and A431 epidermoid carcinoma, cells for injection into mice were obtained from tumor xenografts. In these experiments, tumors of 2- to 4-g weight were removed, minced finely with scissors, and washed with DME:Ham's F-12 medium, and a portion was injected s.c. into athymic mice through a 18-gauge needle. For treatment, varying quantities of MoAb were dissolved in 0.5 ml of PBS, pH 7.4, and injected i.p. into mice. Initially, injections were given 3 times per week, but identical results were obtained in later studies using a twice weekly schedule. Control animals received 0.5 ml of PBS. Each experimental group consisted of 5 or 6 athymic mice unless otherwise described.

Clearance of 125I-labeled MoAb in Athymic Mice Carrying A431 Tumors. 125I-labeled MoAb 528 was prepared from purified MoAb 528 by the procedures described previously (13). A431 cells were injected s.c. into athymic mice. When small tumors had formed, 125I-labeled MoAb 528 (10 μCi/0.6 μg) was injected i.p. into a group of 5 animals. The same amount of 125I-labeled MoAb 528 plus 1.0 mg of unlabelled MoAb 528 was injected into a second group of 2 animals. Ten μl of blood were taken from the tail veins on successive days and mixed with 1 ml of 0.1% albumin solution, and trichloroacetic acid was added. Acid-precipitable radioactivity was counted in a \( \gamma \) counter. The first group of mice were killed, tumors and muscle were removed from each animal and weighed, and the tissue radioactivity was measured in a \( \gamma \) counter. The specific radioactivity of tumor and muscle was calculated for each animal.

RESULTS

Tumorigenicity of Human Tumor Cells in Athymic Mice. In initial experiments, the tumorigenicity of human tumor cells in BALB/c athymic mice was tested by injecting varying numbers of cells s.c. (Table 1). Inoculation of more than 106 A431 cells was needed to produce tumors consistently, and 105 cells were selected for the experiments conducted in this study. HeLa cells were less tumorigenic than were A431 cells, requiring an inoculation of more than 5 × 105 cells for tumor formation. The tumorigenicity of T222 human epidermoid carcinoma cells and Li-7 human hepatoma cells was also tested, and it was found that inoculation of 106 cells was required for tumor formation. Furthermore, tumor growth was too slow for a useful growth assay. In feasible conduction of immunotherapy experiments using T222 and Li-7 cells, an inoculum of finely minced tissue was used in subsequent experiments. The inoculum consisted of 0.2 ml of minced tumor tissue. Since therapy against T222 cells and Li-7 cells was analyzed in a system using minced tumor and HeLa cells in a system using cultured cells, A431 experiments were carried out with both systems.

Growth Inhibition of A431 Cells in Athymic Mice by Administration of Anti-EGF Receptor MoAbs. Three monoclonal antibodies against the EGF receptor were assayed for their capacity to suppress the growth of A431 cells in athymic mice (Chart 1). Treatment 3 times weekly with ascites fluid containing either MoAb 528 or 225 completely inhibited tumor formation when treatment was started on the day of tumor cell inoculation. After stopping the MoAb treatment, the mice were observed for 2 months, and no tumors formed in these animals. Administration of EGF (30 μg/animal, 3 times weekly) did not affect the tumor growth. Treatment with ascites fluid containing control MoAb from ARB229 did not affect the growth of A431 cells in athymic mice (Chart 2). Also, treatment with mouse serum immunoglobulin did not affect the tumor growth (data not shown).

Next, the capacity of MoAb treatment to modify the growth of developed tumors was tested. Seven days after injection of

<table>
<thead>
<tr>
<th><strong>Table 1</strong></th>
<th>Tumorigenicity of A431 and HeLa cells in athymic mice</th>
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</thead>
<tbody>
<tr>
<td><strong>Cells</strong></td>
<td><strong>No. of cells inoculated</strong></td>
</tr>
<tr>
<td>A431</td>
<td>10^7</td>
</tr>
<tr>
<td></td>
<td>3.3 × 10^6</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
</tr>
<tr>
<td></td>
<td>3.3 × 10^6</td>
</tr>
<tr>
<td>HeLa</td>
<td>5 × 10^7</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
</tr>
<tr>
<td></td>
<td>5 × 10^6</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
</tr>
</tbody>
</table>

- Number of animals in which tumors formed per total number of animals studied.
- Average ± S.E. of tumor size for A431 cells and the average size for HeLa cells.
inhibition of tumor formation was obtained by treatment with MoAb 528 at a 0.2-mg dose, whereas the same effect with MoAb 225 and 455 therapy required a 2-mg dose. A significant suppression of tumor formation was evident at 0.2-mg doses of MoAbs 225 and 455 (Table 2). When these experiments were repeated, there was some variation in the rate of tumor growth, but similar responses to MoAb treatment are always obtained. The results of these experiments demonstrate clearly that anti-EGF receptor MoAbs, with different characteristics in terms of IgG isotypes and capacity to block EGF binding, inhibit the growth of A431 tumors in athymic mice.

In a parallel experiment, minced A431 tumor tissue was inoculated into athymic mice, and animals were treated with MoAb 528. Inhibition required higher antibody concentrations with minced tumor than with cultured cells. Complete inhibition of

A431 cells, tumors had grown to an approximate volume of 1 cu cm. At this juncture, the tumor-bearing animals were divided into 2 groups displaying similar size distributions of tumors, and treatment with ascites fluid containing MoAb 528 was started on a twice weekly schedule. As shown in Chart 3, this treatment regimen reduced tumor growth significantly. As previously, the same treatment abolished tumor formation completely when started at the time of tumor cell inoculation (data not shown).

In the experiments described above, ascites fluid was used as the source of MoAbs. To quantitate the amount of antibody needed for inhibition of tumor formation, experiments were performed with purified immunoglobulin.

Cultured A431 cells were inoculated into athymic mice; the animals were treated with various amounts of purified MoAb 528, 225, or 455, and the effects upon tumor formation were tested. Because experiments with T222 cells and Li-7 cells used minced tumor, rather than a suspension of cultured cells, the effect of MoAb treatment on growth of A431 minced tumor was examined also (Table 2). Treatment with increasing concentrations of MoAbs resulted in progressive reduction in mean tumor size in each case.

The inhibitory effect of MoAbs 225 and 455 was somewhat less than that of MoAb 528 on tumors from A431 cells. Complete

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tumor formation was obtained with the 2.0-mg dosage, whereas with tumors from cultured cells, growth was completely suppressed with only 0.2 mg. We estimate that an inoculum of 0.2 ml of minced tumor tissue contains, at most, 1 to 2 x 107 cells, which is comparable to the inoculum of cultured A431 cells. Our estimate is based on the following considerations: a volume of 0.2 ml of packed cultured A431 cells contains about 4 x 107 cells; and microscopic examination shows that approximately 50 to 70% of the A431 minced tumor consists of connective tissue, necrotic tumor tissue, and dead cells. We reason that the weaker inhibitory response with tumors from minced tissue may be attributable to the fact that the minced tumor retains some of the preexisting histological structure of tumor tissue and may be capable of producing tumors more easily than a single-cell suspension. When minced tumor was inoculated s.c., measurable residual tissue always remained even when tumor formation was inhibited, probably due to the connective tissue present in the inoculum. The residual s.c. mass never exceeded 0.3 cu cm. It should be noted that growing tumors did not appear in adequately treated animals, which were observed for 2 months after MoAb treatment was discontinued.

Clearance of 125I-labeled Anti-EGF Receptor MoAb in Athymic Mice Carrying A431 Tumors. To determine the clearance of anti-EGF receptor MoAbs, 125I-labeled MoAb 528 was injected i.p. into athymic mice carrying A431 tumors, and the specific radioactivity in the blood was measured on successive days as described in "Materials and Methods" (Chart 4). When 0.6 μg of 125I-MoAb 528 was administered, most of the acid-precipitable radioactivity was cleared from the blood in 2 to 4 days. When 1 mg of unlabeled MoAb 528 was added as a carrier at the time of 125I-MoAb 528 injection, the radioactivity remained in the blood for up to 8 days. For the former group of mice, the specific radioactivities were determined in the muscle and in the tumor 6 days after injection. The ratio of the specific radioactivity (tumor: muscle) was 3.46 ± 0.43. The results suggest that anti-EGF receptor MoAb is concentrated in the tumors, and the clearance data indicate that treatment on a twice weekly schedule is reasonable. When mice were given injections of 125I-MoAb 528 plus carrier MoAb 528, the specific activity of blood remained much higher than that of tumor and muscle even 9 days after injection.

Growth Inhibition of Other Human Tumor Cells by Administration of Anti-EGF Receptor MoAb. As described above, administration of anti-EGF receptor MoAbs inhibited the growth of A431 cells in athymic mice. To ascertain whether the effect of this MoAb treatment upon tumor xenograft growth is generalized, we tested the effects of MoAb 528 on the growth of Li-7 human hepatoma cells, HeLa cells, and T222 human lung epidermoid carcinoma cells. The results are illustrated in Table 3. The growth of Li-7 human hepatoma cells and HeLa cells was not affected by this treatment. However, the growth of minced T222 tumors was inhibited significantly, even at a 0.002-mg dose. The inhibitory effect of MoAb 528 on this tumor growth was greater than that with minced A431 tumors (Table 2, Experiment 4).

125I-EGF Binding Assay. One reason for selectivity in the antitumor activities of MoAb treatments might be differences in ligand binding to the various target cell populations. One important variable is the number of receptors for EGF on these cells, because it is known that A431 cells have a number of EGF receptors which is far higher than that reported for HeLa cells. We compared the number of receptors and the apparent dissociation constants for 125I-EGF on the cells used in these studies, using the procedures described in "Materials and Methods." The numbers of receptors on A431, T222, Li-7, and HeLa cells (and their Kd values) were: 2.8 x 104/cell (Kd 1.6 x 10^-9 M); 2.9 x 104/cell (Kd 4.2 x 10^-9 M); 2.8 x 104/cell (Kd 2.3 x 10^-9 M); and 2.0 x 104/cell (Kd 7.1 x 10^-9 M), respectively. Thus, A431 cells have a greater number of receptors for this growth factor than do T222, Li-7, and HeLa cells, by a factor of 2 logs.

Using saturating amounts of 125I-MoAb 528, the number of binding sites for MoAb was compared between A431 cells and T222 cells. In this experiment, the numbers of sites on A431 cells and T222 cells were 2.0 x 104/cell and 4.0 x 104/cell, respectively, demonstrating that A431 cells had 50 times more binding sites than T222 cells.

### Table 3

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Tumor cells</th>
<th>No. IgG treat.</th>
<th>Tumor formation</th>
<th>Tumor size (cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Hepatoma (Li-7)</td>
<td>0</td>
<td>6/6</td>
<td>4.5 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>HeLa</td>
<td>0</td>
<td>6/6</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>Lung epidermoid carcinoma (T222)</td>
<td>0.2</td>
<td>6/6</td>
<td>0.2 ± 0.0 (p&lt;0.001)</td>
</tr>
<tr>
<td>4</td>
<td>Lung epidermoid carcinoma (T222)</td>
<td>0.002</td>
<td>5/5</td>
<td>0.6 ± 0.0 (0.001&lt;p&lt;0.01)</td>
</tr>
</tbody>
</table>

* Number of animals in which tumors formed per total number of animals studied.

* Average ± S.E.
binding sites for MoAb 528 than did T222 cells. These data suggest that the number of EGF receptor sites on tumor cell surface membranes does not account for differences in MoAb antitumor activity against the various cell lines examined in these studies.

**DISCUSSION**

The results of our present studies show that the growth of A431 and T222 tumor xenografts in athymic mice is remarkably inhibited by anti-EGF receptor MoAbs. When MoAb 528 is used in therapy against Li-7 hepatoma or HeLa cells, under identical conditions, tumor growth remains unaffected. Both A431 cells and T222 cells were derived from epidermoid carcinomas. HeLa cells were established from a cervical cancer and were considered to be epidermoid carcinoma cells (5). However, a reexamination of the original slides revealed that the tumor is an adenocarcinoma (12). The results suggest that tumor growth suppression by MoAbs against EGF receptors may be limited to epidermoid carcinomas. The fact that A431 cells (vulva source) and T222 cells (lung source) both respond to MoAb therapy indicates that the tissue origin of the EGF receptors used to raise MoAbs is not a relevant factor in the antitumor effect. Presently, we are testing the effect of these MoAbs on the growth of a variety of human tumor xenografts in athymic mice to clarify these points.

The number of EGF receptors on A431 cells is approximately 100-fold higher than that on Li-7 and HeLa cells, but the receptor number on T222 cells is comparable to that on Li-7 and HeLa cells. This suggests that the EGF receptor number on the cell surface membranes is not an important determinant in tumor growth inhibition.

Among the 3 MoAbs utilized in our studies, 2 are capable of competing with EGF for receptor binding and of inhibiting A431 cells in culture; the other is incapable of blocking EGF binding to its receptors and has no significant effect on the growth of cultured A431 cells.7 Despite the different in vitro characteristics, when administered to mice all 3 MoAbs inhibited the tumor formation by A431 cells. This suggests that binding of MoAbs to EGF receptors, and not blocking of EGF from binding to its receptors, is the essential process leading to suppression of tumor growth in the animals. However, it is also possible that binding of MoAbs to EGF receptors could alter the actual function of the receptors, resulting in tumor growth inhibition.

Recently, Koprowski and coworkers (8, 28) proposed that only MoAbs of isotype IgG2a can inhibit the growth of human tumors in athymic mice. As described in our studies, we found that not only MoAb of isotype IgG2a (No. 528), but also MoAbs of IgG1 isotype (Nos. 225 and 455) were capable of inhibiting tumor growth. This difference may be due to the fact that MoAbs used in their studies were raised against tumor-associated antigens, whereas the MoAbs in our studies were raised against a growth factor receptor. These 2 types of MoAbs might exert antitumor activity by different mechanisms.

Data from cell culture studies in our laboratory, as well as many others, suggest that malignant cells have specific requirements for cell proliferation. These include some growth-promoting agents, such as hormones, growth factors, and transport proteins, that often are needed or may be essential for selected cell types (i.e., EGF, transferrin, and insulin) (2). Furthermore, studies in which essential hormones, growth factors, and nutrients are withheld from cultured cells have suggested that tumor cells are more likely to die when deprived of substances essential for growth than are normal cells (21). EGF is known to stimulate proliferation of various types of cells in culture, but when added to the culture medium of A431 cells, it results in growth inhibition. In comparison to the studies with MoAbs conducted in athymic mice inoculated with A431 cells, EGF therapy has no effect on tumor growth. This failure to inhibit growth of A431 cell tumors by EGF is possibly attributed to the short half-life of EGF in animals (3). In contrast, anti-EGF receptor MoAbs have a longer half-life of about 3 days, under the experimental conditions in our studies (Chart 4). Thus, MoAbs may provide an important method for controlling access of malignant cells to growth factors and hormones required for proliferation.

In cell culture, MoAbs 528 and 225 inhibit proliferation of A431 cells, but they do not kill the cells, since the cell number increases slightly in the presence of these MoAbs.7 When mice that received injections of A431 cells were treated with MoAb, more than 90% of the inoculated cells probably were killed by the antibody. The data in Tables 1 and 2 suggest that, if 106 or more cells of the 107 cell inoculum had survived the in vivo MoAb treatment, tumors would have formed. The mechanism by which MoAbs suppress tumor growth in animals remains unclear, but annihilation of A431 tumors by complement-mediated lysis is unlikely, because cultured A431 cells are not lysed in the presence of these MoAbs and complement. Since cytotoxic T-cells are absent in athymic mice, natural killer cells, antibody-dependent killer cells (K), and/or macrophages may have contributed to the inhibition of tumor formation in the animals. Further experiments should clarify the mechanisms by which anti-receptor MoAbs prevent tumor cell growth and explore refinements which could lead to direct application in the treatment of human cancer.

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