Suppression of Solid Tumor Growth by Immunoneutralizing Monoclonal Antibody against Human Basic Fibroblast Growth Factor

Akira Hori, Reiko Sasada, Etsuya Matsutani, Kenichiro Naito, Yasufumi Sakura, Takeshi Fujita, and Yoshio Kozai

ABSTRACT

Basic fibroblast growth factor (bFGF) is a potent angiogenic mitogen. To elucidate the effect of bFGF inhibitors in vivo, anti-bFGF immunoneutralizing monoclonal antibody was prepared. One monoclonal antibody against human bFGF, obtained by cell fusion and designated 3H3, completely inhibited bFGF-induced proliferation of human umbilical vein endothelial cells at a concentration of 100 ng/ml. 3H3 did not bind to acidic fibroblast growth factor or HST1 protein, indicating high specificity for bFGF. Furthermore, the immunoneutralizing activity of 3H3 was examined in vivo. K1000 cells (a BALB/c 3T3 transformant in which the leader sequence-fused bFGF gene was transfected) were transplanted s.c. into BALB/c nude mice. Growth of the tumor cells was inhibited by i.v. treatment with 3H3 at a concentration of 200 μg/mouse. Histological observation showed that the antitumor effect of 3H3 was due to the inhibition of bFGF-induced angiogenesis. This experiment provides direct causal evidence for the hypothesis that tumor growth is angiogenesis dependent. This finding could also have implications for the development of novel therapeutic approaches to angiogenic solid tumors.

INTRODUCTION

bFGF is a strong mitogen for a wide variety of mesoderm- or neuroectoderm-derived cells (1, 2). Recently, several oncogenic characteristics of bFGF have been revealed: (a) bFGF is synthesized by several types of tumor cells such as hepatoma cells, rhabdomyosarcoma cells, and retinoblastoma cells (3–5); (b) several oncogene products such as int-2 (6), HST1/K-fgf (7–9), FGF5 (10), and FGF6 (11) show highly conserved homologous amino acid sequences with bFGF as well as aFGF; (c) when cells are transfected with bFGF cDNA, drastic morphological changes and anchorage-independent growth are observed (12), and when cells were transfected with signal peptide-fused bFGF, these transfected cells were highly tumorigenic in nude mice (13); and (d) bFGF is a potent tumor-derived angiogenic factor which induces the formation of new capillary blood vessels (14, 15).

To study the biological activities of bFGF, monoclonal antibodies with immunoneutralizing activity against bFGF have been reported (16, 17). These antibodies inhibited the bFGF-induced cell proliferation in vitro and the angiogenesis induced by exogenous bFGF in vivo. However, it has not been clearly demonstrated that tumor growth can be suppressed by inactivation of bFGF in vivo. In this work, we present in vivo evidence that the anti-bFGF immunoneutralizing monoclonal antibody shows an antitumor effect mediated by the inhibition of angiogenesis.

MATERIALS AND METHODS

Animals. BALB/c mice and athymic BALB/c nude mice (nu/nu) were purchased from the Charles River Breeding Laboratories (Osaka, Japan). Six-week-old female BALB/c mice were used for immunization. Eight-week-old female BALB/c nude mice were used to investigate the in vivo effect of anti-bFGF antibodies. Experiments were performed according to the NIH Guideline for the Care and Use of Laboratory Animals.

Materials. Recombinant human bFGF (18), bFGF mutein, CS23 (the serine residue is substituted for the cysteine residue at 70 and 88 of bFGF protein) (19), aFGF (20), HST1 protein, and anti-human bFGF nonimmunoneutralizing monoclonal antibody, MAB78 (21), were provided by Dr. K. Igarashi (Biology Research Laboratories, Takeda Chemical Ind. Co., Ltd., Osaka, Japan).

Cell Lines. The mouse myeloma cell line, SP2/0-Ag14, was purchased from the American Type Culture Collection. HUVE cells were kindly donated by Dr. I. Yamane (Institute for Tuberculosis and Cancer, Tohoku University, Sendai, Japan) (22). HUVE cells maintained in GIT medium (Nihon Pharmaceutical Co., Ltd., Osaka, Japan) supplemented with 2.5% heat-inactivated fetal calf serum and 2 ng/ml bFGF in a two-gas incubator containing 5% CO2 and 7% O2. The mouse BALB/c fibroblast 3T3 cell line, A31, was kindly donated by Dr. T. Kakunaga (23). A31 cells were maintained in Dulbecco-Vogt modified Eagle’s minimum essential medium supplemented with 5% calf serum in an incubator containing 5% CO2.

Preparation of Immunoneutralizing Monoclonal Antibody against bFGF. CS23 (50 μg/mouse) was immunized three times i.p. and then once i.v. every 2 weeks. The spleen cells were fused with SP2/0 Ag14 cells 3 days after final immunization. The hybridomas were selected by Dulbecco-Vogt modified Eagle’s minimum essential medium containing 10% fetal calf serum, 1.6 mM thymidine, 10 mM hypoxanthine, and 0.4 mM aminopterin (21).

Culture supernatants from hybridomas were screened for their anti-bFGF immunoneutralizing activity by investigating the effect on the bFGF-induced HUVE cell proliferation as described in the cell proliferation assay. Hybridomas which secreted anti-bFGF immunoneutralizing monoclonal antibodies were cloned by limiting dilution. Anti-bFGF monoclonal antibodies were purified by a protein A Sepharose CL-4B (Pharmacia, Sweden) column and a diethylaminoethanol cellulose (DE52; Whatman, Clifton, NJ) column.

Cell Proliferation Assay. HUVE cells or K1000 cells were plated on 24-well multilanes (Flow Laboratories, McLean, VA) on day 0 (1 × 104 cells/well). On day 1, culture supernatants of hybridomas or various doses of monoclonal antibody were added in the presence or absence of bFGF (2 ng/ml) into the medium simultaneously. On day 4, cell numbers were counted with a Coulter particle counter after trypsinization.

Competition Assay. A polyvinyl microtiter plate (Nunc, Denmark) was coated with bFGF (100 ng/well) for 2 h. 3H3 (100 ng/ml) was preincubated for 1 h with various doses of competitors prior to incubation with the precoated bFGF. The plate was then incubated at 37°C for 3 h and each competitor for 2 h. The plate was then incubated with anti-mouse IgG coupled to alkaline phosphatase (BioRad, Cambridge, MA). After a 2-h incubation, the plate was washed extensively, and p-nitrophenyl phosphate disodium (1 mg/ml; Sigma, St. Louis, MO) was added. The alkaline phosphatase reaction was measured by the increase in absorbance at 405 nm.

Establishment of K1000 Cell Line. The K1000 cell line was established by transfection of an expression vector, pTB1000, coding the signal peptide-fused bFGF. The pTB1000 consists of the following
were counted with a Coulter counter after trypsinization. Added in the presence (•) or absence (○) of 2 ng/ml bFGF. On day 4, the cells containing the long terminal repeat region of murine leukemia virus, a SV40-derived promoter, and the leader sequence of human interleukin-2; a 0.38-kilobase EcoRI-BamHI fragment from pTB732 (12) containing the region coding for the COOH-terminal end of human bFGF and a pBR322-derived plasmid. These three fragments were ligated via the EcoRI, BamHI, and Clal sites. A31 cells were transfected with 10 µg of pTB1000 by the calcium phosphate coprecipitation procedure (25). The transfected cells were selected by focus-forming assay, and the K1000 cell line was cloned by limiting dilution. Secreted bFGF in the K1000 cell culture supernatant was confirmed by Western blotting and enzyme-linked immunosorbent assay. Basic FGF secreted by K1000 cells was confirmed to display biological activity and binding ability with 3H3 and nonimmunoneutralizing monoclonal antibody, MAb78.

Treatement for Tumor-bearing Mice. Monoclonal antibodies were dissolved in phosphate-buffered saline. Groups of 5 mice were each inoculated s.c. with 3 x 10^6 K1000 cells cultured in vitro. Tumor-bearing animals were randomly assigned to different cages. Monoclonal antibodies were injected i.v. from the tail vein in a carrier volume of 0.2 ml. Tumor measurements were made in two directions using calipers, and tumor volume was calculated by using a^2b/2, where a is the width and b is the length of the tumor.

Histology and Hit Point Method. Tumors were fixed in 10% neutral buffered formalin for a minimum of 2 days. These specimens were dehydrated with ethanol, cleared with xylene, and infiltrated with paraffin. Slides were stained with hematoxylin and eosin. The frequency of blood vessels per unit area in the peripheral region of K1000 tumor was evaluated morphometrically using a hit point method (26). Eight different pictures of the histological section were taken at random for each tumor (magnification, ×288). One picture covered 0.285 mm^2 of tumor. To count blood vessels, the picture was overlaid with a colorless sheet containing a dot matrix (28 x 17; 408 total). Dots which overlapped with capillaries including red blood cells were scored.

RESULTS

Effect of 3H3 on the Proliferation of HUVE Cells. To assess the ability of bFGF inhibitors to suppress solid tumor growth, we attempted to prepare anti-bFGF immunonutalizing monoclonal antibodies as bFGF inhibitors. BALB/c mice were immunized with mutein bFGF, CS23, which shows the same biological activity as bFGF. As a result, we obtained one immunonutralizing monoclonal antibody against human bFGF (8), designated 3H3, which belongs to the IgG1 subclass. To determine the immunonutralizing activity of 3H3 exactly, we examined its ability to inhibit the bFGF-induced proliferation of HUVE cells (Fig. 1). For this study, 2 ng/ml bFGF and various concentrations of 3H3 were incubated simultaneously, and the cell number was measured 3 days after the addition of bFGF. In the presence of bFGF, 3H3 inhibited the proliferation induced by exogenous bFGF in a dose-dependent manner. The bFGF-induced proliferation of HUVE cells was completely inhibited by 100 ng/ml 3H3. In contrast, 3H3 showed no effect on the cell number in the absence of bFGF, indicating that the inhibition by 3H3 is not due to nonspecific cytotoxicity.

Binding Specificity of 3H3 to bFGF. Recently, several molecules which are members of the FGF family have been reported to have amino acid sequences which share homology with bFGF (6-11, 20). Acidic FGF and HST1 also belong to the FGF family and have amino acid sequences showing homology with bFGF. To check the binding specificity of 3H3 to bFGF, we examined the effect of aFGF and HST1 as competitors for the binding of 3H3 to bFGF (Fig. 2). The binding of 3H3 to bFGF coated to plastic wells was competitively blocked by bFGF in a dose-dependent manner. However, aFGF and HST1 showed no effect on the binding of 3H3 to bFGF coated to plastic wells even at a concentration of 20 µg/ml.

Antitumor Effect of 3H3 against K1000. To investigate the immunonutralizing activity of 3H3 in vivo, we artificially constructed K1000 cells containing leader sequence-fused bFGF cDNA. K1000 cells demonstrated tumorigenicity in a BALB/c nude mouse model. To elucidate the antitumor activity of 3H3, we examined the effect of 3H3 on the in vitro growth of K1000 cells in this system (Fig. 3/4). Briefly, 3 x 10^6 K1000 cells cultured in vitro were transplanted s.c. into a BALB/c nude mouse on day 0. 3H3, at a dose of 100 µg/mouse/day, was then administered i.v. daily for 5 days starting on day 3. Animals in the control group received injections of nonimmune mouse IgG at the same dose. In addition, the effect of anti-bFGF nonimmunoneutralizing monoclonal antibody, MAb78, was also examined at the same dose. Tumors in the control group doubled in size approximately every 4-5 days. However, successive injections of 3H3 retarded further growth of the tumors (34% of control on day 14). In contrast, the same treatment with nonimmunoneutralizing monoclonal antibody showed no antitumor effect. On the other hand, the prolifera-

![Fig. 1. Immunoneutralizing activity of 3H3. Duplicate samples of HUVE cells (1 x 10^4 cells/well) were seeded on day 0. On day 1, various doses of 3H3 were added in the presence (•) or absence (○) of 2 ng/ml bFGF. On day 4, the cells were counted with a Coulter counter after trypsinization.

![Fig. 2. Binding specificity of 3H3 to bFGF. The effect of aFGF (○), HST1 (■), or bFGF (●) on the binding of 3H3 to bFGF was investigated using competition assay. The binding of 3H3 to the coated bFGF was measured by the increase in absorbance at 405 nm.](cancerres.aacrjournals.org)
overlapped with blood vessels numbered 121.0 ± 42.3 (SD) in was scored using a hit point method (Table 1). Dots which vessels in the 3H3-treated tumor on day 12 was evaluated groups. To confirm quantitatively that blood vessel formation (Fig. 4C). In contrast, in the 3H3-treated sections, vessels were which had arisen from the new blood vessels observed on day 6. In addition, congestion and red cell stasis were recognized sparse, and sinus-like capillaries were not observed (Fig. 4D). On day 6, the mean volumes of 3H3-treated tumors were 36% and 35%, respectively, of those of control tumors. On day 6, hematoxylin-eosin-stained control tumor sections demonstrated that intense neovascularization was induced around the tumor (Fig. 4A). In contrast, neovascularization was clearly suppressed in sections of 3H3-treated tumor (Fig. 4B). On day 12, control tumor sections demonstrated the formation of sinus-like capillaries in the peripheral region of the tumor, which had arisen from the new blood vessels observed on day 6. In addition, congestion and red cell stasis were recognized (Fig. 4C). In contrast, in the 3H3-treated sections, vessels were sparse, and sinus-like capillaries were not observed (Fig. 4D). A further observation was that the degree of infiltration of lymphocytes and neutrophils into the tumor, a marker of immune response, was not different between treated and untreated groups. To confirm quantitatively that blood vessel formation in the 3H3-treated tumor was suppressed, the depletion of blood vessels in the 3H3-treated tumor on day 12 was evaluated morphometrically. The frequency of capillaries per unit area was scored using a hit point method (Table 1). Dots which overlapped with blood vessels numbered 121.0 ± 42.3 (SD) in the untreated control group. On the other hand, there were 19.5 ± 11.8 dots in the 3H3-treated group (16.1% of control group).

**DISCUSSION**

The role of bFGF in tumor growth has been reported previously (1, 2), but it has not been demonstrated that tumor growth can be suppressed by inactivation of bFGF in vitro. To assess the ability of bFGF inhibitors to suppress solid tumor growth, we prepared anti-bFGF immunoneutralizing monoclonal antibody as a bFGF inhibitor. An anti-bFGF monoclonal antibody, 3H3, showed strong immunoneutralizing activity against bFGF-induced HUVE cell proliferation. Competition assays indicated that 3H3 had a high binding affinity for bFGF, although bind to the same receptor (27, 28). Therefore, the effect of 3H3 is to specifically block the biological activity of bFGF not only in vitro but also in vivo. To examine the antitumor effect of 3H3, it was necessary to develop tumor cells that secreted bFGF as their sole angiogenic factor. Because bFGF cDNA lacks a leader sequence for the secretion of translation product (18) and native bFGF cDNA transfectants did not demonstrate tumorigenicity in the BALB/c nude mouse model (12, 13), we artificially constructed K1000 cells containing a leader sequence-fused bFGF cDNA. K1000 cells secreted bFGF and demonstrated tumorigenicity in the BALB/c nude mouse model. Therefore, the tumorigenicity of K1000 cells is acquired by the leader-dependent secretion of bFGF from K1000 cells. These findings indicate that the in vivo growth of K1000 cells depends on bFGF and that K1000 cells are suitable to evaluate the antitumor effect of 3H3.

While examining the antitumor effect of 3H3 against K1000, 3H3 was found to retard the growth of K1000 tumors in vivo. There are several possible explanations for this finding. One possibility is that the K1000 cells are lysed by a nonspecific immune response mediated by complement or activated lymphocyte binding to 3H3 at the tumor site, as may occur with anti-P-glycoprotein monoclonal antibodies MRK-16 and MRK-17 (29). To investigate this, we examined the antitumor effect of the anti-bFGF nonimmunoneutralizing monoclonal antibody against K1000; however, the antibody demonstrated no antitumor effect against K1000 cells in vitro. Since the antitumor effect of 3H3 is due to immunoneutralizing activity and not just binding ability with bFGF, the antitumor effect of 3H3 is probably not due to a nonspecific immune response. Furthermore, the infiltration of lymphocytes at the peripheral region of the tumor was not induced by the 3H3 treatment as judged by histological observation. Therefore, the antitumor effect of 3H3 is not due to a nonspecific immune response. The role of bFGF in tumor growth has been reported previously (1, 2), but it has not been demonstrated that tumor growth can be suppressed by inactivation of bFGF in vitro. To assess the ability of bFGF inhibitors to suppress solid tumor growth, we prepared anti-bFGF immunoneutralizing monoclonal antibody as a bFGF inhibitor. An anti-bFGF monoclonal antibody, 3H3, showed strong immunoneutralizing activity against bFGF-induced HUVE cell proliferation. Competition assays indicated that 3H3 had a high binding affinity for bFGF, although bind to the same receptor (27, 28). Therefore, the effect of 3H3 is to specifically block the biological activity of bFGF not only in vitro but also in vivo. To examine the antitumor effect of 3H3, it was necessary to develop tumor cells that secreted bFGF as their sole angiogenic factor. Because bFGF cDNA lacks a leader sequence for the secretion of translation product (18) and native bFGF cDNA transfectants did not demonstrate tumorigenicity in the BALB/c nude mouse model (12, 13), we artificially constructed K1000 cells containing a leader sequence-fused bFGF cDNA. K1000 cells secreted bFGF and demonstrated tumorigenicity in the BALB/c nude mouse model. Therefore, the tumorigenicity of K1000 cells is acquired by the leader-dependent secretion of bFGF from K1000 cells. These findings indicate that the in vivo growth of K1000 cells depends on bFGF and that K1000 cells are suitable to evaluate the antitumor effect of 3H3.

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Fig. 4. Histological examination of K1000 tumor excised from untreated and 3H3-treated mice. K1000 cells (3 x 10^6) were transplanted s.c. into a BALB/c nude mouse (n = 3) on day 0. 3H3 (100 µg/mouse/day) was injected into the mice i.v. daily for 6 days after starting on day 1. H&E, x 166. Right, tumor; left, muscle. Arrows, representative new blood vessels. A, section of tumor from an untreated mouse on day 6; B, section of tumor from a 3H3-treated mouse on day 6; C, section of tumor from an untreated mouse on day 12; D, section of tumor from a 3H3-treated mouse on day 12.

Table 1 Decrease of blood vessels in 3H3-treated K1000 tumor

The frequency of blood vessels per unit area was scored morphometrically using the hit point method. Untreated and 3H3-treated tumors excised on day 12, shown in Fig. 4, were investigated. Eight areas of the peripheral region for each tumor were observed. The number of dots overlapping with blood vessels is shown below. The differences between the 3H3-treated and untreated groups are significant at P < 0.001 (Student's t test).

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Untreated tumor</th>
<th>3H3-treated tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>134.8 ± 38.5</td>
<td>23.0 ± 13.4</td>
</tr>
<tr>
<td>2</td>
<td>141.4 ± 36.8</td>
<td>20.3 ± 12.9</td>
</tr>
<tr>
<td>3</td>
<td>86.8 ± 27.5</td>
<td>15.3 ± 6.7</td>
</tr>
<tr>
<td>Average</td>
<td>121.0 ± 42.3</td>
<td>19.5 ± 11.8</td>
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</tbody>
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Furthermore, the formation of new blood vessels on day 6 and sinus-like capillaries on day 12 was clearly suppressed in the 3H3-treated group. These results provide strong evidence that the antitumor effect of 3H3 against K1000 tumor growth is due to the inhibition of bFGF-induced angiogenesis.

Dennis and Rifkin (30) reported that anti-bFGF immunoneutralizing polyclonal antibodies showed neither antiangiogenic nor antitumor effects in vivo against several murine tumors. It was unclear whether the angiogenic activity or the growth of the tumors which they used were solely dependent on secreted bFGF. In contrast, we show that the angiogenic activity of K1000 cells and subsequent tumor growth in vivo are dependent...
on the angiogenic effect of bFGF.

In this study, we obtained evidence that a specific inhibitor of bFGF, a potent angiogenic factor, inhibited tumor angiogenesis and tumor growth. In this system, bFGF was the sole mediator of angiogenesis. The inhibition of K1000 tumor growth by 3H3 provides compelling direct evidence for the hypothesis that tumor growth is angiogenesis dependent (31). This finding may have important clinical applications for the treatment of those angiogenic solid tumors for which bFGF is the only angiogenic mediator. For effective therapy, it may be necessary to screen human tumor cells which secrete bFGF and induce angiogenesis and to examine the antitumor effect of 3H3 against them.

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