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Inhibition of cell growth and tumorigenesis of human glioblastoma cells by a neutralizing antibody against human basic fibroblast growth factor

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We report here that a neutralizing mouse monoclonal antibody against basic FGF inhibited both anchorage-dependent and anchorage-independent growth of U-87MG and T98G human glioblastoma cells and HeLa cells, all of which express both the basic FGF and the FGF receptor genes. In addition, the subcutaneous administration of this antibody significantly suppressed the tumor development of these tumor cells in nude mice. Therefore, basic FGF plays an important role in neoplastic growth of these cells. The neutralization of basic FGF will be effective in controlling the growth of tumors, such as glioblastoma and other cancer cells which bear basic FGF and FGF receptors.

Basic fibroblast growth factor; Fibroblast growth factor receptor; Mouse monoclonal antibody; Glioblastoma; HeLa cell

1. INTRODUCTION

Autonomous cell growth and tumorigenesis may result from constitutive interaction of cellular growth factors with their corresponding receptors [1]. Basic fibroblast growth factor (FGF) is a mitogen and a differentiation factor for neuroectoderm-derived cells or mesoderm-derived cells [2] as well as a potent angiogenic factor [2,3]. We recently demonstrated using Northern hybridization that basic FGF was abundantly produced in 94.4% of human glioma tissues in a tumor origin-specific manner [4]. Furthermore, a number of glioma cells are known to bear FGF receptors [5,6], and U-87MG human glioblastoma cells have been reported to produce and release basic FGF extracellularly as an autocrine factor [7]. We have suggested that glioma-derived basic FGF might be involved in their autonomous growth and tumorigenesis as an autocrine growth factor in vivo [4]. In addition, many other tumor cells have been previously reported to produce basic FGF and to be dependent upon their cellular basic FGF [2,8]. In order to determine whether basic FGF is responsible for the autonomous growth and tumorigenesis of human glioblastoma cells and other basic FGF expressing cells, we examined the inhibitory effect of a neutralizing anti-basic FGF IgG on cell growth in monolayer culture, colony formation in soft agar and tumorigenesis in nude mice.

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Abbreviations: FGF, fibroblast growth factor

2. MATERIALS AND METHODS

2.1. *Cells and cell culture*

U-87MG and T98G human glioblastoma cells were obtained from Riken Cell Bank Inc. and Japan Cancer Research Resource Bank Inc., respectively. A431 human epidermoid carcinoma cells and HeLa cells were kindly provided by K. Lee and K. Tsuboi, respectively. All cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS, depleted of complement activity) at 37°C in an atmosphere of 5% CO_2/95% air.

2.2. *Antibody*

As a neutralizing antibody against human basic FGF, 3H3 mouse monoclonal antibody (3H3 MoAb, mouse IgG class 1) was generated by immunizing with basic FGF mutein CS23 (supplied by Takeda Chemical Industries Ltd.), within which two serine residues are substituted for two cysteine residues at positions 70 and 88 in the natural human basic FGF. On a Western blot analysis, 3H3 MoAb crossreacted with basic FGF and basic FGF mutein CS23, but without acidic FGF, HST1, epidermal growth factor or interleukin-2. This antibody can neutralize the effect of exogenous basic FGF in a dose-dependent manner; 100 ng/ml of 3H3 MoAb inhibited entirely the endothelial cell proliferation induced by 2 ng/ml basic FGF [9].

2.3. *Northern blot analysis*

Cells grown to confluence were lysed in 5 M guanidinium thiocyanate and total RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method [10]. Ten micrograms of poly(A)$^+$ RNA selected using oligo(dT) cellulose affinity chromatography were denatured in 1 M glyoxal/50% dimethyl sulfoxide, fractionated by electrophoresis in 1% agarose gels and

transferred to diazophenylthioether paper (Schleicher and Schuell, Inc., Keene, NH). The following cDNA probes were used for hybridization: human basic FGF (a 0.4-kb *Bam*HI fragment [11]) and human FGF receptor (a 2.8-kb *Eco*RI fragment [12]). These probes were labeled with [α-^{32}P]dCTP by random priming and hybridization was carried out as previously described [4]. The final washes were performed twice under stringent conditions using 0.1 × SSC and 0.5% SDS at 65°C for 30 min each time [4]. Filters were then autoradiographed for two days at −70°C using intensifier screens.

2.4. *Immunofluorescence*

Cells were grown on glass coverslips, then fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) for an hour at 4°C and washed with PBS (5 min, twice). The fixed cell monolayers were permeabilized with 0.1% NP-40 in PBS for 10 min at room temperature, washed with PBS three times. After preincubation with normal rabbit serum diluted with PBS, 3H3 MoAb mouse monoclonal antibody against human basic FGF (40 μg/ml) or non-immune mouse serum was applied to coverslips for one hour at 37°C. After washes with PBS (for 15 min, 3 ×), cells were incubated for one hour at 37°C with FITC-conjugated rabbit anti-mouse IgG. Then, coverslips were washed with PBS (for 15 min, 4 ×), mounted on glass slides and examined in a microscope equipped for epifluorescence. Micrographs were obtained after exposure of 40–80 s (Kodak Tmax 800 Asa).

2.5. *Cell growth assays*

Cells (5×10^3 cells/well) were grown in DMEM/10%FCS and seeded in 48-well plates (11.3-mm wells, Costar). After cells had become attached to the substratum, the media was changed to fresh DMEM/10% FCS containing 3H3 MoAb anti-basic FGF IgG_1 (100 μg/ml), normal mouse IgG_1 (100 μg/ml) or no IgG. After three days, triplicate cultures were trypsinized and counted with an improved Neubauer hemocytometer. Cell viability was determined by dye exclusion test with Trypan blue.

2.6. *Soft agar colony assays*

DMEM/10% FCS (2 ml) containing 0.5% agarose (SeaPlaque, FMC) was added to 35-mm dishes and allowed to solidify. The cells of U-87MG, T98G and HeLa cells were trypsinized, suspended in DMEM/10% FCS (1 ml) containing 0.3% agarose and 3H3 MoAb anti-basic FGF IgG_1 (100 μg/ml) or normal mouse IgG_1 (100 μg/ml), and were plated in triplicate at a density of 8×10^3 cells per dish. A-431 cells were suspended in the medium containing 0.2% agarose, 400 pM epidermal growth factor (Receptor grade, Collaborative Research Inc.) and anti-basic FGF antibody (100 μg/m) or normal mouse IgG_1 (100 μg/ml), and plated in triplicate at a density of 2×10^4 cells per dish. These cultures were incubated at 37°C in a 5% CO_2 atmosphere for two weeks. Colonies which were then larger than 60 μm in diameter were counted.

2.7. *The antitumorigenic activity of anti-basic FGF IgG in nude mice*

Cells (6×10^6 cells/mouse) were injected subcutaneously into the back of female BALB/c athymic nude mice (5 to 7 weeks old) in triplicate or quadruplicate. Two, four and six days after transplantation, 3H3 MoAb anti-basic FGF IgG (200 μg/mouse/day) or normal mouse IgG (200 μg/mouse/day) was injected into the subcutaneous space surrounding the tumor mass. Mice were monitored for the appearance of solid tumors, and the length and width of the tumors were measured using a caliper every other day. The volume of tumors was calculated according to the following formula [9]: tumor volume in $mm^3 = \text{length} \times (\text{width})^2 \times 0.5$. After mice were sacrificed, subcutaneous tumors with surrounding tissues were resected and fixed in Bouin's fixative. The histology of subcutaneous tumor and surrounding tissues was examined in paraffin embedded sections with hematoxylin and eosin staining.

3. RESULTS

3.1. *Expression of basic FGF and FGF receptor*

Northern blot analysis of basic FGF in T98G cells and HeLa cells revealed four bands at 7.0, 3.7, 2.3 and 1.5 kb (Fig. 1), corresponding to ones previously reported [13]. In addition, a 4.2-kb transcript for FGF receptor [14] was detected in T98G cells and HeLa cells (Fig. 1). A431 cells yielded no transcript for either basic FGF or FGF receptor.

3.2. *Immunofluorescence*

Bright staining was observed mainly in nuclei of U-87MG, T98G and HeLa cells while it was not detected in A431 cells (Fig. 2). In control experiment, no staining was observed when nonimmune serum was applied.

3.3. *Inhibition of Anchorage-dependent Growth*

Anti-basic FGF IgG significantly inhibited the growth of U-87MG, T98G, HeLa and A431 cells at

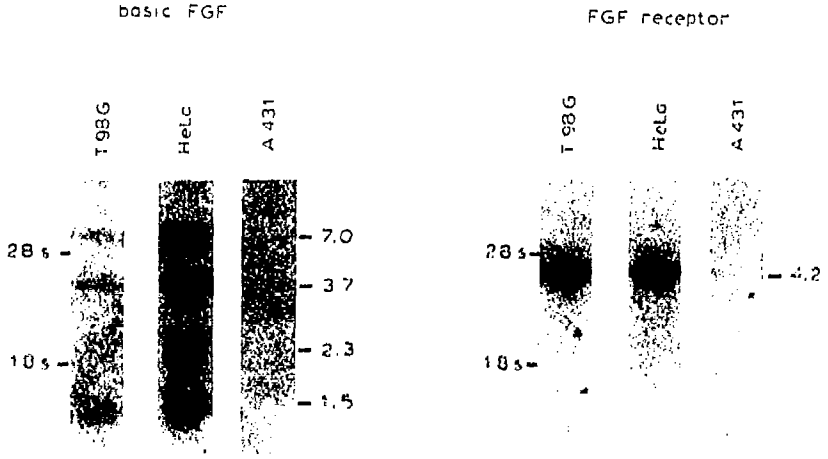

Fig. 1. Northern hybridization of basic FGF and FGF receptor. Ten micrograms of poly(A)$^+$ RNA were loaded in each slot.

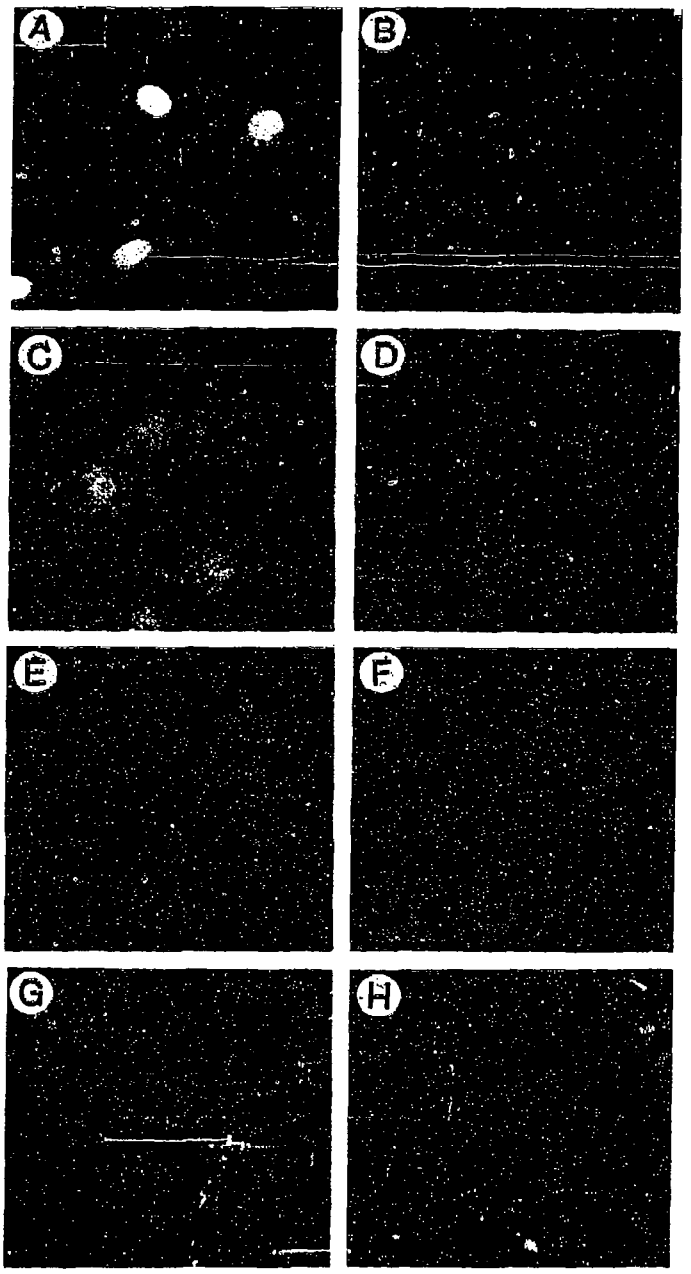

Fig. 2. Immunofluorescence staining with 3H3 MoAb anti-basic FGF antibody. Panels A and B show U-87MG cells; T98G cells, (C,D); HeLa cells, (E,F); A431 cells, (G,H); immunofluorescence applied with anti-basic FGF IgG (A,C,E,G); control with non-immune rabbit serum (B,D,F,H). Staining is mainly detected in nuclei of U-87MG, T98G and HeLa cells while it is not in A431 cells.

rates of 34.0% ($P<0.05$, assessed by t-test), 23.1% ($P<0.05$), 34.4% ($P<0.05$) and 84.6% (not significant), respectively, of cells incubated with normal mouse IgG_1 (Fig. 3). At a concentration of 100 μg/ml, anti-basic FGF IgG remarkably blocked the growth of U-87MG, T98G and HeLa cells, whereas normal IgG at the same concentration did not significantly affect their cell growth.

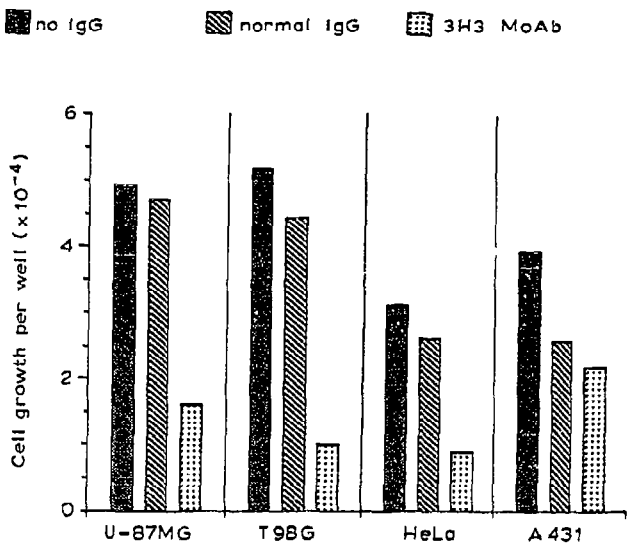

Fig. 3. The inhibitory effects of anti-basic FGF IgG on the anchorage-dependent growth of U-87MG, T98G, HeLa and A431 cells. Cells (5×10^3 cells/well) were seeded in 48-well plates, and incubated in DMEM/10% FCS with 3H3 MoAb (100 μg/ml), normal mouse IgG_1 (100 μg/ml) or no IgG for three days. The cell numbers of U-87MG, T98G, HeLa and A431 cells incubated with 3H3 MoAb for three days decreased at the rates of 34.0% ($P<0.05$, assessed by t-test), 23.1% ($P<0.05$), 34.4% ($P<0.05$) and 84.6% (not significant), respectively, compared to those with normal mouse IgG_1.

3.4. *Inhibition of anchorage-independent growth*

The sizes of U-87MG, T98G and HeLa cell colonies decreased significantly when they were incubated in anti-basic FGF IgG (Fig. 4). The numbers of colonies, which were larger than 60 μm in diameter, of U-87MG, T98G and HeLa cells incubated with anti-basic FGF antibody also decreased at rates of 20.7% ($P<0.05$), 6.6% ($P<0.01$) and 12.9% ($P<0.05$), respectively, compared to those with normal IgG_1 while those of A431 cells did not (Table I). Agarose containing 100 μg/ml of normal mouse IgG_1 did not inhibit colony formation of these cells compared to that containing no IgG (data not shown).

3.5. *Inhibition of tumorigenesis in nude mice*

The tumor development of U-87MG, T98G and HeLa cells in nude mice was apparently suppressed by subcutaneous administration of anti-basic FGF IgG (U-87MG, T98G and HeLa cells, $P<0.05$, assessed by the Wilcoxon rank sum test on the last day indicated in Fig. 5A–C). The mice injected with normal IgG did not show the inhibition of tumor development compared to those without injections (Fig. 5A–C). The tumorigenesis of A431 cells, however, was not suppressed by injections of a neutralizing antibody. A-431 tumors grew at exponential rates in both groups (Fig. 5D).

The sections of tumors of U-87MG, T98G and HeLa cells demonstrated that the surrounding capsules of

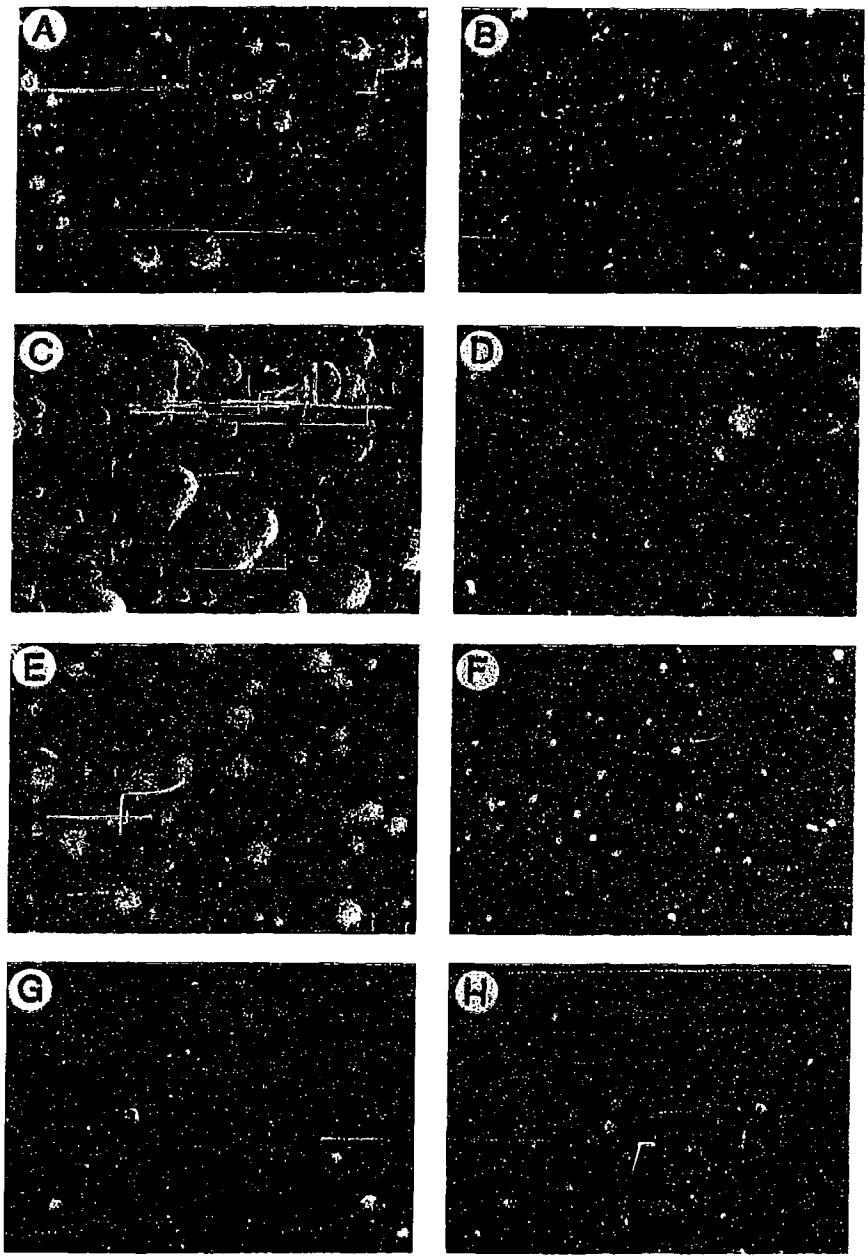

Fig. 4. Effect of anti-basic FGF IgG on the anchorage-independent growth. 8×10^3 Cells per 35-mm dish were suspended in DMEM/10% FCS containing 0.3% agarose and 3'I3 MoAb (100 μg/ml) or normal mouse IgG_1 (100 μg/ml). U-87MG cells (A,B), T98G cells (C,D), HeLa cells (E,F) and A431 cells (G,H) incubated with normal mouse IgG_1 (A,C,E,G) or with anti-basic FGF IgG (B,D,F,H) at a concentration of 100 μg/ml.

Table I

Inhibition of colony formations by anti-basic FGF IgG

Cells	Number of colonies in the presence of	
	Normal IgG	Anti-basic FGF IgG
U-87MG	4700	960
T98G	5100	660
HeLa	6700	440
A431	550	630

Normal mouse IgG_1 (100 μg/ml) or anti-basic FGF IgG was added at a concentration of 100 μg/ml. The colonies, which were larger than 60 μm in diameter, were counted.

tumors, which was composed of collagen fibers and fibroblasts originated from hosts, were thinner in the groups of mice injected with anti-basic FGF IgG than in those injected with normal IgG (Fig. 6).

4. DISCUSSION

Anti-basic FGF IgG was able to inhibit the anchorage-dependent and anchorage-independent growth of U-87MG, T98G and HeLa cells, but was ineffective when administered to A431 cells. We demonstrated us-

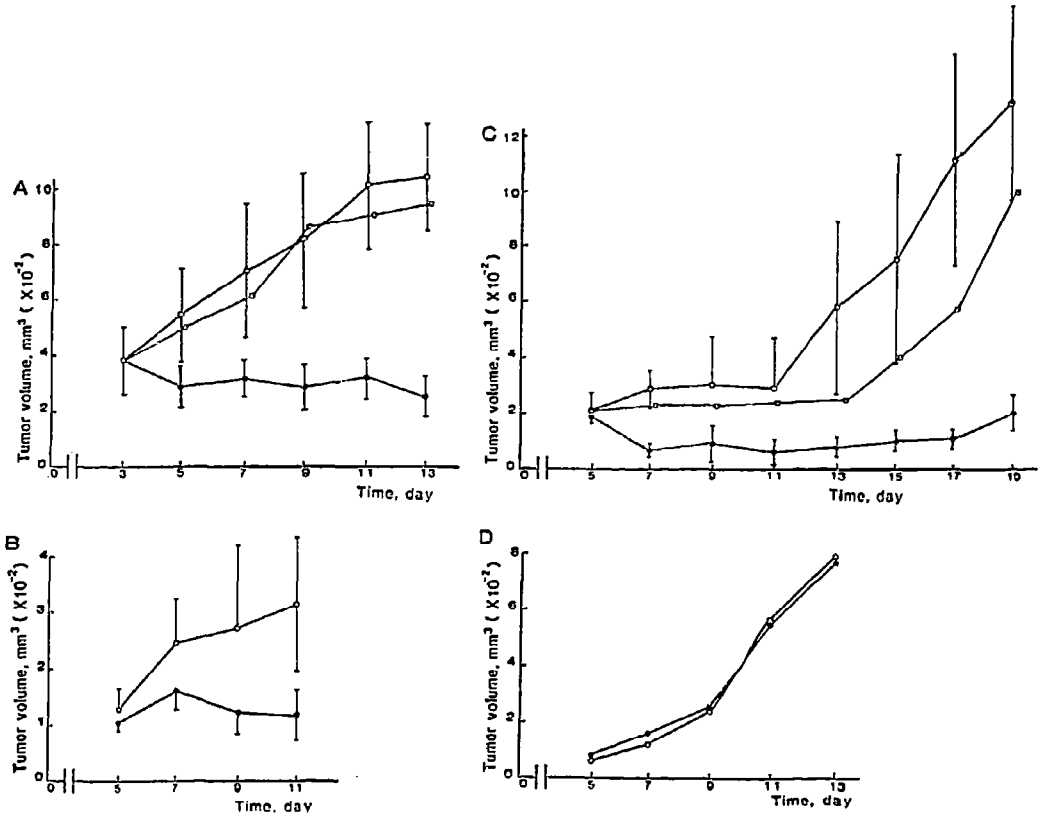

Fig. 5. Inhibitory effect of anti-basic FGF IgG on tumor growth on backs of nude mice. Panels show the profiles of tumor volume of U-87MG (A), T98G (B), HeLa (C) and A431 cells (D). Tumor cells were injected subcutaneously into mice on day 1. On day 3, 5 and 7, mice received the subcutaneous injection of anti-basic FGF IgG (solid circles, ●), normal mouse IgG (open circles, ○) or no IgG (open squares, □) at a concentration of 200 μg/mouse/day. Vertical lines indicate SD.

ing Northern blot analysis that T98G and HeLa cells expressed both the basic FGF and the FGF receptor genes. Immunofluorescence study also revealed the expression of basic FGF peptide in U-87MG, T98G and HeLa cells. However, the expression of either basic FGF or FGF receptor was not detected in A431 cells. These results suggest that, U-87MG and T98G and HeLa cells require cellular basic FGF for their neoplastic growth in vitro.

Tumorigenesis of the xenograft transplantation of U-87MG, T98G and HeLa cells was apparently suppressed by anti-basic FGF IgG while that of A431 cells was not. In addition, the inhibitory effect on the formation of tumor capsules, which were composed of fibroblasts and connective tissues derived from the hosts, was histologically observed in the tumors administered with a neutralizing antibody. These findings suggest that administered anti-basic FGF antibody was active in vivo and was able to inhibit host's reaction as well as tumor cells per se through the inactivation of tumor-derived basic FGF. Recent reports have indicated no suppressing of tumorigenesis in nude mice with the intravenous or intraperitoneal administration of neutralizing antibodies against human basic FGF [15,16]. In those reports, although the bioactivity of administrated antibodies was demonstrated to remain in blood for several days, the amounts and the distribution of neutralizing antibodies in tumor tissues have never been evaluated [15]. The concentration of antibody in tumor tissues is expected to be less than in blood when it is administrated intravenously or intraperitoneally. Therefore, we injected anti-basic FGF IgG into the subcutaneous space surrounding tumors in order to expose tumor mass directly with adequate concentration of a neutralizing antibody. Such local administration will be more suitable in clinical trials of neutralizing antibodies toward brain tumors because blood-brain barriers in the central nervous system may prevent the sufficient distribution of antibody from the blood circulation into the brain parenchyme.

It has been suggested that basic FGF is involved in neoplastic growth from the following evidence; a group of oncogenes, such as HST/K-FGF, INT2, FGF5 and HST2/FGF6, encodes basic FGF-related proteins [17–22]; certain cells can acquire transformed phenotypes after transfection of basic FGF gene [23–26]. Some of these acquired transformed characters, such as colony formation in soft agar, were reversed by the ad-

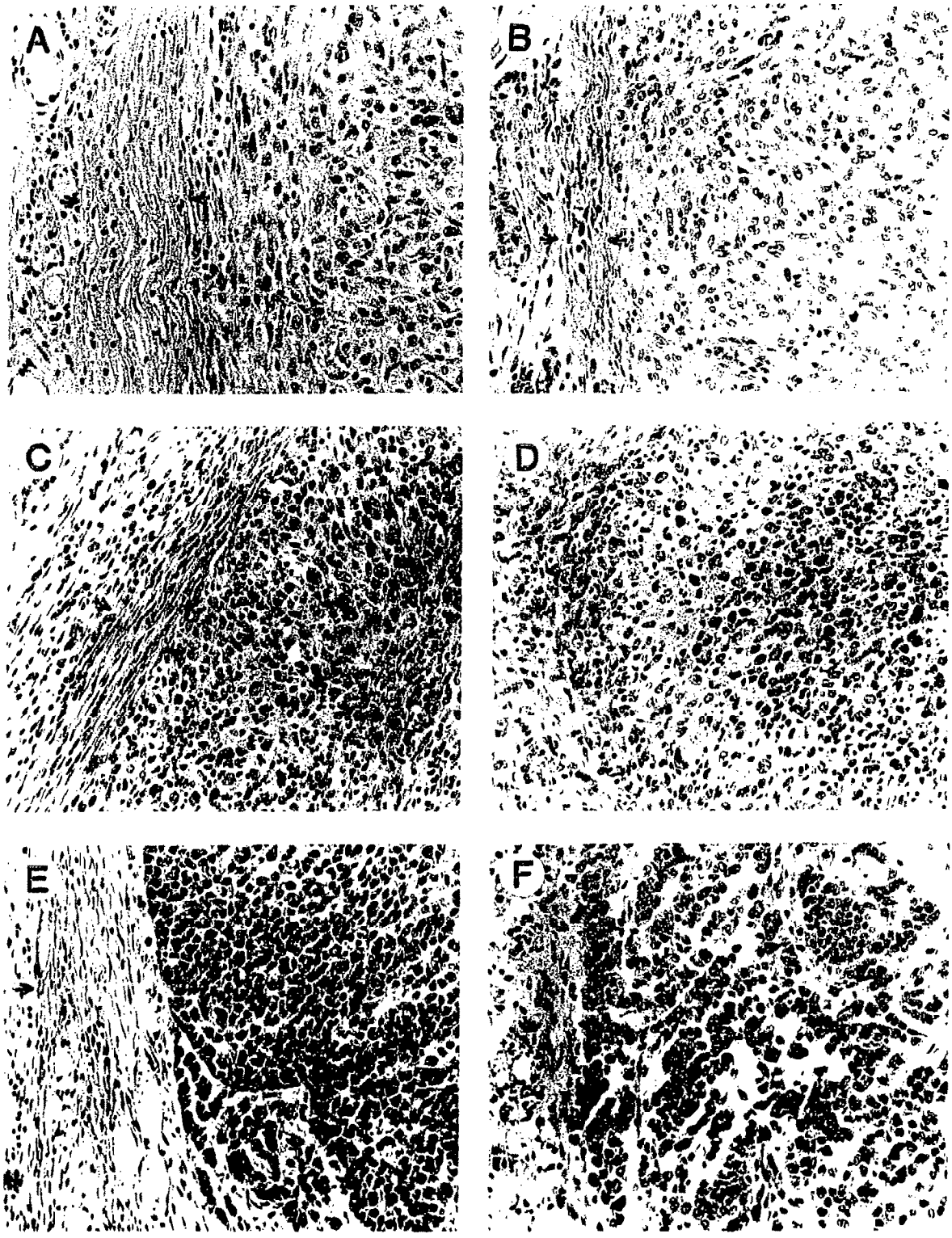

Fig. 6. Hematoxylin and eosin-stained sections of tumors in nude mice. Sections of U-87MG (A,B), T98G (C,D) and HeLa (E,F) were taken from nude mice injected with anti-basic FGF IgG (B,D,F) or normal mouse IgG (A,C,E) (×100). Arrows show the width of the tumor capsule.

dition of anti-basic FGF IgG [23]; and, a number of tumor cells produce basic FGF as an autocrine growth factor. The release of cellular basic FGF and the interaction with its surface receptor may result in the acquisition of the malignant phenotype. For example, exogenous basic FGF is usually required for the growth of normal cells such as normal adrenal cortical cells and myoblast cells [8]. On the other hand, the proliferation

of Y-1 mouse adrenal cortical tumor cells and A204 rhabdomyosarcoma cells become independent of exogenous basic FGF because of the acquisition of the capacity to produce and to respond to endogenous cellular basic FGF [8,27]. A lack of the typical signal peptide in basic FGF remains its release mechanism unknown [2]. However, the actual release of basic FGF into the conditioned media or the extracellular matrix was demonstrated in U-87MG glioblastoma cells [7], PC13 embryonal carcinoma cells [28] and bovine endothelial cells [29]. Such mechanism as cell lysis or leakage may be involved in the release of basic FGF since the existence of similar mechanisms has been proposed for interleukin-1, another growth factor that lacks a signal peptide [30-32].

Our previous study has demonstrated that 94.4% of human gliomas produce basic FGF abundantly in vivo [4], while platelet-derived growth factor A-chain, one of the suggestive autocrine factors, is expressed in 38.9% (data not shown). Glioma cells are also known to bear cell surface receptors of FGF [5,6]. These suggest that basic FGF plays an important role in tumorigenesis of gliomas as an autocrine growth factor. In the present study, we clearly showed that the blockage of an interaction of basic FGF with its receptor resulted in the inhibition of growth in vitro and tumorigenesis in vivo of tumor cells. Thus, the inactivation of basic FGF will bring to control neoplastic growth of tumor cells depending on basic FGF as an autocrine growth factor and/or a paracrine factor.

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