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Transformation of Mouse BALB/c 3T3 Cells with Human Basic Fibroblast Growth Factor cDNA

REIKO SASADA,* TSUTOMU KUROKAWA, MAKOTO IWANE, AND KOICHI IGARASHI

Biotechnology Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Jusohonmachi, Yodogawaku, Osaka 532, Japan

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The expression of human basic fibroblast growth factor (bFGF) cDNA in mouse BALB/c 3T3 clone A31 cells induced morphological transformation. These transformed cells grew well and reached more than a sixfold-higher saturation density than parental A31 cells even in serum-free medium. They were able to form colonies in soft agar. The phenotypic alteration in the transformed cells was reversed by the addition of anti-human bFGF antibodies to the medium. These results suggest that the cellular transformation mediated by bFGF is caused by autocrine stimulation with secreted bFGF molecules.

The production of a growth factor by a cell that responds to the factor has been termed autocrine stimulation of proliferation, and it can lead to uncontrolled growth and cellular transformation. Growth factors are thought to be involved in malignant transformation through autocrine stimulation of growth (19, 36, 37), and examples have been described (4, 8, 10, 20, 25, 31, 33, 38). The growth factors are considered to act from outside cells, but several authors have suggested the possibility that the growth factor binds to its receptor in an intracellular compartment before the receptor reaches the cell surface (4, 32).

Basic fibroblast growth factor (bFGF) is a very potent mitogen for various cells of mesodermal or neurodermal origin in vitro (17) and also induces angiogenesis in vivo (11, 17). Recently, the cDNAs of human and bovine bFGF have been cloned (1, 2, 24): the precursor of bFGF consists of 155 amino acids; 9 amino acids precede the mature peptides. These nine amino acids differ from other typical signal peptides of secretory proteins (9). The mechanism of secretion of bFGF outside cells is not known, although bFGF is considered to interact with membrane-bound receptors on the target cells (29).

Recently, we cloned human bFGF cDNA from a human fibroblast cDNA library and detected a part of synthesized bFGF in the culture medium after transfection of the expression plasmid to COS7 cells (24). In this report, we describe the autocrine growth stimulation and acquisition of malignant phenotypes in mouse BALB/c 3T3 cells in which human bFGF cDNA was expressed. We also present evidence that intracellularly synthesized bFGF is released outside the cells to work and that it may act by binding to its receptor. In addition, the role of bFGF sequestered within cells and the mechanism of release of the molecules are discussed.

MATERIALS AND METHODS

Plasmid construction. Standard procedures for constructing plasmids (28) were used. The expression vector pTB732 was constructed by ligating the following four fragments. The 1.8-kilobase (kb) *Cla*I-*Xho*I fragment containing the promoter sequence of a mouse MT-I gene (18): the *Eco*RI-*Bgl*II fragment from pMK (6) (pMK was provided by R. D. Palmiter) was ligated with synthetic linkers to generate *Cla*I and *Xho*I sites. The 0.17-kb *Xho*I-*Pst*I fragment containing a sequence encoding two functional introns of simian virus 40 (SV40) DNA is derived from pL1 (30). The 1.5-kb *Pst*I-*Eco*RI fragment, containing human bFGF coding sequences flanked by 0.29 kb of 5′ and 0.78 kb of 3′ untranslated regions, is derived from pTB627 (24). The 2.5-kb *Cla*I-*Eco*RI fragment, containing the pBR322 replication origin, the β-lactamase gene, and the SV40 late-region polyadenylation signal, is derived from pcDV1 (30), after modification of the *Bam*HI site to *Eco*RI with a linker. Plasmids pL1 and pcDV1 were obtained from H. Okayama and P. Berg. Since the MT promoter is known to work without induction (26, 35), the inducer $CdCl_2$ was not used unless otherwise mentioned.

Recombinant human bFGF and antibody. Recombinant human bFGF was prepared from *Escherichia coli* carrying a human bFGF cDNA expression plasmid and purified by heparin affinity column chromatography (21). Its biological activities were almost equivalent to those of bFGF purified from bovine pituitary. The immunoglobulin G (IgG) fraction of rabbit anti-human bFGF antibody was provided by K. Kato.

Transfection of cells. Mouse BALB/c 3T3 clone A31-1-1 cells, obtained from T. Kakunaga (23), were cultured in Dulbecco modified Eagle medium (DMEM; Flow Laboratories) supplemented with 10% calf serum (Flow). A31 cells (3×10^5 cells) in 60-mm dishes were cotransfected with 10 μg of expression plasmid, pTB732, and 0.3 μg of pTB6 (34), which carries a neomycin resistance gene, by the calcium phosphate coprecipitation procedure (40). The transfected cells were grown in culture medium for 42 h and were then incubated in selective medium containing G418 (Geneticin; Gibco Laboratories) (500 μg/ml). G418-resistant clones were isolated and transferred into 24-well plates after 2 weeks.

Assay for bFGF activity. Bioassays for bFGF activity were based on the ability to stimulate DNA synthesis in A31 cells maintained at a low serum concentration (14). A31 cells were plated in DMEM with 5% calf serum (2×10^3 cells per well), and the next day, the medium was replaced with 0.2 ml of DMEM containing 0.5% calf serum. Three days later, 0.01 ml of serial 1:5 dilutions of samples (in DMEM with 0.5% crystalline bovine albumin) was added to each well. At 18 h after the sample was added, 1 μCi of [*methyl*-^{3}H]thymidine (5 Ci/mmol; CEA, France) was added. After the cells were incubated for 4 h, they were trypsinized and collected on glass filters with a Titertek cell harvester (Skatron, Lier, Norway). The filters were dried and immersed in scintillation

* Corresponding author.

fluid; radioactivity was measured in a scintillation counter. The FGF activity was calculated by determining the dilution of factor required to give 50% of the maximal stimulation and was indicated as the weight of the standard, bovine pituitary FGF (Takara Shuzo).

Growth of cells in serum-free medium. Cells were plated on 3.5-cm dishes in DMEM containing 5% calf serum (2.5×10^4 cells per dish). The next day the cells were washed once with serum-free DMEM and cultured with 2 ml of DMEM supplemented with ITS$^+$ (Collaborative Research; insulin [6.25 μg/ml], transferrin [6.25 μg/ml], selenium [6.25 ng/ml], bovine serum albumin [1.25 mg/ml], linoleic acid [5.35 μg/ml]). Cell numbers were counted with a Coulter particle counter after trypsynization.

Colony formation in soft agar. MEM (2 ml; Nissui Seiyaku) containing 10% fetal calf serum (Microbiological Associates) and 0.5% Bacto-agar (Difco Laboratories) was added to each 35-mm dish. The cells were trypsinized, counted, suspended in the same medium containing 0.3% Bacto-agar, and then plated at a density of 10^4 cells per dish. Recombinant human bFGF, $CdCl_2$, or anti-bFGF was added to the cell suspension before the cells were plated. These cultures were incubated at 37°C in a 5% CO_2 atmosphere for 2 weeks. Viable colonies were stained by iodonitrotetrazolium and counted as described by Rosenthal et al. (33).

Southern and Northern (RNA) blotting analyses. Polyadenylated [poly(A)$^+$] RNA was prepared by disrupting the cells by guanidinium isothiocyanate and centrifuging them through 5.7 M cesium chloride (7) and was further purified by oligo(dT)-cellulose chromatography. Poly(A)$^+$ RNA was denatured, size-fractionated on a 1.4% formaldehyde–agarose gel, and transferred to a nitrocellulose membrane (27).

High-molecular-weight DNA was prepared by treating cells with 0.5 M EDTA (pH 8.0)–0.5% (wt/vol) Sarkosyl–proteinase K (100 μg/ml) for 3 h at 50°C, followed by phenol extraction (28). DNA digested with the appropriate restriction endonucleases was submitted to 0.6% agarose gel electrophoresis and transferred to a nitrocellulose membrane.

For blot hybridization analysis, probes were prepared by nick-translation of human bFGF cDNA fragments cut out from pTB627. Hybridizations were performed in 50% (vol/vol) formamide–10% (wt/vol) dextran sulfate–5× SSPE–5× Denhardt solution–0.1% sodium dodecyl sulfate (SDS)–sonicated-denatured salmon sperm DNA (100 μg/ml) at 42°C for 16 h. The blots were washed three times in 0.1% SDS–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 20 min at room temperature and then in 0.1% SDS–0.1× SSC for 30 min at 55°C (for RNA blots) or in 0.1% SDS–0.5× SSC for 30 min at 65°C (for DNA blots). The dried filters were exposed to Kodak X-AR film at −70°C with an intensifying screen.

RESULTS

Transformation of A31 cells by a human bFGF cDNA expression plasmid. An expression vector, pTB732, that expresses human bFGF cDNA under the control of mouse MT-I promoter (Fig. 1) was introduced into mouse BALB/c 3T3 clone A31 cells by the calcium phosphate transfection procedure. Cells showed a morphological change suggesting transformation. The morphological change appeared to be dependent on the dose of transfected DNA and was potentiated by the addition of $CdCl_2$.

To isolate cell clones harboring and expressing bFGF cDNA, pTB732 was introduced into A31 cells with pTB6, which carries a neomycin resistance gene (at a molar ratio of 50:1), and clones resistant to 500 μg of G418 per ml were isolated. More than 80% of the G418-resistant clones were morphologically transformed: these cells looked more elongated, spindle shaped, or rounded with long processes and grew in a disorderly criss-cross pattern (Fig. 2A). One of the clones, TCNO523, which showed the most marked morphological changes, was refractile and poorly adherent to the dish and grew somewhat slowly. In contrast, the parental A31 cells showed contact inhibition, with a regular cobblestone pattern in the monolayer culture. A morphological change similar to that of the transformed cells was observed when A31 cells were cultured in the presence of bFGF, as reported by Gospodarowicz and Moran (15).

DNA and RNA analysis of transformed cells. To ascertain the existence of introduced DNA in the transformed cells and to examine their transcriptional levels, DNA and RNA of the transformed cells were analyzed by blot hybridization. After *Cla*I and *Eco*RI digestion of cellular DNA of the transformed cells, 3.5-kb DNA fragments, which represent the length of the bFGF cDNA linked to the MT-I promoter (Fig. 1), were hybridized to the labeled bFGF cDNA probe (Fig. 3A). Other clear bands were not observed in the radioautogram, indicating that the integrated plasmid might be conserved without marked rearrangements. More integrated cDNA could be observed in TCNO522 than TCNO523; the copy number was roughly estimated to be dozens. The integrated cDNA was transcribed in the transformed cells (Fig. 3B). The transcripts were 2 kb long, representing the expected length of the mRNA of the expression plasmid. This indicated that the bFGF mRNA was transcribed in the transformed cells under the control of MT-I promoter. We could not find any remarkable difference between two transformed cell clones in the level of the transcription of the cDNA.

Synthesis and secretion of human bFGF in transformed cells. The amount of bFGF in the culture medium and in the

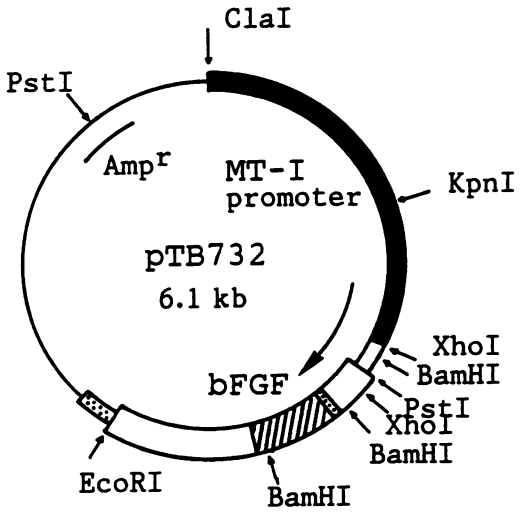

FIG. 1. Schematic presentation of pTB732, the human bFGF expression vector. The MT-I promoter segment is shown as a black box. Within the human bFGF coding sequences, indicated by an open box, the dotted box represents the signal sequence and the dashed area corresponds to the 146-amino-acid human bFGF. pBR322 sequences are shown by a single line, SV40 late-region introns by an open narrow box, and the SV40 late-region polyadenylation signal by a dotted narrow box. The transcriptional orientation is shown by an arrow. The positions of some restriction sites are indicated.

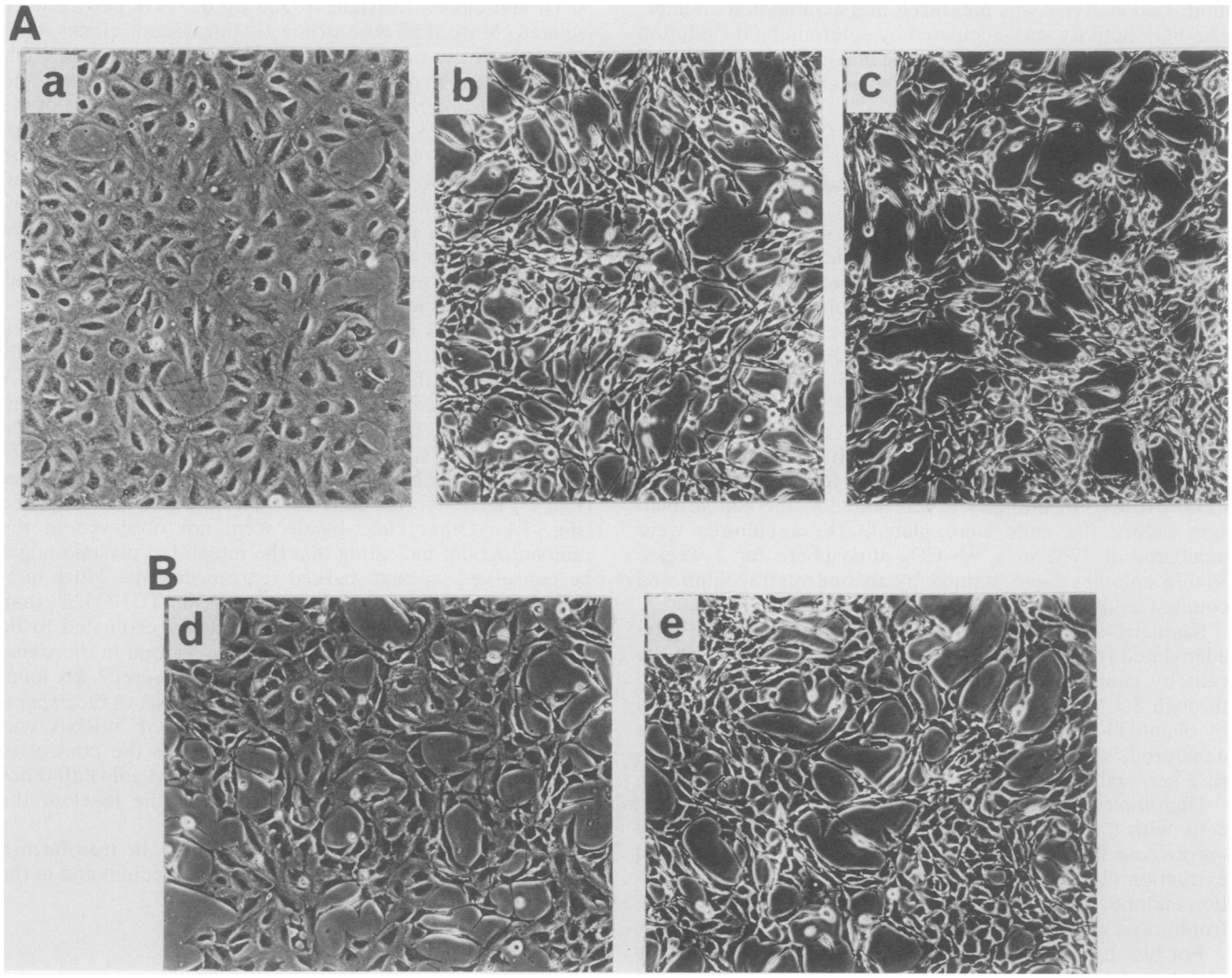

FIG. 2. Morphology of transfected cells. (A) Cells were plated with 0.5 ml of DMEM containing 5% calf serum in 24-well plates and photographed 4 days later. a, A31; b, TCNO522; c, TCNO523. (B) Anti-human bFGF antibody (100 μg/ml) was added to the culture at day 1, and the cells were photographed at day 4. d, TCNO522; e, TCNO523.

cell extract (prepared by sonication) of the transformed clones was assayed by the stimulation of [^{3}H]thymidine incorporation in quiescent A31 cells (Table 1). In the culture medium of TCNO522 or TCNO523 cells, very low mitogenic activity was detected; the level of FGF detected was at most 0.3 ng/ml per about 5×10^5 cells. No significant activity was detected in the culture medium of several other clones that showed slight or moderate morphological changes (data not shown). On the other hand, high FGF activity was detected in the cell extract of two clones at the level of 22 and 138 ng per 60-mm dish culture. These levels of FGF activity were calculated to be more than 50-fold higher than those in the culture medium. These were also confirmed by a transient expression experiment with pTB627 (24) in COS7 cells: about 15 ng in the culture medium and 320 ng of FGF in the cell extract per 60-mm dish were detected 3 days after transfection.

The amount of FGF synthesized and the degree of the morphological changes were correlated: TCNO523, synthesizing a high level of biologically active FGF, showed the most markedly transformed phenotype. When the cells were cultured in medium containing 0.1 μM $CdCl_2$, the FGF activities in the culture medium and in the cells increased to some extent (1.5- to 2-fold), but enhancement of the morphological changes was not observed.

Growth of transformed cells. The growth profile of the two transformed clones, TCNO522 and TCNO523, was examined. When the cells were cultured in DMEM supplemented with 10% calf serum, one of the clones, TCNO523, grew more slowly than the parental A31 cells. However, the saturation densities of the two transformed cells were six- to sevenfold higher than the parental cells (Table 1). When the cells reached confluence, the parental A31 cells showed the cobblestone pattern, but the transformed cells showed a criss-cross pattern.

As serum contains many kinds of growth factors, including FGF (3, 16), we examined the growth of transformed cells in serum-free medium (5) to clarify the differences between the transformants and the parental cells. In serum-free DMEM supplemented with ITS$^+$, the A31 cells ceased to grow at the density of 2×10^5 cells after 2 to 3 days. The transformed clones, in contrast, grew well in the serum-free

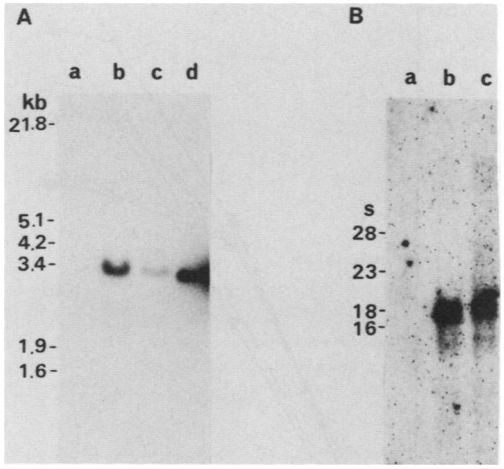

FIG. 3. Southern and Northern blot analyses. (A) High-molecular-weight DNA from transformed cells (10 μg) or plasmid pTB732 (1.25 μg) treated with *Cla*I and *Eco*RI were fractionated on a 0.6% agarose gel, transferred to nitrocellulose, and hybridized to a nick-translated 0.43-kb *Bam*HI fragment of pTB627 cDNA. The molecular sizes (in kilobase pairs) of the standards are indicated. Lanes: a, A31; b, TCNO522; c, TCNO523; d, pTB732. (B) Poly(A)$^+$ RNA (12 μg per lane) was fractionated on a 1.4% formaldehyde–agarose gel, transferred to nitrocellulose, and hybridized to the same probe as in panel A. The migration positions of the 28, 23, 18, and 16S ribosomal subunits are indicated. Lanes: a, A31; b, TCNO522; c, TCNO523.

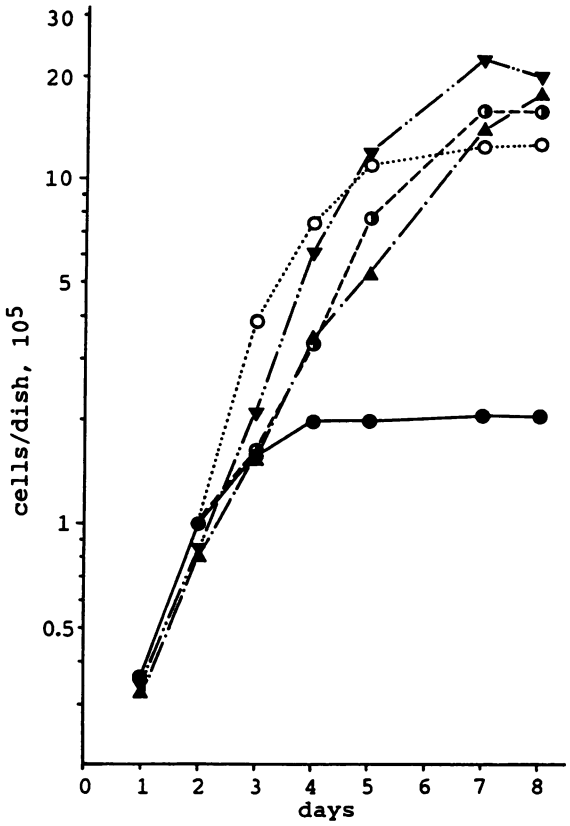

FIG. 4. Growth curves of transformed A31 cells in serum-free medium. Cells (2.5 × 10^4 cells per 35-mm dish) were plated in 2 ml of DMEM containing 5% calf serum on day 0. On day 1 the medium was changed to DMEM supplemented with ITS$^+$. Cell numbers were counted at the indicated day in a Coulter particle counter. Symbols: ●, A31; ▲, TCNO522; ▼, TCNO523; ○,◑, A31 supplemented daily with bFGF at 0.2 ng/ml (○) or 2 ng/ml (◑).

medium and showed 8- to 10-fold-higher saturation densities than A31 cells (Fig. 4). When supplemented with purified bFGF, the A31 cells were able to grow well and reached a high saturation density in the same medium. As shown in Fig. 4, the A31 cells showed slower growth with bFGF at 2 ng/ml than at 0.2 ng/ml. The growth pattern of transformed clones resembled that of A31 cells grown in the presence of 2 ng of bFGF per ml.

Anchorage-independent growth of transformed cells. The transformed cells were examined for their ability to grow in soft agar medium, because the acquisition of anchorage independence is generally considered to be a property concomitant with malignant transformation. Both TCNO522 and TCNO523 cells were able to form colonies in soft agar, in contrast to the parental A31 cells (Table 2). The number of colonies correlated roughly with the amounts of biologically active bFGF synthesized. At least 1 ng of bFGF per ml was needed to form colonies in A31 cells, and the frequency of colony formation (the number of colonies per cells seeded) increased in proportion to the amount of bFGF added (data not shown).

Reversion of transformed phenotypes by antibodies. To test whether the phenotypes expressed by the transformed cells were caused by autocrine stimulation through cell surface receptors, we analyzed the effects of anti-human bFGF antibody on the cells. The anti-bFGF antibody used here neutralized the action of bFGF on A31 cells. As shown in Fig. 2, when antibody was added, the morphology of the transformed cells appeared to revert to normal, which was

TABLE 1. Properties of A31 cells transfected with human bFGF cDNA

Cells	Morphological transformation	FGF activity[a] (ng/dish)		Saturation density[b] (10^5 cells/cm^2)	Doubling time[b] (h)
		Medium	Cell extract		
A31	−	<0.25	<0.25	1.1	15.5
TCNO522	+	0.45	22	6.3	16.0
TCNO523	++	1.30	138	7.3	18.5
TCNO525	+	<0.25	2.9	ND[c]	ND

[a] FGF activity, indicated by the weight equivalent to the standard bovine pituitary FGF, was determined by incorporating [^{3}H]thymidine into quiescent A31 cells. The cells of 80% confluent monolayer cultures in a 60-mm dish were incubated for 2 days in 5 ml of DMEM containing 1% calf serum. The medium was collected to estimate FGF activity. The cells were scraped, washed twice with phosphate buffered saline, suspended in phosphate-buffered saline, and sonicated briefly. After centrifugation at 15,000 rpm for 15 min, the supernatant was assayed for FGF activity.

[b] Cells were seeded in duplicate at 2.5 × 10^4 cells per 35-mm culture dish with 2 ml of DMEM containing 10% calf serum. Cell numbers were counted every 2 days. The cultures were refed at day 5 and day 8. Transfected cells reached saturation on day 8 to 10, while the parental A31 cells reached it on day 4 to 5.

[c] ND, Not determined.

TABLE 2. Colony formation in soft agar by transformed A31 cells and inhibition by anti-bFGF antibody[a]

Cells	No. of colonies	No. of colonies in the presence of anti-bFGF antibody
TCNO522	20	0
TCNO523	>1,000	20
A31	2	2
A31 + bFGF (10 ng/ml)	65	8

[a] Anti-bFGF antibody was added at a concentration of 100 μg/ml. Colony formation was not affected by adding the IgG fraction prepared from a preimmune animal. The colonies formed in the presence of anti-bFGF IgG were smaller than those formed in the absence of anti-bFGF IgG.

similar to the parental A31 cells. The saturation density was lowered (Fig. 6) and colony formation was inhibited (Fig. 5 and Table 2). Anti-bFGF antibody did not revert the transformed phenotypes of A31 cells transformed with the Ki-*ras* gene (data not shown). Preimmune serum had no effect on the reversion of the transformed phenotypes. The phenotypes of the transformed cells could be suppressed by adding the antibody, suggesting that the synthesized bFGF may act extracellularly on the transformed cells after secretion.

DISCUSSION

We have shown that A31 cells change their morphology drastically when they are transfected with human bFGF cDNA. The transformed cells also exhibit a high saturation density and anchorage-independent growth. All these characteristics are typical of malignant transformation. In addition, the phenotypic alterations observed were confirmed to be bFGF specific by the presence of transfected bFGF DNA and the synthesis of bFGF RNA in the transformed cells. Therefore, it was concluded that the transformed phenotypes were all directly or indirectly controlled by the bFGF synthesized. In fact, all the transformed phenotypes were well correlated with the level of biologically active bFGF. Unexpectedly, the expression level of mRNA was not correlated to the production of bFGF between 522 and 523 cells, the reason for which is not obvious at present. In any case, these facts suggest that bFGF as well as platelet-derived growth factor may play a role in stimulating the growth of some tumor cells, possibly by promoting uncontrolled growth.

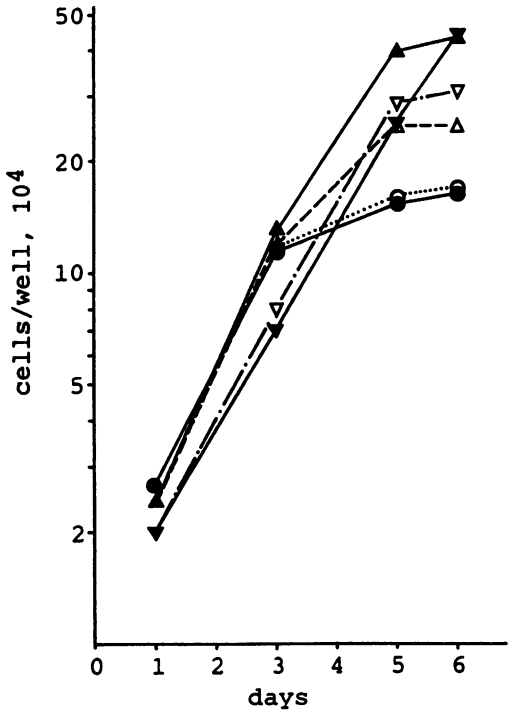

FIG. 6. Growth inhibition of transformed A31 cells by anti-bFGF antibody. Cells (1.5×10^4 cells per well of 24-well plate) were plated in 0.5 ml of DMEM containing 10% calf serum on day 0. On day 1, anti-bFGF antibody was added to the culture at a concentration of 100 μg/ml. Symbols: A31 with (○) and without (●) antibody; TCNO522 with (△) and without (▲) antibody; TCNO523 with (▽) and without (▼) antibody.

Previous reports of experimental reconstitution of some autocrine systems (20, 22, 25, 33, 38) suggested that the growth factor interacts with and activates its receptor extracellularly. In contrast, intracellular interactions of growth factor and the intracellular compartment were also predicted (4, 32). In the present study, the addition of anti-bFGF antibody to the culture of transformed cells resulted in reversion of the malignant morphology to that of normal A31 cells, prevented colony formation in soft agar, and decreased the saturation density. This is the first evidence that bFGF is

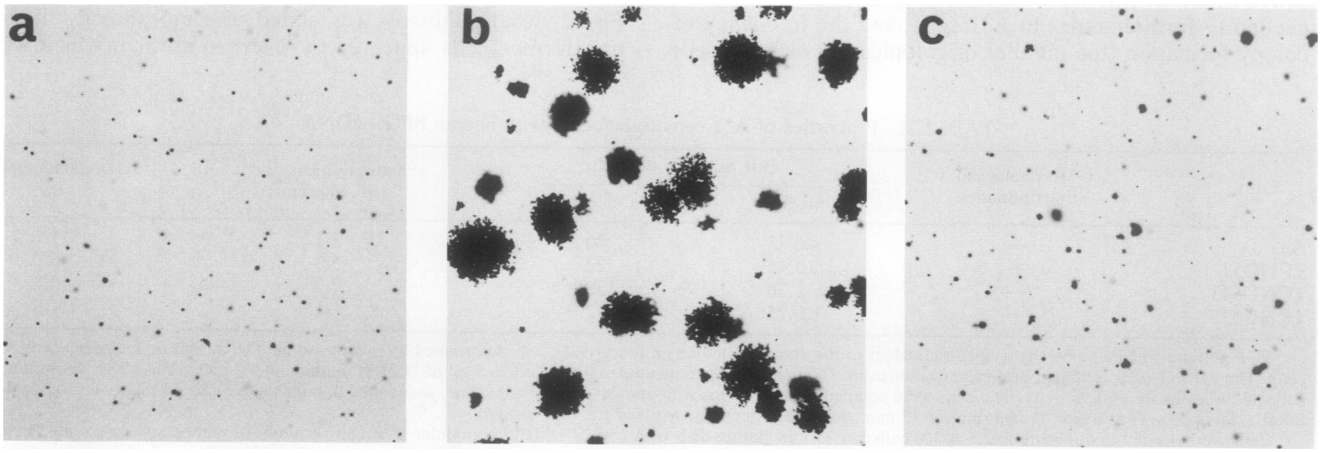

FIG. 5. Colony formation of transformed cells in soft agar. a, A31; b, TCNO523; c, TCNO523 with anti-bFGF antibody (100 μg/ml).

released from the cells and then exerts its effects through receptors on the cell surface, although the possibility that synthesized FGF can act intracellularly is not completely excluded.

As discussed above, bFGF has to be secreted from the cells. But as predicted from the nucleotide sequences of cloned bFGF cDNA, bFGF has no typical signal peptide for secretion. Indeed, only very low bFGF activity was detected in the culture medium of the transformed cells. Moreover, when the leader sequence of the bFGF gene was connected to the 5′ end of the human interleukin 2 (IL-2) gene and expressed in COS7 cells, human IL-2 was not secreted at all. Conversely, when the bFGF gene having the leader sequence of the IL-2 gene at the 5′ terminus was expressed, most of the bFGF was again found within the cells (unpublished observation). These facts clearly show that the signal sequence of bFGF does not work to secrete the protein and that bFGF itself is kept in the cells by some mechanism. One possible mechanism is the binding of bFGF to some cellular components; such a complexed molecule may protect the release of bFGF. The synthesis of bFGF in several cell lines transfected with the cDNA showed that the ratio of bFGF activity detected inside and outside the cells varied among cell lines and also among clones from one cell line. Therefore, bFGF synthesized in cells is not released only by spontaneous disruption of the cells, but may be secreted by an unknown route.

The transformed cells were shown to grow in soft agar, but there was a discrepancy between the amount of bFGF detected in the culture medium of the transformed cells and that of bFGF required for A31 cells to form colonies in soft agar; all the transformed cells secreted bFGF poorly in the culture medium (Table 1), and these low amounts of bFGF, when added exogenously, did not allow A31 cells to form colonies in soft agar. Synthesis of bFGF in transformed cells is thought to be constitutive, and bFGF molecules are continuously synthesized. In contrast, purified bFGF was added only once when A31 cells were seeded in soft agar. It is suggested that the low amount of bFGF is sufficient to cause soft-agar growth of transformed cells from which it is secreted continuously.

The proper concentration (0.1 to 0.2 ng/ml) of bFGF stimulated the growth of A31 cells, but a higher concentration (above 1 ng/ml) exerted some inhibitory effect on the growth of the cells (Fig. 4). Therefore, the transformed cells, producing much bFGF, were expected to have some disadvantage for growth. Our experiments with transformed cells showed that this was the case. These results clearly show that secretion of bFGF outside the cells results in the slow growth of bFGF-producing transformed cells. In addition, these transformed cells had a tendency to lose their transformed phenotypes when they were passaged in nonselective medium. For example, when TCNO523 cells were recloned after 10 passages in nonselective medium, about half the clones lost their resistance to G418 and most of them had the same morphology as that of A31. The inhibitory effect of bFGF on A31 cells may be caused by excessive signal transduction, as observed on A431 cells with epidermal growth factor (13).

In spite of the advances made in biochemical studies of bFGF in recent years, much remains to be learned about the physiological role of this factor. It is now evident that bFGF released from cells induces autocrine growth stimulation, and several cell lines transformed with human bFGF cDNA which are able to produce bFGF can be isolated. It is hoped that further studies on the transformed cell line will help to clarify the fundamental problems in the mechanism of cellular growth stimulation by growth factor.

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