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POSSIBLE ACTIVITY OF ACIDIC FIBROBLAST GROWTH FACTOR AS A PROGRESSION FACTOR RATHER THAN A TRANSFORMING FACTOR

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Summary : Acidic and basic fibroblast growth factors (aFGF and bFGF) are mitogens for mesoderm- and neuroectoderm-derived cells. The facts that FGF-related proteins are oncogenic and that FGFs are expressed in a variety of tumor cell lines or tumor tissues suggest the transforming activities of FGFs. To examine such an activity of aFGF, we introduced a human aFGF expression vector into two populations of Rat-1 cells; one was non-transformed (nRat-1), the other was partially-transformed (tRat-1). tRat-1 cells transfected with aFGF cDNA formed larger colonies in soft agar and produced larger and more malignant tumors in nude mice than those of parental cells. In contrast, nRat-1 cells transfected with aFGF cDNA neither formed colonies in soft agar nor produced tumors in nude mice. Our results suggest that high expression of aFGF may enhance a tumorigenic potential of Rat-1 cells rather than confer such a potential *de novo*. © 1992 Academic Press, Inc.

Acidic and basic fibroblast growth factors (aFGF and bFGF) display a broad spectrum of growth, differentiation and survival activities on a wide variety of mesoderm- and neuroectoderm-derived cells (1) and are angiogenic *in vivo* (2). They belong to a larger heparin binding growth factor family of polypeptides, each of them encodes the protein which shares approximately 30-45 % homology with aFGF and bFGF. The oncogenic properties of these FGF-related proteins, together with the fact that FGFs and FGF mRNAs are found in a large number of human tumor cell lines (3) or human tumor tissues

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Abbreviations
BSA : bovine serum albmin, FGF : fibroblast growth factor, FITC : fluorescein isothiocyanate, HTLV-I : human T-cell leukemia virus type I, LTR : long terminal repeat, PBS : phosphate buffered saline, SDS : sodium dodecyl sulfate, SSC : 0.15M NaCl 15mM sodium citrate, tax: transcriptional trans-activator coded in X region

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(4), suggest that FGF gene expression may play an important role in cellular transformation.

To examine the transforming potential of aFGF, we constructed a human aFGF expression vector and introduced it into Rat-1 cells (Rat fibroblast cell line;5). Although Rat-1 is a well-known cell line, several authors reported that this cell line was sometimes tumorigenic when injected into nude mice or syngeneic Fisher rats (6,7,8). So, we prepared two types of Rat-1 cells. One was non-transformed Rat-1 cells (designated as nRat-1), isolated in our laboratory and maintained carefully. They have been proved not to grow in soft agar nor to produce tumors in nude mice, but they are transformed by the expression of a tax gene. The other was spontaneously-transformed Rat-1 cells. This cell line has characteristics of partial transformation, that is, an ability to form small colonies in soft agar and to make small, slow-growing tumors in nude mice. It was designated as transformed Rat-1 (tRat-1), to distinguish from nRat-1.

In the present study, mixed population and clones of tRat-1 cells transfected with a human aFGF cDNA showed transformed characteristics more distinctly than control tRat-1 cell lines did, while those of aFGF-expressing nRat-1 cells were non-transformed as well as parental nRat-1 cells. These results suggest that aFGF may enhance a tumorigenic potential of Rat-1 cells rather than act as a primary transforming factor.

Materials and Methods

Plasmid construction A 1185-base-pair (bp) Sac I/Hind III fragment including human aFGF coding region from pMJ23 (9) was inserted into an Hind III site of pH2Rneo (10). pH2Rneo contains the SV40 sequences including the SV40 early promoter and the SV40 polyadenylation signal (SV), an R fragment of human T-cell leukemia virus type I (HTLV-I) long terminal repeat (LTR) (R), and neo^r under control of the SV40 early promoter (SV/Neo/SV(A)n).

Cell culture, transfections, and selection Rat-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum (Flow Laboratories). 5 x 10^5 Cells, plated in 10 cm dishes on the previous day, were transfected with 15 μg of plasmid DNA by the calcium phosphate precipitation method. One day later the medium was replaced by selective medium containing 600 μg/ml of G418 (Sigma) and incubated for another 14 days. G418 resistant colonies in one dish were trypsin-treated together and expanded (designated as mix) and were considered to be a polyclonal cell population. Monoclonal colonies were isolated with stainless steel cloning cylinder.

Northern blot analysis Total RNA was prepared by acid guanidinium thiocyanate-phenol-chroloform-extraction method. Twenty μg of RNA was denatured in 1 M glyoxal/50% dimethyl sulfoxide, fractionated by electrophoresis in 1% agarose gel and transferred to a nylon membrane. A 1.2-kb Sac I/Hind III fragment from pMJ23 was labeled with [α-^{32}P]dCTP by random priming and used as a probe. Hybridization was performed in a solution containing 50% formamide/5 x SSC at 42°C. The final washes were done twice under stringent conditions with 0.1 x SSC/0.5% SDS at 50°C for 30 min each time. The same probe was rehybridized with a GAPDH probe.

Immunofluorescence study Cells were cultured on Lab-Tek Chamber Slide (Nunc, Inc.) in conditioned medium, then fixed with 3.5% formaldehyde for 30 min at room temperature. The fixed cells were permeabilized with 0.1% NP-40 for 10 min at room temperature. After preincubation with 1% BSA/PBS for 15 min, cells were incubated with rabbit antiserum raised against bacterially produced human aFGF (11) or non-immune rabbit serum at 37°C for 45 min. The slides were rinsed, and incubated with FITC-conjugated goat anti-rabbit IgG for 30 min at 37°C. Then, the slides were rinsed, mounted with 90% glycerol and viewed by fluorescence microscope (Zeiss).

Assays for growth properties Saturation densities of cells were determined by duplicate counting of cells in 6-cm dishes at 14 days after plating 2×10^5 cells, with the medium changed every 3 days. In anchorage-independent growth assay, 1×10^4 cells were suspended in complete medium containing 0.33% agarose (Sea-plaque, FMC) for 2 weeks. Colonies larger than 50 μm in diameter were counted and their average diameters were calculated.

Tumorigenicity studies Viable cells (5×10^6) suspended in 0.25 ml of medium were injected subcutaneously into 5-week-old BALB/c nude mice. Animals were monitored at regular intervals for tumor formation over a period of 5 or 6 weeks. Histology of the tumors was examined in paraffin embedded sections with hematoxylin and eosin staining.

Results

Transfection and isolation of aFGF-expressing tRat-1 cells : An expression vector, pH2RaFGF, that expresses human aFGF cDNA under the control of SV40 early promotor and R fragment of HTLV-I LTR was introduced into tRat-1 cells by the calcium phosphate precipitation method. One G418-resistant mixed cell population (tRat-aFGFmix) and eight G418-resistant clones were established, and G418-resistant cell population transfected with pH2Rneo (tRat-Neomix) were obtained as a control. Among these eight clones, the clones with low (tRat-aFGF cl.1), middle (tRat-aFGF cl.2) and high (tRat-aFGF cl.3) level expression of aFGF mRNA were chosen for further analyses. Northern blot analysis of these clones (Fig. 1) revealed the presence of transcripts of 2.5-kb in length, equivalent to the expected size of the mRNA, that was not detected in parental tRat-1 cells. Immunofluorescence study using anti-human aFGF antibody demonstrated that aFGF was synthesized in tRat-aFGFcl.3 cells and

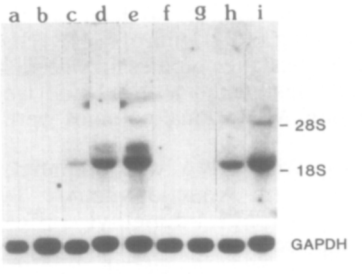

Fig. 1. Northern blot analysis of G418-resistant clones established from tRat-1 and nRat-1 cells transfected with pH2RaFGF. Twenty μg of total RNA per lane was hybridized with a human aFGF probe. a: tRat-1, b: tRat-Neomix, c: tRat-aFGFcl.1, d: tRat-aFGFcl.2, e: tRat-aFGFcl.3, f: nRat-1, g: nRat-Neomix, h: nRat-aFGFcl.1, i: nRat-aFGFcl.2.

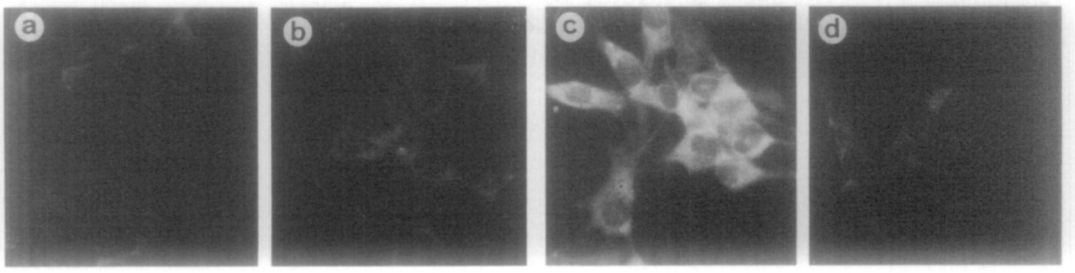

Fig. 2. Immunofluorescence study. tRat-1 cells (**a, b**) or tRat-aFGF cl.3 cells (**c, d**) were incubated with rabbit antiserum raised against human aFGF (**a, c**) or control non-immune rabbit serum (**b, d**). After incubation with goat anti-rabbit IgG conjugated to FITC, the slides were mounted and photographed.

localized mainly in the cytoplasm, while no distinct staining was observed in parental tRat-1 cells as well as in control experiments (Fig. 2).

Growth characteristics and of tRat-1 cells transfected with pH2RaFGF : As shown in Table 1, tRat-aFGFmix and tRat-aFGFcl.2 cells grew slightly denser than control cells did. Especially, tRat-aFGFcl.3 cells showed morphological changes, getting longer, thinner and palisading, and grew to approximately double the cell density of control cells without contact inhibition(Fig. 3, Table 1). When their abilities of anchorage-independent growth were tested, tRat-1 cells formed tiny colonies in soft agar. Among tRat-1 cell lines transfected with pH2RaFGF, tRat-aFGFcl.3 cells formed larger colonies with higher efficiency compared with those of control tRat-1 cells. On nude mice, tRat-1 cells produced small tumors (tumorigenicity at 5 weeks: 4/4, mean volume: 134 mm^3), which were visible 3 weeks after subcutaneous injection. Histological examination revealed that the tumor cells were loosely arranged and myxomatous (Fig. 4). In contrast, tRat-aFGFcl.3 produced larger tumors on 4 out of 4 mice (mean volume: 3538 mm^3), which were visible as soon as 1 week

Table 1. Growth characteristics of tRat-1 and nRat-1 cells transfected with pH2RaFGF

cell line	Saturation density (10^6 cells/ 6cm dish)	Efficiency of colony formation,%	Mean diameter of colonies, μm
tRat-1	8.5	2.0	56.0
tRat-Neomix	7.8	0	
tRat-aFGFmix	9.4	2.8	63.3
tRat-aFGFcl.1	7.5	0	
tRat-aFGFcl.2	9.4	2.6	58.8
tRat-aFGFcl.3	16.2	84.6	161.3
nRat-1	7.2	0	
nRat-Neomix	6.1	0	
nRat-aFGFmix	7.4	0	
nRat-aFGFcl.1	7.8	0	
nRat-aFGFcl.2	6.5	0	

Saturation densities of cells were determined by duplicate counting of cells in 6-cm dishes at 14 days after plating 2 x 10^5 cells. In anchorage-independent growth assay, 1 x 10^4 cells were suspended in medium containing 0.33% agarose and incubated for 2 weeks. Colonies larger than 50 μm in diameter were counted and their average diameters were calculated.

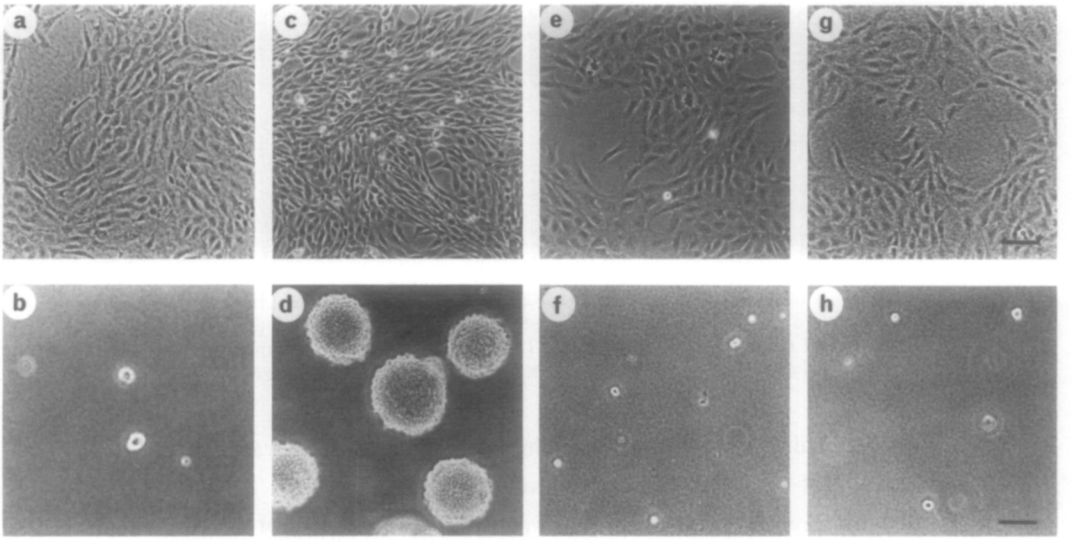

Fig. 3. Cell morphology on monolayer cultures and cell growth in 0.33% soft agar. (**a**, **c**, **e**, **g**) : Cells were incubated in monolayers for 3 days after plating 5 x 10^5 cells onto 10-cm dishes and photographed. (**b**, **d**, **f**, **h**) : 1 x 10^4 cells were suspended in medium containing 0.33% agarose, incubated for 2 weeks and photographed. (**a**, **b**) tRat-1 cells; (**c**, **d**) tRat-aFGFcl.3 cells; (**e**, **f**) nRat-1 cells; (**g**, **h**) nRat-aFGF cl.2 cells. The bars in (**g**) and (**h**) represent 100 μm.

after injection and grew progressively. Histologically, the tumor cells were compact, spindle-shaped and looked like fibroblastic sarcoma with a high mitotic activity and an invasion into surrounding adipose and muscle tissues.

Growth characteristics of nRat-1 cells transfected with pH2RaFGF : The same plasmids used for tRat-1 cells were introduced into nRat-1 cells. One mixed cell population (nRat-aFGFmix), ten clones with pH2RaFGF, and control G418-resistant mixed cell population (nRat-Neomix) with pH2Rneo were established. Among ten clones, the clones with low (nRat-aFGF cl.1) and high (nRat-aFGF cl.2) level expression of aFGF mRNA were chosen for further analyses. When cultured in conditioned medium, nRat-1 cells transfected with pH2RaFGF grew

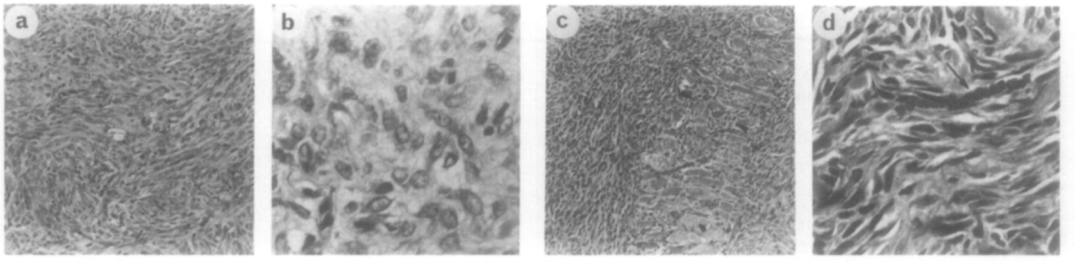

Fig. 4. Hematoxylin and eosin-stained sections of tumors in nude mice. The tumors were excised 5 weeks after subcutaneous injection of tRat-1 cells (**a**, **b**) or tRat-aFGFcl.3 cells (**c**, **d**), and their paraffin embedded sections were photographed with low (100x : **a**, **c**) and high (400x : **b**, **d**) magnification. Arrowheads in (**c**) show the tumor cells infiltrating into muscle layer. An arrow in (**d**) shows a neo-vessel in the tumor.

to the same or slightly higher saturation density than parental nRat-1 or nRat-Neomix cells. They retained contact inhibition without apparent morphological changes. In soft agar assay, the cells tested underwent at most 2 or 3 doublings, but none of them formed colonies (Fig. 3, Table 1). Neither nRat-1 cells transfected with pH2RaFGF nor control nRat-1 cells produced tumors even 6 weeks after subcutaneous injection into nude mice.

Discussion

Several studies have demonstrated that over expression of bFGF in NIH-3T3 (12,13,14), BALB/c 3T3 (15), or BHK-21 (16) cells yields a transformed phenotype. Rogelj *et al.* and Blam *et al.* found that transformation occurred only with the bFGF expression vector that contained an exogenous signal sequence fused 5' to the bFGF coding sequence. Other groups observed the appearance of transformed phenotype with cells transfected with bFGF expression vector lacking a secretory signal sequence. With regard to aFGF, 3T3 NR6 cells transfected with a human aFGF cDNA showed characteristics of partial transformation (11). Since both FGFs lack a signal sequence and are inefficiently released from cells, very large amount of FGFs may be required to fully activate their cell surface receptors. In contrast, *hst*-1 which is efficiently released and binds to its receptors was able to transform NIH 3T3 cells at low levels (14). Therefore, these differences in the transforming potential of FGFs observed by various groups may reflect differences in expression levels. Furthermore, the observation that very high expression of FGFs are required for transformation suggests that the activation of FGF expression is not a primary event in the etiology of spontaneous neoplasms (1,14).

The tumors derived from tRat-1 cells were slow-growing, and the tumor cells were loosely arranged and myxomatous. In contrast, the tumors derived from tRat-aFGFcl.3 cells grew progressively, and were fibroblastic-sarcomatous. The tumors of tRat-aFGFcl.3 cells were richer in blood supply than those derived from tRat-1 cells, although no remarkable differences of the vessels in number were noted. These results indicated that tRat-aFGFcl.3 cells progressed to malignancy *in vivo* from the state of tRat-1 cells. An invasive activity of tRat-aFGFcl.3 cells was consistent with the results that expression of aFGF in the rat epithelial cell line induced morphological changes and invasive potentials *in vitro* (17).

In general, it has been hypothesized that the clinical and biological events of "tumor progression" represent the result of sequential selection of variant subpopulations within one or a small number of altered cells (18). Several combinations of genetic lesions have been observed to produce the malignant phenotype in the human cancer, and the accretion of genetic alteration is likely to play an important role in that event (19). Some agents, however, are reported

to augment tumorigenesis in an epigenetic manner (20). And, a number of experiments have shown that two or more oncogenes cooperate in the tumorigenic transformation of cells (21). Recently, it is reported that actions of FGFs may not be entirely dependent on interaction with cell surface FGF receptors, but they are capable of directly influencing intracellular processes (22,23). Our results raise the possibility that aFGF may cooperate with some kind of intracellular- or intranuclear-located oncogene products which do not exist in nRat-1 cells but do in tRat-1 cells. Further analysis of biochemical changes of Rat-1 cells transfected with aFGF cDNA in our system may provide a clue to understand a role of aFGF in the progression of natural tumors.

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