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Secreted or nonsecreted forms of acidic fibroblast growth factor produced by transfected epithelial cells influence cell morphology, motility, and invasive potential

(cancer metastasis/protein secretion/signal peptide/gelatinases)

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ABSTRACT **Addition of exogenous acidic fibroblast growth factor (aFGF) to NBT-II epithelial carcinoma cells results in fibroblastic transformation and cell motility. We have generated aFGF-producing NBT-II cells by transfection with recombinant expression vectors containing human aFGF cDNA, or the human aFGF cDNA coupled to a signal peptide (SP) sequence. The effects of the nonsecreted and the secreted 16-kDa growth factor on the morphology, motility, and cell invasive potential (gelatinase activity) were compared. aFGF coupled to a SP was actively secreted out of the producing cells. The secretion of aFGF was not necessary for induction of gelatinase activity, as this was observed in NBT-II cells producing aFGF with or without SP. Production of aFGF, whether secreted or not secreted, resulted in increased *in vitro* motility of most isolated clones; however, there was no correlation between aFGF level and motility rate. The data suggest that expression of aFGF in NBT-II cells induces metastatic potential through an autocrine or intracrine mechanism.**

The cellular and molecular mechanisms responsible for the induction of epithelial cell dispersion and cell motility are still unclear. Epithelial cells can move *en bloc* as an epithelial sheet or as individual cells after a scattering event (1).

Several factors have been reported to promote cell dispersion and cell motility. A 55-kDa autocrine motility factor responsible for tumor cell kinesis was described (2). A scatter factor produced by mesenchymal cells was reported to induce epithelial cell dispersion *in vitro* (3, 4).

A rat bladder carcinoma cell line (5) was found to be a suitable model system to address molecular mechanisms of epithelial to mesenchymal cell conversion, particularly for the search of scatter factors. Based on the original observation that type I collagen induced motility in this cell line (6), a detailed analysis of the effect of various components of the extracellular matrix demonstrated that several collagen types could induce the dissociation and the motility of tumor cell aggregates (7). A heparin-binding growth factor present in brain extract and subsequently identified as acidic fibroblast growth factor (aFGF) was also able to convert the bladder carcinoma cell monolayer into motile fibroblast-like cells (8, 9).

aFGF and basic FGF (bFGF) belong to a family of heparin-binding growth factors or related oncogene products. Both aFGF and bFGF are potent mitogens for a wide variety of cells (10). The putative murine oncogene *int-2* (11), the human oncogenes hst/KFGF (12) and FGF5 (13), as well as FGF6 (14) and keratinocyte growth factor (KGF), specific for epithelial cells (15, 16), have been assigned to the FGF family based on similarities in their genomic organization and nucleotide and amino acid sequences. Unlike aFGF and bFGF (17, 18), their corresponding translation products contain a hydrophobic N-terminal region that is probably a signal sequence leading to secreted products (16) as demonstrated for hst/KFGF, FGF5, and KGF. Cloned FGF receptors bind several members of the FGF family with high affinity, including KFGF, aFGF, and bFGF (19, 20). Since FGF receptors bind with high affinity both secreted FGFs such as KFGF and nonsecreted FGFs such as aFGF and bFGF, the lack of signal peptide (SP) in the latter may be fundamental in regulation of their activity.

NBT-II cells originated from a chemically induced rat bladder carcinoma. These cells have an epithelial phenotype. Upon addition of aFGF, but not bFGF, cells start to dissociate and progressively acquire a fibroblastic appearance and become motile (8). This phenomenon is fully reversible and involves high-affinity receptors for aFGF expressed at the NBT-II cell surfaces (9).

The NBT-II cell model system allows *in vitro* study of the conversion from an epithelium to a mesenchyme; it may provide insight into the very early phase of carcinoma, invasion and metastasis.

To study the potential autocrine or intracrine role of aFGF in carcinoma dissociation and the acquisition, at the single-cell level, of motile, invasive, and metastatic properties, we used transfection to generate clones of NBT-II cells expressing either human aFGF or SP-aFGF conjugate. Cells producing human aFGF with or without a SP were converted morphologically to mesenchymal cells and produced endogenous metalloprotease activities that were secreted into the medium. Most of the aFGF-producing clones became motile *in vitro*. However, the absence of correlation between cell motility and level of expression of either the secreted or the nonsecreted form of aFGF indicates that aFGF synthesis may not be sufficient to convert carcinoma cells to the fully migratory phenotype.

MATERIALS AND METHODS

Reagents. Monoclonal antibodies against desmosomal proteins (8) were a gift of W. Franke (German Cancer Research Center, Heidelberg). Monoclonal anti-vimentin antibodies were purchased from Amersham. Rabbit polyclonal antiserum to recombinant human aFGF has been described (21). Fluorescein-conjugated goat anti-rabbit IgG (Pasteur Insti-

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Abbreviations: FGF, fibroblast growth factor; aFGF, acidic FGF; bFGF, basic FGF; SP, signal peptide; EIA, enzyme immunoassay.

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tute, Paris) and Texas Red-coupled goat anti-mouse IgG (Immunotech, Marseille, France) were used as second antibodies.

Recombinant Plasmids. The pAG60 plasmid, containing the neomycin-resistance (Neo^R) gene (22), was a gift of A. Garapin (Pasteur Institute). The recombinant expression vector p267 (paFGF), containing the human aFGF cDNA, encodes the 16-kDa aFGF (21). A similar construct, pMJ35 (pSP-aFGF), containing 5′ to the aFGF cDNA a heterologous SP sequence coding for 21 amino acids was constructed and also used for transfection. This construct joins the SP of human fibroblast interferon (23) to the first residue (methionine) of the encoded aFGF (Fig. 1); after translation, this chimeric protein should be inserted in the endoplasmic reticulum and a 16-kDa protein identical to the one produced by paFGF should be secreted after cleavage of the SP. Control experiments were performed with a SP-aFGF recombinant (pMJ31) containing a stop codon between the SP and the aFGF sequence, leading to aFGF production without a SP (Fig. 1).

Transfection. NBT-II cells were transfected by the calcium phosphate co-precipitation technique (24) with 1 μg of pAG60 per dish or were cotransfected with pAG60/paFGF, pAG60/pSP-aFGF, or pAG60/pMJ35 in a 1:10 or a 1:20 molar ratio. The cells were seeded (0.8×10^6 cells per 90-mm-diameter dish), 24–36 hr before transfection. Twenty-four hours after transfection, cells were trypsinized and diluted 1:4 in selective medium containing the neomycin analogue G418 (400 μg/ml). After 2–3 weeks, visible resistant colonies were picked and expanded in selective medium for the two first passages and then passaged in standard medium.

Cell Motility Assay. Cell motility was assessed as described (7). Time-lapse video cinematography was performed over a 16-hr period, and the average speed of locomotion (μm/hr) was calculated.

aFGF mRNA Expression. Total RNA was prepared (25) from untransfected and transfected cells, and samples (10–15 μg) were analyzed by Northern blot hybridization (26) with the aFGF *Xho* I–*Bgl* II cDNA insert as probe. Labeled plasmid containing rat glyceraldehyde-3-phosphate dehydrogenase cDNA was used as an internal control for expression (27).

Cell Extracts. Extracts were prepared from subconfluent cells on 90-mm dishes after 4 days in culture; cells were scraped, centrifuged, resuspended in 2 ml of 100 mM phosphate, pH 7.4/2 M NaCl/0.1% bovine serum albumin, and disrupted with a homogenizer. Homogenates were centrifuged for 30 min at 19,000 rpm (SW41 Beckman rotor). Supernatants were adjusted to 100 mM phosphate, pH 7.4/0.65 M NaCl/0.1% bovine serum albumin and applied to a heparin-Sepharose column (Pharmacia). Bound growth factor was then eluted with 100 mM phosphate, pH 7.4/1 M NaCl quantitated by enzyme immunoassay (EIA), and tested for biological activity, as described below.

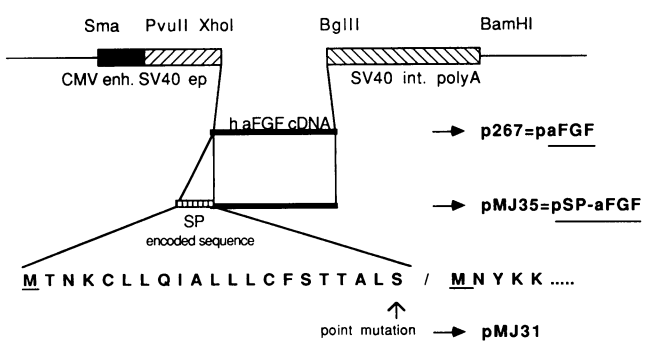

FIG. 1. Recombinant expression vectors. The human (h) aFGF cDNA sequence cloned in p267 (paFGF) has been reported (21). The 422-base-pair cDNA sequence was cloned under the control of the simian virus 40 early promoter (SV40 ep) and the cytomegalovirus enhancer (CMV enh.), with downstream SV40 regulatory sequences. The pMJ35 (pSP-aFGF) recombinant contains upstream of the first methionine codon of aFGF a heterologous sequence coding for a 21-amino acid SP. A mutation in the codon of the 21st amino acid generates a growth factor without a leader sequence (pMJ31 construct).

Immunoassay for aFGF. Production of aFGF by transfected clones was assayed by a solid-phase EIA specific for quantitation of aFGF (28). Standard curves were generated with bovine aFGF and recombinant human aFGF. The limit of detection of aFGF was 200 pg/ml (10 pg per assay). Assays were done either on total cellular extracts or on conditioned media after concentration by heparin-Sepharose affinity chromatography.

Immunoblot Analysis. Samples corresponding to aFGF eluted from the heparin-Sepharose column were first desalted with diatomaceous earth (acid-washed, catalogue no. D5384, Sigma). Each sample (1 ml), in 1 M NaCl, was mixed with 250 μl of a 10% (wt/vol) suspension of diatomaceous earth in water at 4°C for 1 hr, using a rotating rack. After centrifugation the supernatant was discarded and the pellet was washed twice with 1 ml of 10 mM phosphate (pH 7). The final pellet was boiled 5 min with 25 μl of 2× reducing sample buffer (0.2 M Tris, pH 6.8/4% SDS/10% 2-mercaptoethanol/20% glycerol/0.4% bromophenol blue); the supernatant was adjusted to 50 μl with water and loaded on a 12.5% or 15% polyacrylamide gel. After electrophoretic separation and transfer onto nitrocellulose, the proteins were incubated overnight with rabbit polyclonal anti-aFGF serum (1:500 dilution) in 10 mM Tris, pH 7.4/0.15 M NaCl/1% Triton X-100/3% ovalbumin at 4°C. Bound antibodies were detected with affinity-purified ^{125}I-labeled protein A (10 μCi/μg, Amersham; 1 μCi = 37 kBq).

Biological Activity of Culture Medium Conditioned by aFGF-Producing Clones. Clones of cells expressing aFGF or SP-aFGF were cultivated for 3 days; conditioned media were collected, centrifuged, and tested for induction of motility and epithelial–mesenchymal conversion of NBT-II cells. Internalization of desmosomal proteins was scored and percentage of vimentin-positive cells was determined (8, 9). To confirm that the biological effect of the conditioned medium was due to the production of secreted aFGF, growth factor content was determined by EIA and tested for biological activity on NBT-II cells.

Zymography. Collagenase activities secreted from epithelial NBT-II cells and aFGF-producing clones were tested by zymography (29). Cells (10^6) were grown for 2 days in standard medium in a 90-mm culture dish and then incubated for 24 hr in 10 ml of serum-free medium. Samples (20 μl) of conditioned media were electrophoresed at 4°C under nonreducing conditions in a 9% polyacrylamide gel containing gelatin (0.5 mg/ml). The gel was incubated in 2.5% Triton X-100 for 30 min at room temperature, rinsed with water, and then incubated overnight at 37°C in 100 mM Tris, pH 7.4/30 mM $CaCl_2$. The gel was stained with Coomassie blue and destained to visualize unstained areas corresponding to zones of proteolysis.

RESULTS

Expression of aFGF by NBT-II Cells Transfected with Human aFGF cDNAs. In preliminary experiments, control NBT-II cells were analyzed for their ability to produce endogenous aFGF. Neither aFGF nor its transcript could be detected by Western blot or EIA and Northern blot analysis.

After cotransfection with paFGF/pAG60 or pMJ31/pAG60 (see Fig. 1) and selection with G418-containing medium, 80% of the selected clones expressed the expected 1.4-kilobase aFGF transcript; no concomitant expression of

endogenous rat aFGF was detected (data not shown). Total cell extracts as well as 1 M NaCl heparin-Sepharose column eluates were prepared from G418-resistant clones and analyzed for aFGF. All the clones that expressed the human aFGF mRNA contained significant amounts of the growth factor, ranging from 4.5 to 166.7 ng per 10^7 cells (Table 1). The 16-kDa aFGF in the extracts was detected by immunoblotting (Fig. 2); no aFGF was found in the media of aFGF clones or of control Neo or NBT-II clones.

Ninety percent of the selected clones of cells transfected with the SP-aFGF plasmid produced the growth factor in amounts ranging from 2.9 to 208.4 ng per 10^7 cells; the 16-kDa protein was revealed by immunoblotting and found in the conditioned medium of the pSP-aFGF-transfected cells, indicating proper functioning of the SP construct (Fig. 2).

aFGF-Producing Clones Have Lost Polarized Epithelial Markers. NBT-II clones expressing human aFGF with or without SP were totally or partially converted from an epithelial to a fibroblastic morphology. Morphologically transformed cells and control cells were assayed for characteristics of fibroblasts such as immunoreactivity for desmosomal proteins and vimentin. Immunoreactivity for desmosomal proteins was largely distributed within the cytoplasmic compartment in transfected cells, whereas in control cells the desmosome-specific proteins were typically confined to the plasma membrane in punctuate arrays along lateral cell–cell boundaries (8, 9). The aFGF-transfected cells had internalized the desmosomal proteins and expressed vimentin intermediate filaments at different levels depending on the clones (Fig. 3).

Table 1. Properties of aFGF-transfected clones

Cells*	aFGF,† ng per 10^7 cells: Extract	Medium	Motility,‡ μm/hr	Gelatinase§
Control				
NBT-II	0	0	<2	No
Induced NBT-II	0	—	30.0	Yes
Neo4	0	0	<2	No
Neo3	0	0	0	No
Transfected				
SP6	2.9	1.9	<2	Yes
NSF7	4.9	0	<4	Yes
NSF28	17.4	0	<4	Yes
NSF16	56.6	0	<2	Yes
NSF13	4.5	0	24.5	NT
NSF46	10.7	0	9.7	Yes
NSF22	11.6	0	55.3	Yes
NSF32	13.1	0	10.1	NT
NSF27	13.9	0	15.1	NT
NSF15	16.2	0	35.5	NT
NSF9	21.0	0	23.0	Yes
NSF5	77.3	0	20.6	Yes
NSF37	112.9	0	4.8	NT
NSF14	166.7	0	25.0	Yes
SP32	7.3	2.3	NQ	NT
SP27	8.5	8.3	NQ	Yes
SP29	18	14.9	NQ	Yes
SP44	23.8	17.8	NQ	NT
SP18	30.9	28.9	NQ	Yes
SP19	47.2	17.2	NQ	Yes
SP37	121.6	171.9	10.6	Yes
SP30	208.4	305.0	23.2	Yes

*Induced NBT-II cells were incubated with aFGF at 20 ng/ml. Neo4 and Neo3 are clones obtained after transfection with the pAG60 vector alone. NSF clones were obtained after transfection with aFGF with no SP (paFGF or pMJ31). SP clones were obtained after transfection with pSP-aFGF plasmid.

†aFGF content of cell extract or conditioned medium was determined by EIA of the growth factor eluted from a heparin-Sepharose column.

‡Clones with cell motility <4 μm/hr were considered nonmotile. NQ, not quantitated (the motility of these motile clones has not been quantitated).

§As detected by gelatin zymography. NT, not tested.

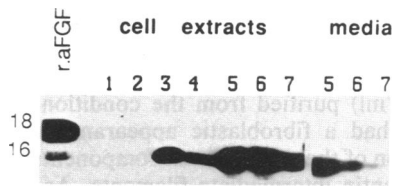

FIG. 2. Immunoblot analysis of aFGF content of cell extracts and conditioned media of control and producing clones. Lane r.aFGF: 25 ng of control recombinant aFGF of 18 and 16 kDa. Cell extracts of NBT-II (lane 1), a Neo clone (lane 2), NSF14 (lane 3), NSF9 (lane 4), SP29 (lane 5), SP32 (lane 6), and SP30 (lane 7) and conditioned media from the same SP clones (media lanes 5–7) were purified by heparin-Sepharose affinity chromatography.

SP-Coupled aFGF Expression Allows Secretion of Biologically Active Growth Factor. Conditioned media of 3-day-cultured clones of NBT-II cells expressing the transfected "native" aFGF (NSF clones) were tested for biological activity on control epithelial NBT-II cells. These media did not contain any scattering or motility-inducing activity; when assayed on untransfected NBT-II cells, desmosomes remained at the cell surface and vimentin expression was not detected. Further, the growth factor was not detected by EIA in the 1 M NaCl eluate of the conditioned media passed through a heparin-Sepharose column, indicating that the growth factor is not secreted.

In contrast, the clones producing aFGF coupled with a heterologous SP secreted the growth factor in amounts that correlated with the quantity found in the total cell extract (Table 1). The biological activity of the secreted aFGF was tested. The conditioned medium of clone SP30, which secreted as much as 305 ng of SP-aFGF per 10^7 cells during a 5-day culture of subconfluent cells, induced vimentin expression and desmosome internalization in 10–15% of the control NBT-II cells. To ascertain that the biological activity contained in the conditioned medium of SP30 was due to the

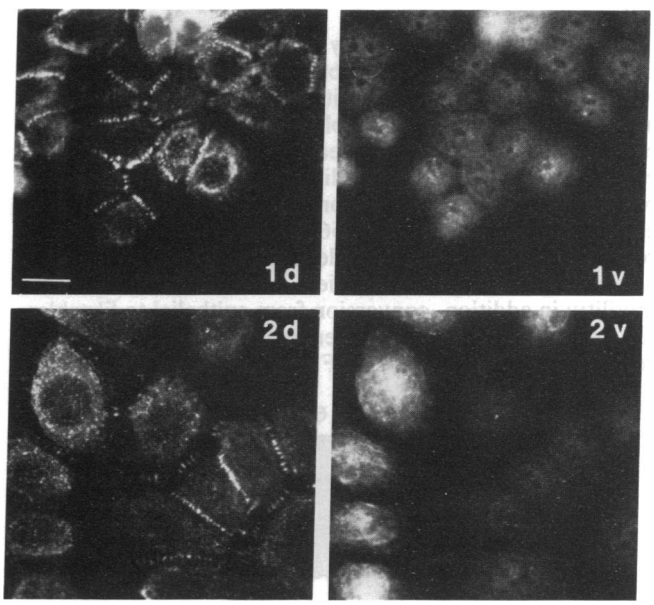

FIG. 3. Immunofluorescence of desmosomal junctions (*d*) and vimentin intermediate filaments (*v*) of NBT-II control cells (*1*) and clone NSF32 (*2*). (Bar = 10 μm.)

secreted aFGF, material eluted from the heparin-Sepharose column in 1 M NaCl was also tested. After 4 days of culture, 65% of control NBT-II cells grown in the presence of aFGF (300–500 ng/ml) purified from the conditioned medium of clone SP30 had a fibroblastic appearance as defined by internalization of the desmosomal components and the presence of vimentin intermediate filaments. As expected, the conditioned medium of clone SP6, a poor producer, did not induce expression of vimentin or desmosomal internalization.

aFGF-Producing NBT-II Cells May Acquire Motility *in Vitro*. In the presence of exogenous aFGF (10–20 ng/ml), normally stationary NBT-II cells become motile, lose cell cohesiveness, and change morphology (9). Similarly, most NBT-II cells expressing the transfected human aFGF cDNA, either with or without a SP, acquired motility *in vitro*. However the production of aFGF appeared not to be sufficient to induce cell motility, as clone NSF16, which produced as much as 56.6 ng of aFGF per 10^7 cells, was completely stationary (Table 1).

Expression of aFGF by NBT-II Cells Induced Secretion of Metalloproteases. The motile clones NSF5, -9, -14, -22, and -46 (Table 1) secreted predominant gelatinase activities at 64 kDa and 90–92 kDa as visualized by zymography (Fig. 4, lane 2). Cell clones producing SP-aFGF secreted essentially identical gelatinase activities (lanes 8–12). Nonmotile clones NSF7, NSF16, and NSF28 also secreted the 90- and 64-kDa gelatinase activities (lanes 3–5), but clones that did not produce the growth factor, control NBT-II cells, or Neo clones did not secrete protease activities. Addition of 10 mM EDTA to the incubation medium of zymography inhibited the enzyme activities, indicating that they were metalloproteases (data not shown).

DISCUSSION

The results demonstrate that nonsecreted human aFGF synthesized after transfection of epithelial NBT-II cells may induce (*i*) a change in cell morphology from epithelial to fibroblastic, (*ii*) an induction of cell motility, and (*iii*) a production of secreted gelatinase activities. Similar results were obtained upon transfection of cells with a chimeric aFGF cDNA specifying secretion of aFGF from transfected cells.

aFGF Synthesis Is Necessary But Not Sufficient to Induce Cell Motility. The clones obtained after transfection of NBT-II epithelial cells with either aFGF or SP-aFGF were mostly (80% and 90%) fibroblastic. The amount of aFGF produced in these clones ranged from 2.9 to 208.4 ng per 10^7 cells, but the expression level did not correlate with the speed of locomotion (Table 1). Clones NSF28 and NSF16, for example, produced 17.4 and 56.6 ng of aFGF per 10^7 cells, respectively, but were immobile. Thus the presence of aFGF was necessary but not sufficient for the induction of cell motility; in addition, conversion from epithelial to fibroblastic morphology, although dependent on aFGF, did not correlate with cell migration. NSF13 cells were motile whereas SP6 and NSF7 cells were stationary (Table 1), despite the observations that these two clones had a fibroblastoid morphology with 70% of the desmosomes internalized and 50–60% of the cells expressing vimentin filaments (data not shown). Therefore, epithelial cells constitutively producing aFGF can convert to fibroblastic cells without induction of motility. Among several mechanisms that could be invoked is that, in some clones, down-regulation of the aFGF receptors impairs the growth factor activity or that additional factors may be involved in this process.

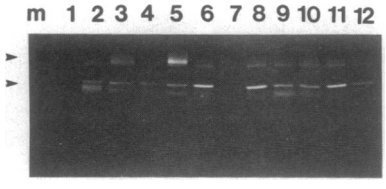

FIG. 4. Zymogram of gelatinase activities. Lane m, size markers (200, 116, 92.5, 66, and 45 kDa); lane 1, NBT-II control cells; lanes 2–5, NSF46, NSF28, NSF16, and NSF7; lane 6, SP6; lane 7, Neo clone; lanes 8–12, SP19, SP29, SP30, SP37, and SP44.

Production of aFGF Induces Invasive Potential. The production of gelatinolytic activities was in all cases linked to the synthesis of aFGF, whether clones were motile or stationary. Since gelatinases may contribute to the *in vivo* invasion of cells through stromal extracellular matrix, their induction by aFGF and the induction of cell migration by the same growth factor may be particularly significant in some cases. It is important to emphasize that all the aFGF-producing cells secreted gelatinolytic activities but that aFGF did not induce motility in all clones, indicating that these two cell properties, motility and gelatinase production, may use different signal-transduction mechanisms (for review see ref. 30).

It is possible that, *in vivo*, aFGF can initiate the dispersion of tumor cells from the primary site. The aFGF could be produced nearby the tumor cells, inducing cell scattering and cell motility via a paracrine mechanism; alternatively a subset of cells of the tumor may become able to synthesize aFGF and thus can detach and migrate through an autocrine and/or intracrine mechanism(s). The concomitant secretion of gelatinases capable of degrading type IV collagen (29) would facilitate the migration of the cells. bFGF induces also collagenase production in endothelial cells (31); FGFs may enhance tumor growth and the invasiveness of the cells by stimulating tumor angiogenesis (32). The synthesis of metalloproteases may be correlated with the process of invasion as demonstrated for metastatic colon carcinoma (33) or melanoma (34) cells.

Biological Status of Growth Factor-Producing Cells. The SP clones actively secreted aFGF into the medium. However, only 10% of this growth factor was active in the NBT-II cell morphology conversion assay. The remaining 90%, despite being retained on heparin-Sepharose, was apparently inactive, perhaps because of instability of the secreted growth factor; as reported for both aFGF and bFGF the presence of heparin in the medium prevents the growth factor from degradation (35).

It has been suggested that aberrant expression of FGFs may cause cell transformation by an autocrine mechanism (21, 36, 37). The transforming potential of bFGF cDNA was greatly enhanced by fusing bFGF sequence to an immunoglobulin SP. Despite the presence of the SP the bFGF was mostly associated with the cell and not secreted into the medium (36). Further, the conditioned medium exhibited no biological activity as measured by induction of proliferation of endothelial cells. The authors suggested that the growth factor could be immediately sequestered at the cell surface, activating a mitogenic pathway by an autocrine loop. A bFGF cDNA fused to the growth hormone SP cDNA sequence gave similar results for cell transformation, but in this case aberrant forms of the growth factor were secreted (38). The sum of the data obtained thus far does not allow elimination of the possibility that transformation by FGFs may result from the protein acting intracellularly as reported for the platelet-derived growth factor B chain (39). Similarly, we found that when cells were transfected with plasmids encoding aFGF with no SP, the cell clones became fibroblastic and secreted metalloproteases, and the majority acquired significant motility. Since no aFGF was detected in the conditioned media of these clones, it appears that aFGF may be active without being secreted. On the other hand, aFGF can be detected in

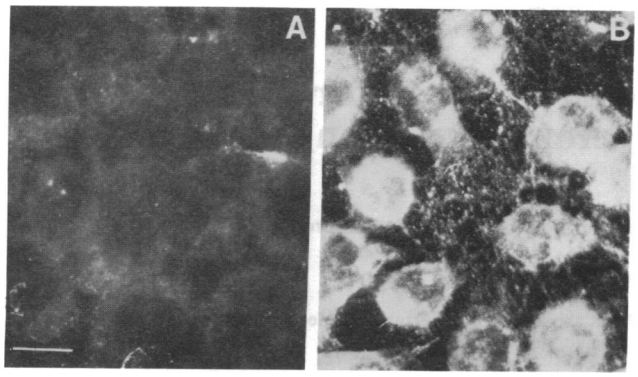

FIG. 5. Extracellular matrix immunoreactivity for aFGF. (*A*) NBT-II control cells. (*B*) NSF14 aFGF-producing clone. (Bar = 10 μm.)

the extracellular matrix by immunofluorescence (Fig. 5), indicating that the aFGF may be exported out of the cell by mechanisms other than the conventional secretory pathways. As NBT-II cells possess ≈5000 high-affinity aFGF receptors per cell (9), it is tempting to conclude that aFGF is acting via an autocrine loop in growth factor-producing cells. We cannot exclude the possible involvement of an intracrine mechanism, by direct coupling of the growth factors to their receptors at the cell surface or even intracellularly.

In conclusion, transfected NBT-II cells expressing human aFGF either with or without a SP acquired properties of potentially metastatic cells: morphological transformation, motility, and active metalloprotease production. Although motility was not completely correlated with the level of aFGF production, aFGF synthesis was necessary but not sufficient for this to be manifested. It is possible that this was due to qualitative and/or quantitative differences in the NBT-II growth factor-producing cells, with regards to aFGF signal transduction. Extrapolated to the *in vivo* situation, the results suggest that increased aFGF signaling—either paracrine, autocrine, or intracrine—has the potential to convert a subset of cells from a noninvasive to a potentially metastatic phenotype.

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