Growth Advantage and Vascularization Induced by Basic Fibroblast Growth Factor Overexpression in Endometrial HEC-1-B Cells: An Export-dependent Mechanism of Action

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ABSTRACT

The human endometrial adenocarcinoma HEC-1-B cell line was transfected with an expression vector harboring the human basic fibroblast growth factor (bFGF) cDNA under the control of the human β-actin gene promoter. Stable transfectants were obtained in which a constitutive, limited overexpression of M̄ 24,000, 22,000, and 18,000 bFGF isoforms was observed. When transfectedants were screened for the capacity to release the growth factor, significant amounts of bFGF were present in the conditioned medium and extracellular matrix of the bFGF-B9 clone but not of the bFGF-A8 clone, even though both cell lines produced similar levels of intracellular bFGF. When compared to parental cells, bFGF-B9 cells showed down-regulation of tyrosine kinase fibroblast growth factor receptors along with up-regulation of urokinase-type plasminogen activator expression which was abolished by incubation of the cell cultures with neutralizing anti-bFGF antibody. In vivo, bFGF-B9 cells formed highly vascularized tumors growing faster than parental cells when injected s.c. in nude mice. Also, they were more potent than nontransfected cells in inducing an angiogenic response in the rabbit cornea assay. In contrast, the bFGF-A8 cell phenotype was indistinguishable from parental cells both in vitro and in vivo. In conclusion, clonal differences exist within the HEC-1-B cell line in the capacity to release bFGF. bFGF export by human endometrial adenocarcinoma cells results in autocrine and paracrine effects that confer a growth advantage in vivo associated with increased neoangiogenesis.

INTRODUCTION

bFGF belongs to the family of the heparin-binding growth factors (1). This molecule has been isolated from a variety of tissues and cell lines (2). In vivo, bFGF exerts an angiogenic activity in different experimental models (3). In vitro, bFGF induces both mitogenic and nonmitogenic functions on different cell types (4). bFGF is thought to play a role in the growth and/or neoangiogenesis of solid tumors. Indeed, various tumor cell lines express bFGF in vitro (5–9). In situ hybridization and immunolocalization experiments have shown the presence of bFGF mRNA and/or protein in neoplastic cells, endothelial cells, and infiltrating cells within tumors of different origin (10–14). Also, a significant correlation between the presence of bFGF in cancer cells and advanced tumor stage has been reported (15). Even though bFGF lacks a leader sequence for secretion (16), data suggest that bFGF is secreted from bFGF-producing cells by an alternative secretion pathway (17, 18) and accumulates in the ECM, from where it can be released by ECM-degrading enzymes (19). Interestingly, the appearance of an angiogenic phenotype correlates with the export of bFGF during the development of fibrosarcoma in a transgenic mouse model (20). These data suggest that bFGF release may occur in vivo and may influence solid tumor growth and neoangiogenesis by autocrine and paracrine modes of action. Accordingly, anti-bFGF antibody affects tumor growth under defined experimental conditions (21–23).

We have shown that human endometrial adenocarcinoma cell lines produce limited amounts of bFGF in vitro (24). On the other hand, human recombinant bFGF induces PA production in the same cells (25), suggesting that bFGF may exert autocrine and paracrine roles in cultured tumor endometrial cells. In vivo, we have observed that bFGF is present in the extracts of biopsies of normal human cycling endometrium at levels higher than those found in other tissues, including myometrium, brain, and placenta (26). Also, bFGF has been isolated from porcine and bovine uterus (27, 28), and bFGF mRNA has been detected in rat uterus (29) and human endometrium (30). The cellular source of bFGF in normal uterus is not defined and epithelial cells, as well as smooth muscle cells and endothelial cells, may be involved in bFGF production. bFGF accumulates in the ECM of the endometrial tissue (31), and it is detectable in uterine fluids (32, 33).

Recently, the presence of a biologically active, immunoreactive bFGF-like protein has been reported in biopsies of well-differentiated adenocarcinomas of the endometrium from cycling and postmenopausal patients (34), and bFGF immunostaining has been observed in the glandular epithelium in complex hyperplasia and carcinoma of the endometrium (35). These findings raise the hypothesis that bFGF may be involved in the growth and/or neoangiogenesis of endometrial cancer. To assess this hypothesis, the human endometrial adenocarcinoma HEC-1-B cell line (36), which synthesizes low levels of bFGF under standard culture conditions (24), was transfected with an expression vector harboring a human bFGF cDNA under the control of the human β-actin gene promoter. Stable transfectants, characterized by a constitutive, limited overexpression of M̄ 24,000, 22,000, and 18,000 bFGF isoforms and a different capacity to secrete the growth factor were investigated for their biological behavior in vitro and for their angiogenic and tumorigenic potential in vivo.

MATERIALS AND METHODS

Cell Cultures and Transfection. HEC-1-B human endometrial cells were obtained from American Type Culture Collection (Rockville, MD) and were grown in MEM supplemented with 1% nonessential amino acids, 1% sodium pyruvate, and 10% FCS.

A 1108-bp human bFGF cDNA (38) was cloned into BamHI-SalI into pHBAPr-1 (39) to give the expression vector pHBAA-bFGF. To obtain stable transfectants, HEC-1-B cells were plated at 8.0 × 10⁴ cells/100-mm plate and were cotransfected with a calcium phosphate precipitate containing 20 μg pHBAA-bFGF, 2 μg pSV2Neo (Clontech, Palo Alto, CA), and 40 μg salmon...
sperm DNA. After 20 h, G418 sulfate antibiotic (Sigma) was added at 500 μg/ml to the culture medium. After 3 weeks of selective pressure, the G418-resistant clones were isolated, expanded, and tested by immunoblot and immunocytochemical analysis.

Fetal bovine aortic endothelial GM 7373 cells were obtained from the NIGMS Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ). They were grown in MEM supplemented with 10% FCS, vitamins, and essential and nonessential amino acids.

**bFGF and Antibodies.** Human recombinant M, 18,000 was expressed in Escherichia coli and purified by heparin-Sepharose affinity chromatography as described (37). Rabbit polyclonal antihuman recombinant bFGF antibody was kindly provided by D. B. Rifkin (New York University Medical Center, New York, NY). The IgG fraction was precipitated from serum with 50% saturated ammonium sulfate and purified by bFGF-Affi-Gel affinity chromatography. The flow-through IgG of the bFGF-affinity column will be referred to as irrelevant IgG. Affinity-purified antibody, but not irrelevant IgG, specifically quenches the biological activity of bFGF and recognizes the growth factor in immunodot blot and Western blot assays (26). The rat mAb antimurine CD31 antibody was kindly provided by A. Vecchi (Istituto Mario Negri, Milano, Italy).

**Immunocytochemical Analysis.** Cells were plated at the density of 16 × 10^5 cells/cm² on 12-mm glass coverslips coated with 100 μg/ml poly-L-lysine. After 16 h, medium was removed and cell cultures were fixed in PBS containing 4% paraformaldehyde and 0.1 M sucrose for 5 min at 37°C and for an additional 10 min at room temperature. Cells were washed twice with 0.1 M sucrose in PBS, twice with PBS, and were incubated for 15 min at room temperature with GS diluted 1:100 with PBS containing 0.3% Triton X-100 (GS/PBS/Triton X-100). For bFGF immunolocalization, cells were incubated for 1 h in a humid atmosphere with either irrelevant rabbit IgG or with affinity-purified rabbit anti-bFGF antibody diluted 1:50 in GS/PBS/Triton X-100. After three washes with PBS/Triton X-100, cells were incubated with affinity-purified biotinylated antirabbit IgG in GS/PBS/Triton X-100. After 30 min at room temperature, cells were rinsed three times with PBS and incubated for another 30 min with streptavidin-peroxidase (Società Prodotti Antibiotici, Italy) in PBS. After three washes with PBS, the peroxidase activity was detected by adding 1.5 mg/ml 3-chloroindomobenzidine and 0.3% H₂O₂. For vimentin immunolocalization, cells were incubated for 2 h with a mAb antivimentin antibody (DAKO) diluted 1:10 in GS/PBS/Triton X-100. After three washes with PBS/Triton X-100, cells were incubated for 30 min in the dark with a 1:10 dilution of FITC-conjugated antimouse IgG antibody (Sigma). Coverslips were washed three times with PBS and mounted.

**Immunoblot Analysis.** Confluent cultures were washed with serum-free medium and incubated for 24 h with fresh medium. At the end of the incubation, conditioned medium was collected. Cell monolayers were washed twice with PBS and once with 2 M NaCl in PBS. The 2 M NaCl wash was collected and diluted with PBS to 0.5 M NaCl. Conditioned medium and diluted 2 M NaCl wash were loaded onto 100-μl heparin-Sepharose columns. The resin was transferred to an Eppendorf tube, washed twice with PBS, and added with SDS-PAGE reducing sample buffer. In the meantime, cells were scraped from the dish and sonicated in SDS-PAGE reducing sample buffer with three bursts of 10 s each at 80 W. Cells extracts and resin beads were boiled, run on SDS-15% PAGE, and proteins were electrophoretically transferred to nitrocellulose membranes in 20% methanol, 190 mM glycine, and 25 mM Tris·HCl. After transfer, membranes were saturated with PBS containing 3% BSA and 10% FCS and probed for 2 h at room temperature with affinity-purified, polyclonal anti-bFGF antibody diluted 1:50 in PBS/0.5% BSA. After a 30-min wash with PBS/0.5% BSA/0.2% Tween 20 and a second wash with PBS/0.5% BSA/0.2% Tween 20 and with 50 mM Tris·HCl (pH 7.6), immunocomplexes were visualized by incubation with 5-bromo-4-chloro-3-indol phosphate and nitroblue tetrazolium (Sigma). In some experiments, bFGF was visualized by chemiluminescence Western blotting utilizing the ECL Western blotting kit (Amer sham Life Sciences) according to the manufacturer’s instructions.

**Cell Growth Assay.** Cells were seeded at 10,000 cells/cm² and grown in MEM containing either 10% or 0.4% FCS in the absence or presence of the molecules to be tested. On the days indicated, duplicate cultures were trypanotyped, and the cell number was determined with a Burker chamber.

**125I-bFGF Binding Assay.** Human recombinant M, 18,000 bFGF was labeled with 125I (37 GBq/ml; Amersham International, Amersham, England) using Iodogen (Pierce Chemical Co., Rockford, IL) as described (40) at a specific radioactivity equal to 600 cpm/fmol. Cells were seeded in 24-well dishes at the density of 100,000 cells/cm². After 24 h, cells were washed three times with cold PBS and incubated for 2 h at 4°C in binding medium (serum-free medium containing 0.15% gelatin, 20 mM HEPES buffer, pH 7.5) in the presence of increasing concentrations of 125I-bFGF. Then, after a PBS wash, cells were washed twice with 2 M NaCl in 20 mM HEPES buffer (pH 7.5) to remove low-affinity binding to heparan sulfate proteoglycans and twice with 2 M NaCl in 20 mM sodium acetate (pH 4.0) to remove high-affinity binding to tyrosine kinase FGFRs (41). Nonspecific binding was measured in the presence of a 100-fold molar excess of unlabeled bFGF and subtracted from all of the values. Data were analyzed using the Scatchard plot procedure (42).

**Northern Blot Analysis.** Northern blot analysis of total RNA (20 μg/sample) was performed according to standard procedures (43). Uniform loading of the gels was assessed by ethidium bromide staining of the gel or by methylene blue staining of the filter. FGFR-1 and FGFR-2 probes were kindly provided by A. Mansukhani (New York University, New York, NY), FGFR-3 and FGFR-4 probes by J. Partanen (University of Helsinki, Helsinki, Finland), and the uPA probe by P. Mignatti (University of Pavia, Pavia, Italy).

**Plasminogen Activator Assay.** Confluent cultures of parental and bFGF-transfected HEC-1-B cells were incubated in fresh medium containing 0.4% FCS in the absence or presence of human recombinant M, 18,000 bFGF. After incubation at 37°C for 18 to 20 h, conditioned media were collected, and cell layers were washed twice with PBS. Then PA activity was measured in the conditioned media and cell extracts (25) by using the plasmin chromogenic substrate H-D-norleucyl-hexahydroxylysyl-lysine-p-nitroanilide-acetate (American Diagnostica, Greenwich, CT) according to the manufacturer’s instructions. Human uPA (60,000 IU/mg protein, Calbiochem, San Diego, CA) was used as a standard. One IU corresponds to 0.7 Plug units.

For the determination of the PA-inducing activity of HEC-1-B cell extracts, endometrial GM 7373 cells were seeded at the density of 70,000 cells/cm² (44). After 24 h, endothelial cell cultures were washed twice with MEM and incubated in fresh medium containing 0.4% FCS and increasing concentrations of the endometrial cell extracts. After incubation at 37°C for 24 h, the endothelial cell layers were washed twice with cold PBS. Then PA activity was measured in the endothelial cell extracts by using the plasmin chromogenic substrate as described above. In these experimental conditions, bFGF exerts a 10-fold increase in GM 7373 cell-associated PA activity with a half-maximal concentration of 3 ng/ml (40).

**Tumorigenicity Assay.** Female NCr-nu/nu mice were obtained from the National Cancer Institute Animal Program (Frederick, MD) and used when 6–8 weeks old. Transfected HEC-1-B cells were harvested by a brief exposure to 0.25% trypsin/0.02% EDTA, washed twice, and resuspended in HBSS. Mice were given a s.c. injection of 2 × 10³ or 2 × 10⁴ cells suspended in 0.2 ml HBSS in the dorsal scapular region (five animals per group). The tumor mass was measured once a week with calipers, and tumor weight (in g) was estimated by the formula: (length × width)/2. In some experiments, frozen biopsies of the xenografts corresponding to 500 μg protein were sonicated in SDS-PAGE reducing sample buffer, boiled, and proteins were analyzed by Western blotting for bFGF content.

**Immunohistochemistry.** Xenografts removed 5 weeks after transplantation were embedded in OCT compound, frozen, and 5-μm sections were obtained with a cryostat microtome. Then sections were processed for immunohistochemical analysis by using a rat antiamidine CD31 antibody for the detection of blood vessels (45). To this purpose, sections were fixed in PBS and incubated for 20 min with 0.3% H₂O₂ in absolute methanol to block endogenous peroxidase and for another 20 min with 0.2% Triton X-100 in PBS. Then a 30-min preincubation with diluted normal serum was followed by incubation at 4°C with undiluted antici-CD31 antibody in a humidified chamber. Sections were then exposed to biotinylated secondary antibody (Vector Laboratories) and to avidin-biotin-peroxidase complex (DAKO ABComplex HRP) for 30 min. Peroxidase color reaction was developed with 3-aminobenzidine (Sigma), and the sections were lightly counterstained with Mayer’s hematoxylin.

**Assessment of Microvessel Density.** Two cross-sections (100 μm apart) were examined for each tumor at × 312.5, and the number of CD31-positive blood microvessels per field (0.12 mm²) were counted. The whole surface area
of each section was examined, and microvessels were defined as any immuno-reactive endothelial cell(s) that were separate from adjacent microvessels. Vessel lumens were not necessary for a structure to be defined as a microvessel. Vascular counts for individual sections were then produced using the mean of all of the field counts. No significant differences in microvessel counts were observed between paired sections of individual tumors.

Rabbit Cornea Assay. The capacity of transfected HEC-1-B cells to induce an angiogenic response was tested in vivo using the rabbit cornea assay (46). Each New Zealand White rabbit (Charles River, Calco, Como, Italy) received parental HEB-1-B cells in the left cornea and bFGF-transfected cells in the right cornea. Cell suspensions were obtained by trypsinization followed by serum neutralization and centrifugation at 1400 rpm for 10 min at 4°C. Cell suspensions were diluted in DMEM plus 10% FCS to obtain 3 \times 10^7 cells in 5 µL. Following pentobarbital anesthesia (30 mg/kg), a microsurgical pocket (1.5 mm \times 3 mm) was produced in the lower half of the eye using a pliable 1.5-mm wide iris spatula. Each pocket, located in the transparent avascular corneal stroma, received 5 µL of cell suspension. The observation of the implants, started 48 h after surgery and performed every other day for 21 days, was made with a slit lamp stereomicroscope without anesthesia. An angiogenic response was scored positive when budding of vessels from the limbal plexus was observed and capillaries progressed to reach the implanted pellet according to the scheme previously reported (46). Angiogenic activity was expressed as the number of implants exhibiting neovascularization over the total implants studied. Potency was scored by the number of newly formed vessels and by their growth rate. Data were expressed as the angiogenesis score, calculated as vessel density \times distance from the limbus in mm as measured by an ocular grid. Vessel density values ranging from 1 to 5 corresponded to 0–25, 25–50, 50–75, 75–100, and 100–200 vessels/cornea, respectively.

RESULTS

bFGF cDNA Transfection of HEC-1-B Cells. HEC-1-B cells originate from an endometrial papillary adenocarcinoma of a 71-year-old patient (36). The cell line is transplantable into the hamster cheek pouch where it originates tumors with papillary and adenomatous structure which resembles the original tumor. At chromosomal analysis, HEC-1-B cells have a modal chromosomal number of 46 and show a large marker chromosome (36). HEC-1-B cells produce low levels of endogenous bFGF (20) and express 125I-bFGF binding sites on their cell surface (25).

To investigate the biological consequences of bFGF up-regulation in human endometrial tumor cells, we transfected HEC-1-B cells with an eukaryotic expression vector harboring a human bFGF cDNA which encodes for all of the bFGF isoforms. To obtain levels of expression of bFGF close to those observed under natural conditions, a 1.1-kb bFGF cDNA (38) was cloned into pHßA-Pr-1 under the control of the human ß-actin gene promoter (39), originating pHßA-bFGF.

After transfection and selection, several G418-resistant clones were obtained. Clones were analyzed for bFGF expression by Western blot analysis of the cell extracts. Some of the selected clones are shown in Fig. 1A. pHßA-bFGF induces the expression of M, 24,000, 22,000, and 18,000 bFGF isoforms. The various clones express bFGFs at different levels, bFGF-B9 and bFGF-A8 cells being the most effective. Similar results were obtained whether the amount of bFGF present in the cell extracts was quantitated using a biological assay based on the capacity of bFGF to induce PA production in cultured endothelial cells (14, 26, 34). Indeed, when cell extracts were added to endothelial GM 7373 cells they induced PA up-regulation with a different potency (Fig. 1B). Again, bFGF-B9 and bFGF-A8 cell extracts were the most effective. From the biological and immunological assays, it could be calculated that bFGF is present in the extracts of bFGF-B9 and bFGF-A8 cells at the concentration of 300 ng/mg protein. This concentration is 10 times higher than in parental HEC-1-B cells and close to that observed in different nontransfected tumor cell lines, including SK-Hep-1 cells (5). On this basis, bFGF-B9 and BFGF-A8 cells were utilized for additional studies. Since preliminary experiments demonstrated that the biological behavior of the G418-resistant clones that do not show bFGF overexpression was indistinguishable from that of parental cells, the biological properties of bFGF-B9 and bFGF-A8 cells described hereafter are compared to those shown by nontransfected cells unless specified otherwise.

bFGF lacks a classical signal peptide for secretion (16). However, an alternative mechanism of exocytosis, independent of the endoplasmic reticulum/Golgi pathway, has been proposed for bFGF (17, 18). Accordingly, bFGF has been found to be associated with ECM of cell cultures in vitro (47, 48), from where it can be eluted by a neutral 2 M NaCl wash (49). Since the appearance of an angiogenic phenotype in tumor development has been shown to be associated with a switch to the export of bFGF from neoplastic cells (20), we attempted to identify, among the various HEC-1-B transfectants, bFGF-overexpressing clones with significant differences in the capacity to export bFGF. To this purpose, Western blot analysis of the conditioned medium and of a 2 M NaCl wash of the cell monolayers was performed for bFGF-B9, bFGF-A8, and parental cells (Fig. 2). Samples

Fig. 1. bFGF expression in transfected HEC-1-B cells. Cells were transfected with pHßA-bFGF. Cell extracts of G418-resistant clones were prepared and assessed for bFGF expression by Western blot analysis with affinity-purified anti-bFGF antibody (A). Increasing concentrations of the cell extracts were also added to endothelial GM 7373 cells. After 24 h, GM 7373 cell-associated PA activity was measured by using a chromogenic PA assay (B). GM 7373 cells incubated with 10 ng/ml human recombinant M, 18,000 bFGF, were used as positive controls (arrow). Molecular weights are in thousands. Lane a, parental HEC-1-B cells ( ); Lane b, bFGF-625 clone; Lane c, bFGF-B9 clone ( ); Lane d, bFGF-A8 clone ( ); Lane e, bFGF-B8 clone ( ); Lane f, bFGF-613 clone ( ); and Lane g, bFGF-616 clone.
equally susceptible to the growth inhibitory activity exerted by 10 ng/ml transforming growth factor α (data not shown). In conclusion, bFGF overexpression does not confer any apparent in vitro growth advantage to HEC-1-B cells.

When NIH 3T3 cells are transfected with the same bFGF cDNA utilized in the present study, down-regulation of tyrosine kinase FGFRs is observed, related to the presence of bFGF in the extracellular compartment (52). The different capacity of bFGF-A8 and bFGF-B9 clones to release bFGF prompted us to investigate the ability of 125I-bFGF to bind to parental and bFGF-transfected HEC-1-B cells. As predicted, a dramatic decrease in the capacity of 125I-bFGF to bind to high-affinity FGFRs was apparent in bFGF-B9 cells when compared to parental and bFGF-A8 cells. In contrast, the binding of 125I-bFGF to low-affinity heparan sulfate proteoglycans was not affected by bFGF transfection in both clones (Fig. 5A). Scatchard plot analysis of the 125I-bFGF binding data confirmed the down-regulation of FGFRs in the bFGF-B9 clone (Fig. 5B).

Four distinct FGFR genes have been identified (for a review, see Ref. 53). To rule out the possibility that the decrease in the number of high-affinity 125I-bFGF binding sites observed in the bFGF-B9 clone was dependent on clonal variability of FGFR expression rather than on receptor down-regulation, total RNA extracted from parental and bFGF-transfected HEC-1-B cells was hybridized with specific probes for FGFR-1, -2, -3, and -4 in a Northern blot (Fig. 5C). The results demonstrate that all of the cell lines investigated expressed FGFR mRNAs at similar levels. FGFR-4 mRNA is the most abundant, while intermediate levels of FGFR-1 mRNA, low levels of FGFR-3 mRNA, and undetectable levels of FGFR-2 mRNA are present in the three cell lines. These data support the hypothesis that the decrease in number of high-affinity sites observed in bFGF-B9 cells is due to receptor down-regulation and also rule out the possibility that bFGF overexpression may affect transcriptional and posttranscriptional modulation of FGFR gene expression in HEC-1-B cells.

Taken together, these data suggest that an autocrine loop of stimulation may exist in bFGF-B9 cells. This loop should not be apparent in the bFGF-A8 clone which releases lower amounts, if any, of the growth factor. To assess this hypothesis we took advantage of the capacity of bFGF to induce up-regulation of uPA expression in HEC-1-B cells (25). On this basis, a higher uPA production under standard culture conditions is anticipated for bFGF-B9 cells when compared to bFGF-A8 cells and parental HEC-1-B cells. Indeed, the basal levels of PA activity in bFGF-B9 cells were approximately five times higher than in the other cell lines (Table 1). Addition of 30 ng/ml human recombinant M1 18,000 bFGF to the culture medium had no effect on the levels of PA activity in bFGF-B9 cells while it induced a significant up-regulation of PA activity in bFGF-A8 and parental cells (Fig. 6A). These data were validated by Northern blot analysis, which demonstrated that the steady-state levels of uPA mRNA were higher in bFGF-B9 cells than in the other cell lines and were not affected by bFGF treatment (Fig. 6B).

Finally, bFGF-B9 and parental cells were incubated for 48 h with a 1:25 dilution of neutralizing anti-bFGF antibody, and then PA production was evaluated. Under these experimental conditions bFGF-B9 cell-associated PA activity was decreased to 1.2 ± 0.8 IU/mg of protein, corresponding to 20–25% of the activity measured in cells incubated with irrelevant IgG or left untreated (4.6 ± 0.8 and 4.9 ± 1.7 IU/mg of protein, respectively). No significant modification of cell-associated PA activity was observed instead in parental cells incubated with the anti-bFGF antibody. These observations confirm the hypothesis that uPA up-regulation in the bFGF-B9 clone is due to an autocrine loop of stimulation consequent to an increase in bFGF release.

Tumorigenic and Angiogenic Potential of bFGF-transfected HEC-1-B Cells. To evaluate the capacity of bFGF overexpression and secretion to influence HEC-1-B tumorigenicity, transfected cells were normalized according to cell number to allow a direct quantitative comparison among the different cell lines. Also, trypan blue staining indicated that cell viability was higher than 98% in all cell lines tested. Negligible amounts of bFGF immunoreactive material were detectable in the conditioned media and ECM of bFGF-A8 and parental HEC-1-B cells. On the contrary, both low and high molecular weight bFGF isoforms were present in the conditioned medium and in the 2 M NaCl wash of bFGF-B9 cells, indicating that these cells export significant amounts of the growth factor that becomes associated with ECM after release. Thus, even though bFGF-A8 and bFGF-B9 cells overexpress bFGF at similar levels, only bFGF-B9 cells show an increased capacity to release the growth factor.

In Vitro Biological Properties of bFGF-transfected HEC-1-B Cells. Monolayered cultures of parental and bFGF-transfected cells showed a similar morphology when observed under light and phase-contrast microscopy (Fig. 3A). Parental and transfected HEC-1-B cells appear to be epithelial, showing a pavement arrangement. Individual cells revealed anaplasia as well as nuclear and nucleolar pleomorphism; multinucleated giant cells were also seen (see Ref. 36 for a detailed description of HEC-1-B cell morphology). bFGF-A8 and bFGF-B9 cells showed a strong nuclear immunoreactivity and a weaker cytoplasmic positivity when stained with affinity-purified anti-bFGF antibody, as demonstrated for NIH 3T3 cells transfected with the same bFGF cDNA (50). Faint bFGF immunoreactivity was visualized by chemiluminescence Western blotting. Cell viability was higher than 98% for each cell line.

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were injected s.c. into nude mice. The injection of bFGF-B9 cells results in the formation of fast-growing tumors in all of the animals. After the injection of $2 \times 10^6$ cells, xenografts reached a mean weight of approximately $1 \text{ g}$ in 3 weeks (Fig. 7A). In contrast, bFGF-A8 cells induced tumors characterized by a slower rate of growth that reached $1 \text{ g}$ in approximately 9 weeks. A delay in tumor growth was also evident when mice were given an injection of a lower number of cells ($2 \times 10^5$). Under these conditions the appearance of tumors after injection of bFGF-A8 cells was delayed from 4 to 30 days when compared to bFGF-B9 cells (Fig. 7A). Nevertheless, Western blot analysis of the tissue extracts confirmed the presence of high levels of all bFGF isoforms in both bFGF-B9 and bFGF-A8 tumors when compared to parental HEC-1-B cell-derived tumors (Fig. 7B). In a previous experiment we found that mice given injections of parental HEC-1-B cells or control clones bFGF-625 and bFGF-B8, which express low levels of bFGF (see Fig. 1), grew slowly with kinetics similar to that of bFGF-A8 cells (data not shown).

The histological analysis showed that all of the xenografts were poorly differentiated adenocarcinomas with papillary and adenomatous patterns (Fig. 8A). When tissue slides were immunostained using
an antibody to platelet/endothelial cell adhesion molecule CD31 to highlight blood microvessels (46), bFGF-B9 tumors showed an intense neovascularization both at the margin and within the tumor mass (Fig. 8B). Neovascularization of tumors originating from bFGF-A8 cells and control clones bFGF-B8 and bFGF-625 appeared to be less intense (data not shown). Microvessel density was evaluated by counting the number of CD31-positive structures per field present in two serial cross-sections of each tumor. It must be pointed out that the whole surface area of each section was examined. Vascular counts for individual sections were then produced using the mean of all of the field counts. When microvessel density was measured in xenografts at 5 weeks after injection, the number of microvessels per 0.12-mm² field was 2.9 ± 1.2 (mean ± SD) for bFGF-B9 tumors, significantly higher (ANOVA, P = 0.002) than in xenografts formed by bFGF-A8 cells (1 ± 0.7 microvessels/0.12-mm² field). Control clones bFGF-B8 and bFGF-625 had 1.4 ± 0.8 microvessels/0.12-mm² field. Since bFGF transfection does not affect the proliferative capacity of HEC-1-B cells in vitro (see Fig. 4), these data suggest that the intense neovascularization of bFGF-B9 tumors may account, at least in part, for the faster rate of growth of these lesions in vivo (54).

To evaluate more directly the angiogenic potential of bFGF transfectants, we compared the capacity of bFGF-B9, bFGF-A8, and parental HEC-1-B cells to induce an angiogenesis response in the rabbit cornea. To this purpose, 3 × 10³ bFGF-transfected or parental HEC-1-B cells were implanted into the cornea of a rabbit eye, and implants were observed for the appearance of an angiogenic response. Angiogenic potency of the different cell lines was expressed by an angiogenic score which takes into consideration both the density and the rate of growth of the newly formed blood vessels (see “Materials and Methods”). The results indicate that both parental and bFGF-transfected HEC-1-B cells induce angiogenesis in the rabbit cornea (Table 2). For all of the cell lines, 100% of the eyes scored positive 20 days after implantation, suggesting that more factors, besides bFGF, are involved in neovascularization of endometrial adenocarcinomas. However, when the time lapse for occurrence, intensity, and progression of neovascular sprouting were compared among the implants of the three endometrial cell lines, bFGF-B9 cells were more potent. Indeed, bFGF-B9 cell implants anticipated angiogenesis by 4 days (Table 2) and had an angiogenesis score three times higher than that of the other cell implants at 18 days (Fig. 9). No macroscopic signs of an accompanying inflammatory reaction were observed in any of the cell lines. Taken together, our results strongly support the hypothesis that bFGF overexpression confers an increased angiogenic and tumorigenic potential in HEC-1-B cells only when paralleled by an increased export of the growth factor.

DISCUSSION

Previous observations had shown that NIH 3T3 cell transfectants expressing very high levels of bFGF fused with a signal peptide

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<th>Table 1</th>
<th>PA activity in parental and bFGF-transfected HEC-1-B cellsa</th>
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<tbody>
<tr>
<td>Cell type</td>
<td>Cell-associated PA (IU/mg protein)</td>
</tr>
<tr>
<td>HEC-1-B</td>
<td>1.3 ± 0.5</td>
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<tr>
<td>bFGF-A8</td>
<td>0.8 ± 0.3</td>
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<tr>
<td>bFGF-B9</td>
<td>4.9 ± 1.7</td>
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</tbody>
</table>

a Confluent cultures were maintained for 24 h in fresh medium containing 0.4% FCS. Then cell-associated and secreted PA activity was measured using a plasmin chromogenic assay. Values are the mean ± SE of four independent observations.

Fig. 5. ¹²⁵I-bFGF binding and FGFR expression in transfected HEC-1-B cells. A, parental HEC-1-B cells (●, ○). bFGF-B9 cells (■, □), and bFGF-A8 cells (▲, △) were plated at 100,000 cells/cm². After 24 h, cells were incubated for 2 h at 4°C with increasing concentrations of ¹²⁵I-bFGF, and the binding to low-affinity sites (●, □, ▲) and high-affinity sites (○, △, △) was evaluated. B, Scatchard plot analysis of the high-affinity binding data shown in A. bFGF bound/free, total RNA (20 µg/sample) isolated from HEC-1-B cells (Lane a), bFGF-B9 cells (Lane b), and bFGF-A8 cells (Lane c) was probed with FGFR-1, -2, -3, and -4 cDNAs in Northern blot. Uniform loading of the samples was checked by ethidium bromide staining of the gel. 28S and 18S, ribosomal RNAs.

Fig. 4. Growth properties of transfected HEC-1-B cells. Parental HEC-1-B cells (●, △), bFGF-B9 cells (○, ■), and bFGF-A8 cells (▲, △) were plated at 10,000 cells/cm² in medium containing 10% FCS (●, ○, △) or 0.4% FCS (▲, ■, △). Medium was changed every other day, and cell numbers were counted at the indicated times in a Burker chamber.
bFGF OVEREXPRESSION IN ENDOMETRIAL ADENOCARCINOMA CELLS

Fig. 6. uPA expression in transfected HEC-1-B cells. A, parental HEC-1-B cells (△), bFGF-B9 cells (●), and bFGF-A8 cells (○) were incubated with increasing concentrations of human recombinant M, 18,000 bFGF. After 24 h, cell-associated PA activity was evaluated by a chromogenic PA assay. B, parental HEC-1-B cells (Lane a), bFGF-A8 cells (Lane b), and bFGF-B9 cells (Lane c) were incubated for 8 h at 37°C in the absence or presence of 30 ng/ml human recombinant M, 18,000 bFGF. Then total RNA was extracted and probed by Northern blot hybridization with uPA cDNA (upper panel). Uniform loading of the samples was checked by methylene blue staining of the filter (lower panel). 28s and 18s, ribosomal RNAs.

sequence under the control of a viral promoter produce vascularized tumors in nude mice (23). Similar results were observed for human breast carcinoma MCF-7 cells transfected with fibroblast growth factor-4 (55), a member of the fibroblast growth factor family endowed with a signal peptide sequence (1). In the present article, we report for the first time that wild-type bFGF devoid of a signal peptide sequence enhances the angiogenic and tumorigenic potential of stable human endometrial adenocarcinoma HEC-1-B cell transfectants when expressed at levels close to those observed under natural conditions. The effects induced by the growth factor are observed only in those cells that export significant amounts of bFGF (bFGF-B9 clone) while bFGF-producing HEC-1-B cells which do not release increased amounts of the growth factor (bFGF-A8 clone) are indistinguishable from the parental cell line.

At present, the molecular bases responsible for the clonal differences in bFGF release observed among HEC-1-B cell transfectants are unknown. Even though bFGF lacks a leader sequence for secretion, evidence from several experiments indicate that bFGF can be released from producing cells by an alternative secretion pathway, thus inducing autocrine and paracrine functions (17, 56–59). Relevant to our findings is the observation that the discrete switch to the angiogenic phenotype which occurs in the multistep development of fibrosarcoma correlates with the export of bFGF (20). Thus, bFGF overexpression in tumors does not necessarily result in an increase in the release of the growth factor which will occur only in those cell clones that have acquired a heretofore unrecognized capacity to export bFGF during tumor progression. It is interesting to note that the capacity of transfected bFGF-B9 cells to export bFGF is not restricted to a specific isoform of the growth factor, as shown by the presence in the conditioned medium and ECM of this clone of approximately equimolar amounts of all bFGF forms. Similar results were reported for bFGF-transfected NIH 3T3 cells expressing very high levels of the growth factor (52). In contrast, bFGF release appears to be restricted to the M, 18,000 isoform in transfected COS cells (60).

As observed for bFGF overexpressing NIH 3T3 cells (52), released bFGF isoforms interact with FGFR in bFGF-B9 cells, causing the internalization of the FGFR/bFGF complex and receptor down-regulation. Consequent to receptor activation is the up-regulation of uPA gene expression which is prevented by the addition of neutralizing anti-bFGF antibody to the cell culture medium. It should be noted that recombinant low and high molecular weight bFGF isoforms interact with FGFR in a similar manner and exert a similar biological activity in vitro and in vivo (37). Taken together, these data indicate that an autocrine loop of stimulation is activated by bFGF only in those tumor

Fig. 7. Effect of bFGF transfection on the tumor growth of HEC-1-B cells in nude mice. bFGF-B9 cells (●, ○) and bFGF-A8 cells (△, △) were injected s.c. in nude mice at a concentration of 2 × 10⁶ (●, △) and 2 × 10⁵ (○, △) cells/implant. Mice were monitored twice a week, and tumor size was measured with calipers. The experiment was repeated twice with similar results, and the results of a representative experiment are shown in A. Each point is the mean ± SD of five animals. B, extracts (500 m.g of protein) obtained from tumors removed 3 weeks after injection of parental HEC-1-B cells (Lane a), bFGF-B9 cells (Lane b), or bFGF-A8 cells (Lane c) were analyzed for bFGF content by chemiluminescence Western blotting.

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endometrial cells that are able to secrete the growth factor in significant amounts and that bFGF overexpression is not sufficient per se to elicit a biological response in our experimental system. As predicted for a paracrine mechanism of tumor neovascularization, we have observed also that the potentiation of the angiogenic capacity of transfected HEC-1-B cells is restricted to the bFGF-B9 clone. Both the increased fibrinolytic activity and angiogenic potential of this clone, along with other possible autocrine and paracrine effects consequent to bFGF overexpression and export, may contribute in determining the capacity of bFGF-B9 cells to induce the early appearance of fast-growing neovascularized tumors when injected in nude mice (Fig. 10). Relevant to this point is the fact that bFGF transfection does not confer any apparent growth advantage to bFGF-B9 cells in vitro, suggesting that neovascularization can be rate limiting for bFGF-B9 tumors. Similar conclusions have been drawn for MCF-7 human breast carcinoma cells transfected with the angiogenic vascular endothelial growth factor (61).

Our findings implicate that the extrapolation of the autocrine and paracrine properties of a neoplastic tissue from the data obtained in situ on bFGF production and localization in tumor biopsies must be carefully considered. bFGF overexpression has been demonstrated in a variety of tumors of different origins (10-14), and in one study bFGF production has been associated with a more advanced tumor stage (15). Even though the evidence for an increased production of bFGF by immunohistochemical and/or in situ hybridization techniques does not provide information on the capacity of tumor cells to secrete the growth factor, some experimental data indicate that bFGF export may occur in vivo. For instance, bFGF is detectable in uterine fluids (32, 33), in urine of patients with a wide spectrum of cancers (62, 63), and in cerebrospinal fluid of children with brain tumors (64). Preliminary studies on biopsies of human endometrial adenocarcinoma indicate that bFGF immunostaining of tumor cells is related to histological grading of the lesion and that bFGF immunoreactivity is also frequently associated with ECM in poorly differentiated tumors. These data suggest that bFGF overexpression and release from neoplastic cells occur in human endometrial cancer and may contribute to its progression.

Induction of protease production by tumor and endothelial cells may enhance tissue invasiveness, metastasis, and neovascularization (65). Up-regulation of uPA production is observed in carcinomas of human endometrium (66-68). A coordinate modulation of tumor

Table 2. Angiogenic activity of parental and bFGF-transfected HEC-1-B cells in the rabbit cornea assay: time course

<table>
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<td>2/4</td>
<td>4/4</td>
</tr>
<tr>
<td>bFGF-A8</td>
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<td>2/4</td>
<td>3/4</td>
<td>4/4</td>
</tr>
<tr>
<td>bFGF-B9</td>
<td>2/4</td>
<td>3/4</td>
<td>3/4</td>
<td>4/4</td>
</tr>
</tbody>
</table>

* Number of positive implants/total number of implants performed.

Fig. 8. Histological appearance of bFGF-B9 cell-induced tumors. Tumors from nude mice inoculated with $2 \times 10^6$ bFGF-B9 cells were removed 5 weeks postinjection and processed as described in "Materials and Methods." A, poorly differentiated adenocarcinoma with papillary and adenomatous patterns. H & E staining. B, tumor section immunostained with antimouse endothelial CD31 antibody. No counterstaining was performed to highlight the numerous blood vessels. X20.

Fig. 9. Angiogenic activity of transfected HEC-1-B cells in the rabbit cornea assay. Three $\times 10^5$ parental HEC-1-B cells (C), bFGF-B9 cells (F), or bFGF-A8 cells (A) were implanted in the avascular cornea of rabbit eyes. Daily observations of the implants were then made with a slit-lamp stereomicroscope, and angiogenic potency was scored. Data were expressed as angiogenesis score, calculated as vessel density $\times$ distance from the limbus in mm. Each value is the mean $\pm$ SE of the scores obtained from four rabbits. **, $P < 0.01$, bFGF-B9 vs HEC-1-B, Student’s $t$ test.
ACKNOWLEDGMENTS

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REFERENCES


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