Cell Density-dependent Regulation of Basic Fibroblast Growth Factor Expression in Human Renal Cell Carcinoma Cells

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Abstract

We investigated some of the mechanisms that regulate the expression of basic fibroblast growth factor (bFGF) in human renal cell carcinoma (HRCC). HRCC SN12PM6 cells were cultured as adherent monolayers. The expression of steady-state bFGF mRNA (measured by in situ hybridization and Northern blot) and protein (measured by immunohistochemistry and ELISA) correlated inversely with the culture density. Tumor cells harvested from dense cultures (low bFGF expression) and plated under sparse conditions expressed high levels of bFGF mRNA and protein prior to cell division, suggesting that bFGF may be a competence factor. Similar data were obtained with human vascular endothelial cells. The expression of bFGF was not regulated by spent culture medium, cell cycle, or rate of cell division but was down-regulated by contact inhibition. These data show that the expression of bFGF in HRCC is cell density dependent.

Introduction

The growth, invasion, and metastasis of neoplasms all depend on the development of an adequate blood supply via angiogenesis (1–4). The induction of angiogenesis is mediated by several molecules released by both tumor cells and various host cells (1, 2). Studies using light microscopy in different human neoplasms concluded that the number and density of microvessels in and around the lesions directly correlate with their potential to invade and produce metastasis (5–10). These studies also revealed zonal heterogeneity in vascularization of the neoplasms (5–10). Whether this heterogeneity was due to heterogeneous expression of angiogenic molecules by cells populating different zones of the neoplasms (11, 12) remained unclear.

We have shown recently that the in vivo production of an angiogenic molecule, bFGF, by HRCC is influenced by the organ microenvironment. Specifically, HRCC growing s.c. or in the kidney of nude mice expressed low and high levels of bFGF, respectively (13). Subsequent data suggested that the difference in expression level was due, in part, to down-regulation of bFGF expression by IFN-β (14). Immunohistochemical analyses of both human and rodent neoplasms have revealed that many human neoplasms exhibit zonal heterogeneity in expression of P-glycoprotein (15, 16), collagenase type IV (17, 18), and bFGF (19), with the higher levels of expression being found along the invasive edge of the lesion. Whether this is due to cell proliferation or closer contact with the host microenvironment is unclear. The expression of several genes has been shown to be cell density dependent (20–24). For example, the expression level of collagenase type IV in human tumor cells decreases with contact inhibition of growth (25), and similar data suggest that the expression of bFGF is regulated by cell-cell interaction (26, 27). We investigated some of the mechanisms that regulate the expression of bFGF in HRCCs growing in culture. We found that the expression of bFGF is regulated by cell-cell contact (cell density) in HRCC cells and endothelial cells.

Results

Expression of bFGF in Sparse and Confluent HRCC Cultures. In the first set of experiments, SN12PM6 cells were plated at different densities into culture dishes. Sparse (140.7 cells/mm²; <30% confluence) and dense (1407.0 cells/mm²; >80% confluence) monolayer cultures were analyzed in parallel for bFGF protein (immunohistochemistry) and mRNA (ISH and Northern blot analysis). Cells growing under sparse conditions showed intense staining with the anti-bFGF antibody (Fig. 1A), whereas cells growing under confluent conditions did not (Fig. 1B). The level of bFGF mRNA in sparse and confluent cultures of SN12PM6 was first analyzed by ISH. As was the case in the immunohistochemical analyses, HRCC cells growing as sparse monolayer cultures expressed high levels of bFGF mRNA (Fig. 1C), whereas those in confluent cultures did not (Fig. 1E). No discernible differences were found in the level of poly(d)T reactivity between the sparse (Fig. 1D) and confluent (Fig. 1F) cultures, confirming the integrity of the mRNA. To further examine whether the level of bFGF mRNA in SN12PM6 cells was influenced by cell-cell contact (density), HRCC cells from stationary

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3 The abbreviations used are: bFGF, basic fibroblast growth factor; HRCC, human renal cell carcinoma; HUVEC, human umbilical vein endothelial cells; ISH, in situ hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PTP, protein tyrosine phosphatase.
Cell Density-dependent Regulation of bFGF

Fig. 1. Expression of bFGF in sparse and dense cultures of SN12PM6 cells. Sparse (<30% confluent) and dense (>90% confluent) monolayer cultures of SN12PM6 cells were examined for bFGF expression on the protein (immunohistochemistry) and mRNA (ISH) levels. Sparse cultures were highly positive for bFGF protein (A), whereas dense cultures were not (B). Sparse cultures also expressed higher levels of bFGF mRNA (C) than dense cultures (E). Poly d(T) reactivity in the sparse (D) and dense (F) cultures confirmed the integrity of the mRNA. Cells harvested from dense cultures were replated under sparse (G) or dense (I) conditions. Four h later, bFGF mRNA was vividly expressed in the sparse (G) but not densely (I) plated cells. At the same time, no discernible differences were found between poly d(T) reactivities (control) of sparse (H) and dense (J) cultures.
confluent cultures (low expression of bFGF) were harvested by trypsinization, and single-cell suspensions were plated at low (113.2 cells/mm²) and high (1132.3 cells/mm²) densities into different culture dishes. ISH was performed 4 h after plating. The SN12PM6 cells in sparse monolayer cultures showed intense reactivity with the bFGF probe (Fig. 1G), whereas the cells growing in dense cultures did not (Fig. 1F). The level of poly(d)T reactivity did not differ between the sparse (Fig. 1H) and confluent (Fig. 1J) cultures. These results suggest that trypsinization per se did not affect bFGF expression, whereas density, a measure of cell-cell contact, did. Similar data were obtained in Northern blot analysis.

HRCC cells cultured in sparse (140.7 cells/mm²) monolayers expressed four times as many bFGF-specific mRNA transcripts as HRCC cells growing in confluent (1407.0 cells/mm²) cultures (Fig. 2, Lanes A and B).

The density-dependent expression of bFGF was not unique to the SN12PM6 cells. In experiments using HUVEC cultured as sparse (384.2 cells/mm²; <30% confluence) or dense (4484 cells/mm²; >90% confluence) monolayers, bFGF mRNA (Fig. 2, Lanes C and D) expression was higher in the sparse cells. Quantitative data agreed with the level of mRNA. Cellular bFGF protein was 12.7 pg/10⁵ cells in sparse cultures, whereas in the confluent HUVEC cultures, it was 2.4 pg/10⁵ cells. Thus, in both HRCC and HUVEC cultures, the expression of bFGF was cell density dependent.

To determine whether medium conditioned by confluent SN12PM6 cells contained factors that can inhibit expression by bFGF, we measured the level of cellular bFGF in HRCC cells growing under sparse conditions incubated in control medium or medium collected from sparse or confluent HRCC cultures. The conditioned medium did not affect the level of bFGF expression (data not shown).

Kinetics of bFGF Expression in SN12PM6 Cells. We next examined the early changes in bFGF expression and its kinetics. Confluent cultures of SN12PM6 cells (low expression of bFGF) were trypsinized, and single cells were plated to produce sparse cultures (140.7 cells/mm²). mRNA was isolated from confluent cultures (baseline, low level of expression) and from the HRCC cells growing as sparse cultures at different times after plating (30 min for 4 days). The level of bFGF-specific mRNA transcripts began to rise by 1 h after plating, reaching the maximum by 4 h (Fig. 3). As soon as the cultures neared confluence (4 days), the expression of bFGF declined (Fig. 3), and by day 7 after plating, the level of bFGF mRNA was at the low level found in confluent cultures (data not shown).

Cell Division and bFGF Production. Confluent cultures of SN12PM6 cells were harvested, and 5000 viable cells were plated into 38-mm² wells of 96-well plates at a density of 131.5 cells/mm². After 0–96 h, different plates were harvested, and the cells were counted (hemocytometer). Cultures were also pulse-labeled with [³H]thymidine or [³H]leucine, and the amount of cellular bFGF/10⁵ cells was determined by ELISA. The data in Table 1 show that the increase in bFGF protein (from 5.0 pg/10⁵ cells to 17.35 pg/10⁵ cells) occurred at least 24 h before a significant increase in [³H]thymidine incorporation. The increase in bFGF protein was independent of metabolic activity, as measured by uptake of [³H]leucine (Table 1).

**In Vitro Wounding Experiments.** In the next set of experiments, we used a rubber policeman to scrape off a lane of cells through a dense culture of SN12PM6 cells. The “wounded” cell was then incubated in MEM for 48 h. Immunohistochemistry and ISH analysis were performed to detect bFGF protein and mRNA, respectively. Cells growing on the edge of the wound or in the wounded area showed intense staining for bFGF protein, whereas those in the confluent, intact monolayer did not (Fig. 4). A similar pattern was observed for bFGF mRNA (data not shown). This finding of bFGF-positive cells on the edge of a bFGF-negative confluent culture ruled out that the medium per se controlled expression of bFGF and suggested that dividing cells preferentially produce bFGF.
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**Table 1** Correlation between culture density, protein synthesis, DNA synthesis, and bFGF protein in SN12PM6 cells incubated for different times.

<table>
<thead>
<tr>
<th>Duration of incubation (h)</th>
<th>Density* (cells/mm²)</th>
<th>[³H]Thymidine cpm/10⁶ cells</th>
<th>[³H]Leucine cpm/10⁶ cells</th>
<th>Cellular bFGF pg/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>131</td>
<td>47</td>
<td>450</td>
<td>5.00</td>
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<td>4</td>
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<td>529</td>
<td>17.35</td>
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</tr>
<tr>
<td>96</td>
<td>389</td>
<td>189</td>
<td>500</td>
<td>16.04</td>
</tr>
</tbody>
</table>

* Five thousand HRCC cells/38-mm² well were plated in 38-mm² wells of 96-well plates at the density of 131 cells/mm². The cells were counted by hemocytometer.

**Cell Density and Production of bFGF Protein.** In this set of studies, we harvested confluent cultures of SN12PM6 cells (low bFGF expression), and SN12PM6 cells were plated at different densities (41.1–1315.7 cells/mm²) into 38-mm² wells of 96-well plates (Table 2). Cellular bFGF level, determined 72 h later, was inversely proportional to the culture density; cells plated at low density expressed higher levels than cells plated at high densities. In the next set of experiments, we plated 10,000 cells into 38-mm² wells (263.1 cells/mm²) of 96-well plates and analyzed bFGF protein 1–7 days later. The level of bFGF protein reached 33 pg/10⁶ cells by 24 h, remained there at 3 days, and began to decrease by 5–7 days of culture (Table 3). The decrease in the bFGF level in the confluent cultures was not due to nonspecific inhibition of protein synthesis. We base this conclusion on the results showing similar incorporation of [³H]leucine into SN12PM6 cells growing as sparse or confluent cultures (Tables 2 and 3). To examine bFGF expression at different stages of the cell cycle, we performed fluorescence-activated cell sorting analysis using an antibody to bFGF and propidium iodide. The bFGF protein was expressed at all stages of the cell cycle (data not shown).

**Cell-Cell Contact and bFGF Expression.** The increased expression of bFGF in sparse cultures might have been due to increased cell proliferation or to contact inhibition of growth. To distinguish between these possibilities, we
treated the cells with mitomycin C for 1 h (cell division inhibitor; Ref. 28) and plated the cells under sparse (131.5 cells/mm²) or confluent (1315.0 cells/mm²) conditions for 96 h. Cell proliferation was monitored by uptake of [³H]thymidine and viable cell counts. The metabolic state of the cells was determined by incorporation of [³H]thymidine. The sparse culture of mitomycin C-treated cells expressed a higher level of bFGF protein (36.8 pg/10³ cell) than cells growing under confluent conditions (7.1 pg/10³ cells; Table 4). Neither sparse nor confluent cultures incorporated [³H]thymidine.

**Discussion**

The present results demonstrate that the expression of bFGF by HRCC SN12PM6 cells is regulated by cell density; confluent cultures of SN12PM6 cells expressed low levels of bFGF, and cells growing in sparse monolayers expressed high levels of bFGF. Data obtained with human glioma cells (26) and retinal pigment epithelium (27) are in good agreement with our finding. The respective high and low expression of bFGF in sparse and confluent cultures of SN12PM6, however, was not simply due to high and low rates of cell proliferation. We base this conclusion on the results showing that sparse cultures of SN12PM6 cells treated with mitomycin C expressed high levels of bFGF, despite total cessation of cell division. Confluent cultures expressed low levels of bFGF regardless of the mitomycin C treatment.

Contact inhibition of growth has been shown to regulate autocrine growth factors and their receptors (29–33), and a recent report from our laboratory showed that the production
of collagenase type IV (matrix metalloproteinase-9) is down-regulated in contact-inhibited human squamous cell carcinoma cells (25). The entry of cells into the stationary-quiescent growth phase that follows confluence is associated with major alterations in cell surface receptors, expression of transcription factors, cytochemical enzymes, oncogenes, growth factors, signal transduction pathways, and cellular architecture (20, 25, 27, 29, 34-38). One report suggested that confluent cultures produce factors that down-regulate gene expression (39). In our study, however, conditioned medium from confluent SN12PM6 cells did not inhibit bFGF expression in sparse cultures of SN12PM6 cells or in SN12PM6 cells on the edge of a wound (in a dense culture). Contact inhibition of growth (40) has also been associated with modulation of such signal transduction pathways (41, 42) as phosphorylation of protein kinase C and protein tyrosine kinase (41, 42). Phosphorylation-dephosphorylation of protein kinases regulates gene expression in tumor cells (25, 43, 44). Increased activity of PTP can inhibit the expression/response of growth-promoting signals (38), and a recent report concluded that the expression of a PTP receptor (DEP-1) is enhanced 10-fold in cells approaching confluence (38). Whether this balance between PTP and protein tyrosine kinase is as involved in the control of bFGF expression as it is involved in the general mechanism of contact inhibition of cell growth (45) is unclear.

Previous reports have suggested that bFGF can stimulate the transition of quiescent cells from G0 to G1 of the cell cycle (46). In 3T3 cells, bFGF has been shown to transiently activate c-fos and c-myc (47) and, hence, cell proliferation (37). The rapid increase in bFGF mRNA and protein in the SN12PM6 cells subsequent to their release from confluent culture conditions also suggests that bFGF may be an autocrine signal that precedes cell division. Interestingly, the analysis of the bFGF promoter suggests that bFGF gene expression is under strong negative regulatory control, consistent with its potential functions as a stress-induced mitogenic factor (48). The similarities in the promoter region between c-myc and bFGF (48-50) may explain why growth arrest in vascular smooth muscle cells is accompanied by down-regulation of c-myc protein and mRNA; thus, down-regulation can be maintained by the addition of bFGF (48-50). The rapid decline of bFGF mRNA in cells that enter the confluent-stationary phase also supports the suggestion that the long half-life of bFGF may allow it to act as a competence factor.

In summary, the present findings indicate that the expression of bFGF is regulated by cell density and cell-contact inhibition. Future studies should elucidate which of the various host cells or factors regulate the expression of bFGF in neoplasms.

### Materials and Methods

**Cells and Culture Conditions.** The metastatic variant SN12PM6 cell line was established in culture from a HRCC as described previously (51, 52). The cell line was maintained as a monolayer culture in modified Eagle's medium (M. A. Bioproducts, Walkersville, MD) supplemented with 10% feline bovine serum, sodium pyruvate, nonessential amino acids, l-glutamine, and a 2-fold vitamin solution (GIBCO-BRL, Grand Island, NY). HUVEC were obtained from the American Type Culture Collection and maintained in the above medium supplemented with 0.01 ng/ml bovine bFGF. The cell lines were free of Mycoplasma and pathogenic mouse viruses (assayed by BioWhittaker, Walkersville, MD). For in vivo injections, the SN12PM6 cells were harvested from subconfluent cultures by a 1-min treatment with 0.25% trypsin and 0.02% EDTA. The dislodged cells were washed in medium and then resuspended in HBSS. Only single-cell suspensions with a viability of more than 90% were used for in vivo injections.

**Northern Blot Analysis.** Poly(A)+ mRNA was extracted from 1 × 10⁶ cultured cells growing as sparse (140.7 cells/mm²; 30% confluence) or dense (1407.0 cells/mm²; 85-90% confluence) cultures using the Fast-Track mRNA isolation kit (Invitrogen, San Diego, CA). mRNA was electrophoresed on 1% denaturing formaldehyde agarose gel, electrotransferred at 0.6 A to a GeneScreen nylon membrane (DuPont, Boston, MA), and UV cross-linked with 120,000 μJ/cm² using a UV-Stratallinker 1800 (Strategene, La Jolla, CA). Hybridization was performed as described previously (53). Nylon filters were washed three times with 30 mmol NaCl/3 mmol sodium citrate (pH 7.2) and 0.1% SDS (w/v).

The cDNA probes used in this analysis were a 1.3-kb PstI cDNA fragment corresponding to rat GAPDH (54) and a bovine bFGF (55). Each cDNA fragment was purified using GeneClean (BIO 101, Inc., La Jolla, CA) and radiolabeled with the random primer technique using [α-32P]dCTP deoxyribonucleotide triphosphate (56). Expression of the bFGF gene was quantitated by densitometry of autoradiograms using the ImageQuant software program (Molecular Dynamics, Sunnyvale, CA). The value for each sample was expressed as the ratio of the average areas between bFGF-specific mRNA transcripts and 1.3-kb GAPDH mRNA transcript in the linear range of the film.

**ELISA for bFGF.** Expression of cellular bFGF protein (cell lysate) was analyzed by ELISA using the Quantikine bFGF ELISA kit (R&D Systems, Minneapolis, MN) as described previously (13, 14). The concentration of the bFGF in unknown samples was determined by comparing the absorbance of the samples to the standard curve and was normalized with the viable cell number.

**Immunohistochemistry of Antithuman bFGF.** Cryostat sections of small and large kidney tumors were treated sequentially with cold acetone (−20°C) for 5 min, followed by cold chloroform:acetone (1:1) for 5 min, and rinsed with cold acetone for 5 min, followed by two rinses in PBS. In vitro cultured cells (sparse and confluently growing cultures) were washed twice with PBS, fixed with cold acetone, rinsed with PBS, and stored in PBS at 4°C until processed. Sections were processed for indirect immunoperoxidase assay, where the primary antibody was a polyclonal rabbit anti-human-bFGF (Sigma Chemical Co.) and the second antibody was a peroxidase-conjugated goat antirabbit IgG Fab fragment (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA; Ref. 13). Monoclonal antihuman bFGF antibodies obtained from Wako Pure Chemical Industry (Richmond, CA) and American Diagnostica (Greenwich, CT) showed activity similar to that of the polyclonal antibody (13).

### Table 4. Expression of bFGF in contact-inhibited SN12PM6 cells

<table>
<thead>
<tr>
<th>Cells/mm²² at:</th>
<th>[³H]Thymidine cpm/10⁶ cells</th>
<th>[³H]Leucine cpm/10⁶ cells</th>
<th>bFGF pg/10⁶ viable cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plating</td>
<td>Harvesting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
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<tr>
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<td>1315</td>
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<td>Mitomycin C-treated</td>
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<td>207</td>
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<tr>
<td>1315</td>
<td>960</td>
<td>1.4</td>
<td>150</td>
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</table>

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ISH for bFGF. ISH for bFGF mRNA in cultured cells was performed as described previously (14). Briefly, a bFGF-specific oligonucleotide probe was derived complementary to the 5' end of a human bFGF mRNA transcript (14). The test probe was the DNA oligonucleotide sequence in the antisense orientation and control sense probe (14). A d(T)15 oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. The oligonucleotide probes were hyperbiotinylated (6 biotins at the 3' end; Research Genetics, Huntsville, AL). ISH was carried out using the microprobe system (Fisher Scientific). Control for endogenous alkaline phosphatase includes treatment of the sample in the absence of the biotinylated probe and use of chromogen alone.

[^{2}H]Thymidine Uptake. SN12PM6 cells were plated at different cell densities into 38-mm² wells of 96-well culture plates and incubated for up to 96 h. At different time points, the cultures were pulse labeled with 0.2 μCi/ml[^{2}H]thymidine (specific activity, 2 Ci/mmole) for 4 h. The cultures were then washed four times with ice-cold PBS and lysed with 0.1 N KOH. The residual radioactivity was monitored in a liquid scintillation counter.

[^{3}H]Leucine Incorporation. Different numbers of SN12PM6 cells were seeded into 38-mm² wells of 96-well culture plates and incubated at 37°C in 200 μl medium containing 0.1 μCi/ml[^{3}H]leucine. After different times, the cultures were washed with ice-cold PBS and then lysed in 1 N KOH. The radioactivity was measured in the cell lysate and normalized with viable cell number. Cells incubated in ice-cold medium containing 0.1 μCi/ml[^{3}H]leucine at 4°C for 2 h served as baseline controls.

Cell Cycle and bFGF Expression. To determine whether expression of bFGF depends on different stages of the cell cycle, we performed fluorescence-activated cell sorting analysis by using double-staining techniques. Sparse or dense cultures of SN12PM6 cells were harvested by a 1-min treatment with 0.25% trypsin and 0.02% EDTA. The single cells were washed with medium and then with 1% BSA. The cells were pelleted (250 g) in 0.1% sodium oxide in PBS, gently fixed for 5-7 min with prechilled 70% methanol at 20°C, washed twice with ice-cold PBS/BSA, and resuspended to 5 x 10⁵ cells/ml in ice-cold PBS/BSA. The antibodies and reagents were reconstituted in deionized water to the required concentrations specified by the manufacturer’s instructions. Five μg of a 1:10 dilution of bFGF (Ab) and/or a polyclonal antibody that reacts with residues 147-153 of bFGF and shows no reactivity to acidic bFGF; Oncogene Science, Uniondale, NY) was incubated with 100 μl of cell suspension for 45-60 min at 4°C. The cells were washed once with PBS/BSA and incubated with 5 μl of a 1:10 dilution of FITC-conjugated goat antirabbit F(ab)², total IgG (Oncogene Science) in 100 μl PBS/BSA for an additional 45-50 min. The samples were then washed twice in ice-cold PBS/BSA. These cells were incubated with propidium iodide (50 ng/ml) 1 ml in each sample, with 1 ml of RNase A (20 μg/ml), incubated for 30 min at 37°C, and analyzed by EPICS profile flow cytometer (Coulter Corp., Hialeah, FL).

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References


