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Organ Site-Dependent Expression of Basic Fibroblast Growth Factor in Human Renal Cell Carcinoma Cells

Rakesh K. Singh, Corazon D. Bucana, Mordechai Gutman, Dominic Fan, Michael R. Wilson, and Isaiah J. Fidler

From the Department of Cell Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

We investigated the influence of organ microenvironment on the angiogenic phenotype in human renal cell carcinoma (HRCC) cells. HRCC line SN12C was established in vitro from a surgical specimen, and metastatic line SN12PM6 was isolated from a lung metastasis produced by parental cells implanted into the kidney of nude mice. SN12C (low metastasis) and SN12PM6 (high metastasis) cells were injected into the kidney or subcutis of nude mice. The kidney tumors were highly vascularized (as revealed by immunohistochemistry using antibodies against factor VIII), and metastatic, whereas the subcutaneous tumors were not. The expression of mRNA for basic fibroblast growth factor (bFGF) in kidney tumors was 10 to 20 times that found in subcutaneous tumors. Similar data were obtained at the protein level by using fluorescence activated cell sorting, immunohistochemistry, and enzyme-linked immunosorbent assay. bFGF was detected in the urine of mice with tumors in the kidney but not subcutaneous tumors. The level of bFGF in the serum of mice with kidney tumors was two to three times that in mice with subcutaneous tumors. The changes in bFGF expression in the tumors was transient. Collectively, these data indicate that the organ microenvironment can influence the expression level of bFGF in HRCC. (Am J Pathol 1994, 145:365–374)

The process of cancer metastasis consists of a series of sequential interrelated steps, each of which is rate-limiting inasmuch as a failure at any of the steps aborts the process.[1] Metastasis is a highly selective process that is regulated by a large number of mechanisms,[1] and the outcome of this process depends on both the intrinsic properties of tumor cells and the responses of the host.[2–4] Recent studies have suggested that metastatic cells can usurp host homeostatic mechanisms to survive and grow preferentially in particular organ environments.[1,5–7] Furthermore, organ-specific host factors have been shown to enhance or suppress the growth, invasion, and metastasis of human tumors implanted into nude mice.[5–7] These environmental differences can also influence the ability of human tumor cells to produce degradative enzymes[8] and sensitivity to chemotherapeutic agents.[9]

A crucial step in the process of metastasis is the production of vascularization in and around tumors.[10] Tumors that are <2 mm in diameter can receive nutrients by diffusion, but further growth depends on the development of an adequate blood vasculature.[10] The induction of vascularization is mediated by several angiogenic molecules released by both tumor cells and host cells.[11,12] The prevascular stage of a tumor is usually associated with local nonmetastatic tumors, whereas the vascular stage precedes the processes of invasion and metastasis.[10,13,14] The intensity of angiogenesis in neoplasms such as breast and prostate carcinoma has been correlated with their potential for invasion and metastasis.[15–17] However, whether the organ microenvironment directly contributes with the induction and maintenance of angiogenic factor and angiogenesis has remained unclear.

The production of angiogenic factors such as basic fibroblast growth factor (bFGF) by tumor cells or host cells (macrophages) or the release of bFGF from the extracellular matrix in the absence of angiogenesis

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Address reprint requests to Dr. Isaiah Fidler, Department of Cell Biology, Box 173, University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030.

inhibitors[10–12] leads to growth of endothelial cells and hence vascularization.[13,18] Because the host microenvironment varies among different organs,[1,19] we wished to determine whether bFGF expression (at the mRNA and protein levels) is influenced by the organ microenvironment. We implanted human renal cell carcinoma (HRCC) cells into the subcutis (SC) or the kidney-renal subcapsule of nude mice.[20,21] The HRCC tumors in the kidney were highly vascularized and produced a high incidence of systemic metastasis. In contrast, in the SC of nude mice, the tumors were poorly vascularized and produced few metastases. The expression of bFGF mRNA and protein in HRCC tumors in the kidney was enhanced, whereas in HRCC growing SC, it was diminished. The data clearly demonstrated that the organ site of tumor growth strongly influences the expression of the angiogenic factor bFGF.

Materials and Methods

Animals

Male athymic BALB/c nude mice were purchased from the Animal Production Area of the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when 8 weeks of age. Animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and National Institutes of Health.

Cells and Culture Conditions

The parental SN12C and metastatic variant SN12PM6 cell lines were established in culture from a human renal cell carcinoma as described previously.[20,21] The cell lines were maintained as monolayers in modified Eagle medium (M. A. Bioproducts, Walkersville, MD) supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and a twofold vitamin solution (GIBCO BRL, Grand Island, NY). Both cell lines were free of *Mycoplasma*, reovirus type 3, pneumonia virus of mice, mouse adenovirus, murine hepatitis virus, lymphocytic choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (Microbiological Associates, Bethesda, MD). For *in vivo* injections, the SN12C or SN12PM6 cells were harvested from subconfluent cultures by a 1-minute treatment with 0.25% trypsin and 0.02% EDTA. The dislodged cells were washed in medium and then resuspended in Hanks' balanced salt solution (HBSS) for injection. Only single-cell suspensions with viability of more than 90% were used for *in vivo* injections.

Subcutaneous Inoculation and Growth in Vivo

Subcutaneous tumors were established by the injection of 1×10^6 cells. Tumor growth was monitored every 3 days. The tumors were resected when they reached 15 to 20 mm in diameter (4 weeks after injection). The excised tumors were dissociated for establishment in cell culture or quickly frozen in liquid nitrogen for mRNA extraction (see below). Tumors were also processed for histological and immunohistochemical studies.

Renal Subcapsule Injection

After anesthesia with methoxyflurane, a small incision was made in the mouse's right flank. The kidney was lifted out of the peritoneum, and a 27-gauge needle was inserted into the lower pole and advanced until its point reached just below the renal subcapsule. Tumor cells (1×10^6) were injected in a volume of 0.05 ml HBSS. Visible bulla formation between the renal parenchyma and capsule was the criterion for a successful injection. After the injection, the kidney was returned to the abdominal cavity, and the abdominal wall was closed with metal wound clips. In this orthotopic animal model the implantation of HRCC cells into the kidney of nude mice yields progressively growing tumors and pulmonary metastasis, whereas the implantation of the same cells into the subcutis of nude mice results in only local tumors.[7,20,21] The production of lung metastasis after orthotopic implantation is not caused by an artifact of the injection, given that nephrectomy even 14 days after tumor cell injection cures the mice.[7] This pattern of spontaneous metastasis is specific to HRCC, inasmuch as the injection of human colon cancer cells into the kidney of nude mice results in only local tumors.[3]

Four weeks after HRCC cell injection the mice were killed and the kidneys with tumors were removed and processed for histology and immunohistochemistry. The excised tumors weighing between 250 and 400 mg were either quickly frozen in liquid nitrogen for mRNA extraction or enzymatically dissociated for establishment into cell culture.[3]

Northern Blot Analysis

Poly(A)$^+$ mRNA was extracted from cultured cells (1×10^8) or from tumor tissue using Fast Track mRNA isolation kit (Invitrogen Co., San Diego, CA). mRNA was electrophoresed on 1% denaturing formaldehyde/agarose gel, electrotransferred at 0.6 A to a GeneScreen nylon membrane (DuPont Co., Boston, MA), and UV cross-linked with 120,000 μJ/cm^2 using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). Hybridizations were performed as described previously.[22] Nylon filters were washed three times at 60 C with 30 mmol/L NaCl/3 mmol/L sodium citrate, pH 7.2/0.1% sodium dodecyl sulphate (w/v).

The cDNA probes used in this analysis were a 1.3-kb *Pst*I cDNA fragment corresponding to rat GAPDH[23] and a 1.4-kb *Eco*RI cDNA fragment of bovine bFGF.[24] Each cDNA fragment was purified by agarose gel electrophoresis, recovered using GeneClean (Bio 101, Inc., La Jolla, CA), and radiolabeled with the random primer technique using [α^{-32}P]deoxyribonucleotide triphosphate.[25]

Densitometric Quantitation

Expression of the bFGF gene was quantitated by densitometry of autoradiograms using the Image Quant software program (Molecular Dynamics, Sunnyvale, CA). Each sample measurement was calculated 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.

Enzyme-Linked Immunosorbent Assay (ELISA) for bFGF

Samples that had been stored at −70 C were thawed at room temperature. Expression of cellular bFGF protein was analyzed by ELISA using the Quantikine bFGF ELISA kit (R&D System, Minneapolis, MN). The concentration of bFGF in unknown samples was determined by comparing the optical density of the samples to the standard curve.

FACS Analyses for bFGF

The level of intracellular bFGF was determined in target cells by using polyclonal rabbit anti-bFGF immunoglobulin G (IgG) (bFGF Ab2, catalog no. PC16, Oncogene Science, Uniondale, NY), which reacts with the "residues 147–153" epitope of human bFGF and shows no reactivity with acidic FGF. For *in vitro* studies, semiconfluent cultures that had been incubated for 48 hours were harvested at semiconfluence (30%) into single-cell suspensions with a 1-minute 0.25% trypsin and 0.02% EDTA treatment, washed once with culture medium and then once with ice-cold 1% bovine serum albumin (BSA) and 0.1% sodium azide in phosphate-buffered saline (PBS) by centrifugation at 250 *g*. The cells were gently fixed with prechilled 70% methanol at −20 C for 5 to 7 minutes, washed twice with ice-cold PBS/BSA, and resuspended to 5×10^6 cells/ml in ice-cold PBS/BSA. Antibodies and reagents were reconstituted in 1 ml of deionized water or as specified by the manufacturer, and 5 μl of a 1:10 dilution of the bFGF (Ab2) was incubated with 100 μl samples of cells at 4 C for 45 to 60 minutes. The cells were washed once with PBS/BSA and incubated with 5 μl of a 1:10 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit F(ab′)$_2$ total IgG (catalog no. DC25L, Oncogene) in 100 μl PBS/BSA for an additional 45 to 50 minutes. The samples were then washed twice in ice-cold PBS/BSA, resuspended in 1 ml of PBS/BSA, and analyzed with an Epics Profile flow cytometer (Coulter Corp., Hialeah, FL). The mean channel fluorescence of intracellular bFGF was analyzed by a computer, and the level of bFGF was calculated as net relative fluorescence unit by normalizing with cells stained only with the second fluorescein isothiocyanate-conjugated IgG.

For *in vivo* studies, necrosis-free tumor samples were minced and rinsed with iced HBSS to remove blood elements. The tissues were minced and dissociated with a mixture of collagenase (100 units/ml; Type I, catalog no. C-0130, Sigma Chemical Co., St. Louis, MO) and deoxyribonuclease (300 Kunitz units/ml; Type I, catalog no. D4263, Sigma Chemical Co.) in culture medium containing 1% fetal bovine serum. The samples were incubated at 37 C for 10 minutes with continuous agitation. Tumor cells were separated from aggregates and tissue matrix by static sedimentation at room temperature for 15 to 30 seconds, collected by centrifugation at 250 *g* for 1 minute, and analyzed for bFGF.

Immunohistochemistry of Anti-factor VIII and Quantification of Microvessel Density

Cryostat sections of tissues were fixed with 2% paraformaldehyde in PBS, pH 7.5, for 10 minutes at room temperature, washed twice with PBS, and treated with 1% Triton X-100 for 5 minutes. The sections were washed three times, and endogenous peroxidase was blocked with 3% hydrogen peroxide in methanol for 12 minutes. The samples were then washed three times with PBS and incubated with a

Table 1. *Tumorigenicity and Production of Spontaneous Metastases by SN12C and SN12PM6 Cells Injected into the Subcutis or Renal Subcapsule of Nude Mice*

Cell line*	Injection site	Tumorigenicity†	Lung metastasis	
			Incidence	Median (range) no. of metastases‡
SN12C	Subcutis	8/10	1/10	0 (0–3)
	Kidney	10/10	8/10	6 (0–10)
SN12PM6	Subcutis	9/10	3/10	6 (0–14)
	Kidney	10/10	10/10	80 (10–200)

* 1×10^6 viable tumor cells were injected into the kidney or SC of nude mice. The mice were killed when they became moribund.
† Number of mice with tumors/number of injected mice.
‡ The number of metastases was determined with a dissecting microscope.

protein-blocking solution consisting of PBS containing 1% normal goat serum and 1% horse serum for 20 minutes at room temperature. Excess blocking reagent was drained off, and the samples were incubated with the appropriate dilution of HRP-conjugated rabbit anti-factor VIII-related antigen, (catalog no. P226, Dako Corporation, Carpinteria, CA) for 15 to 18 hours at 4 C. The samples were rinsed four times with PBS, rinsed briefly with distilled water, and incubated with DAB (Research Genetics, Huntsville, AL) for 20 minutes at room temperature. The sections were then washed three times with distilled water, counterstained with aqueous hematoxylin, washed, mounted with Permount, and examined in a bright-field microscope. The positive reaction was indicated by a reddish-brown precipitate.

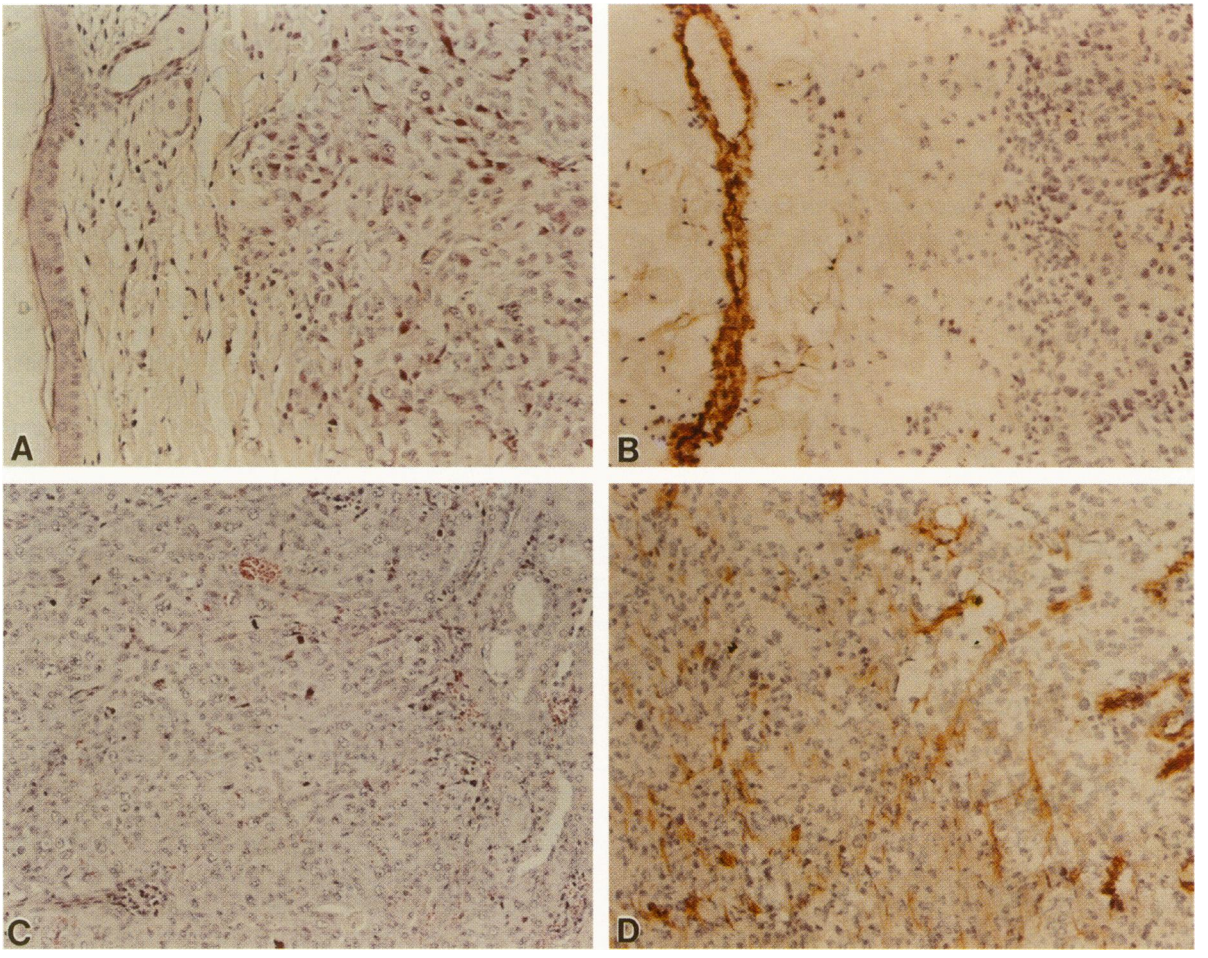

Figure 1. *Histology of SN12PM6 tumors growing in nude mice.* A: *SC tumor 4 weeks after implantation (H&E, ×200).* B: *Immunohistochemical staining of an HRCC tumor growing SC with anti-factor VIII antibodies. Note immunoperoxidase staining in a blood vessel distant from the neoplasm (positive control).* C: *HRCC tumor in the kidney of nude mice. Note numerous blood vessels (H&E, ×200).* D: *Immunohistochemical staining of an HRCC tumor in the kidney with anti-factor VIII antibodies. Note intensive immunoperoxidase staining throughout the neoplasm.*

Microvessel density was determined by light microscopy according to the procedure of Weidner et al.[15] The areas with most intense blood vessels were evaluated. Any brown-staining endothelial cell cluster distinct from adjacent microvessels, tumor cells, or other stroma cells was considered a single countable microvessel. The images were projected and recorded by digitizing the image in a cooled CCD Optronics Tec 470 camera (Optronics Engineering, Goletha, CA) linked to a computer and a digital printer (Sony Corporation, Tokyo, Japan). Each count was expressed as the highest number of microvessels identified within a selected 200× field. All counts were performed by two investigators who had to agree on what constituted a single microvessel. The results are expressed as the highest number of microvessels identified within single 200× fields.[15]

Immunohistochemistry of Anti-Human bFGF

Cryostat sections of tumors growing SC or in the kidney were treated sequentially with cold acetone (−20 C) for 5 minutes followed by cold chloroform: acetone (1:1) for 5 minutes and rinsed with cold acetone for 5 minutes followed by two rinses in PBS. Sections were processed for indirect immunoperoxidase assay where the primary antibody was a polyclonal rabbit anti-huFGFb (Sigma Chemical Co.), and the secondary antibody is a peroxidase-conjugated goat anti-rabbit IgG, $F(ab)_2$ fragment (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA). The buffers and blocking solution are as described above. Monoclonal anti-human bFGF antibodies obtained from Wako Pure Chemical Industry, Ltd. (Richmond, VA) and American Diagnostica (Greenwich, CT) gave reactivity similar to that of the polyclonal antibody.

Results

Tumorigenicity and Production of Metastasis by HRCC Cells Implanted into the Kidney or Subcutis of Nude Mice

In the first set of experiments, we implanted the parental SN12C and metastatic variant SN12PM6 cells into ectopic (subcutaneous) and orthotopic organs of nude mice. In close agreement with previous publications[7,20] HRCC cells implanted into the kidney produced a high incidence of lung metastasis. In contrast, SC tumors produced a low incidence of systemic metastasis (Table 1). As reported previously,[21] after orthotopic implantation (but not ectopic implantation) the highly metastatic SN12PM6 cells produced more metastases than did the SN12C parental cells (Table 1).

Histopathological examination of the tissues stained with hematoxylin and eosin revealed that the HRCC growing in the subcutis of nude mice had few blood vessels (Figure 1A). In contrast, the tumors in the kidney had many blood vessels vascularized (Figure 1C). Neither the SC nor the kidney tumors contained infiltrating host leukocytes.

To determine more specifically the extent of vascularization in the HRCC kidney and skin tumors, we used immunohistochemistry techniques to identify cells reacting with antibodies against factor VIII, which specifically stains endothelial cells.[15–17] Microvessel density was evaluated in multiple sections of at least five SC and five kidney tumors. The average number of microvessels/200× field in the HRCC kidney tumors was 28.8 ± 6.4, and in the HRCC SC tumors it was 4.6 ± 1.5 ($P < 0.003$). A representative example of the differences in microvessel density is shown in Figure 1, B and D.

Expression of bFGF by Cultured HRCC

In the next set of experiments, we determined whether HRCC cells growing in culture express bFGF mRNA and produce bFGF protein. Cultures in their exponential growth phase were used. We analyzed the level of bFGF mRNA by Northern blot analysis and

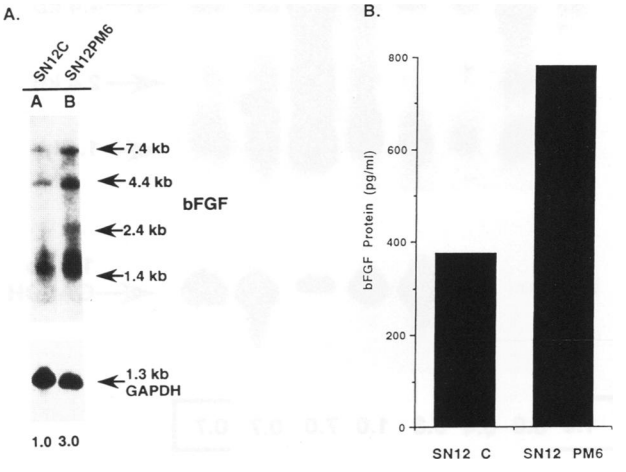

Figure 2. *Expression of bFGF mRNA and protein in HRCC cells.* **A:** *Northern blot analysis. 2.5 μg mRNA/lane was electrophoresed on 1% denaturing formaldehyde/agarose gel, electrotransferred, and hybridized with 1.4-kb EcoRI gene fragment corresponding to bovine bFGF cDNA and 1.3-kb PstI gene fragment corresponding to rat GAPDH cDNA.* Lane A, *SN12C cells in culture;* lane B, *SN12PM6 cells in culture. Densitometric quantitation was performed as described in Materials and Methods.* **B:** *bFGF cellular protein level in HRCC cells as assayed using ELISA technique. 2 × 10^4 cells were washed in cold PBS and cellular bFGF protein was assayed in cell lysates after one freeze-thaw cycle. This is one representative experiment of three.*

cellular bFGF protein by ELISA. These data are summarized in Figure 2. Both parental SN12C and metastatic SN12PM6 cells expressed mRNA for bFGF (Figure 2A) and contained cellular bFGF protein (Figure 2B). The metastatic SN12PM6 cells expressed three times the higher steady-state levels of bFGF-specific mRNA transcripts than did the SN12C cells (Figure 2A). The level of cellular bFGF protein was also higher in continuously cultured SN12PM6 cells than in the continuously cultured SN12C cells (Figure 2B), indicating stability for this expression.

Differential Expression of bFGF in HRCC Cells Growing in the Kidney and Subcutis of Nude Mice

We next investigated the expression of bFGF in the HRCC tumors. Tumors growing in the subcutis, muscle (quadricep femoris), or kidney were harvested and analyzed for the expression of bFGF-specific mRNA. Tumor cells growing in culture were used as a control for baseline expression of bFGF.

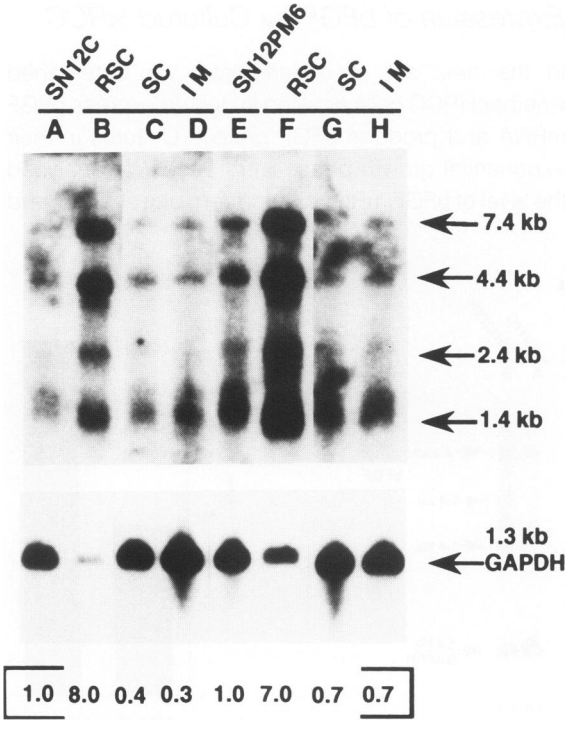

Figure 3. *bFGF mRNA expression level by HRCC cells growing in culture or in the subcutis, muscularis, or kidney of nude mice. 2.5 μg mRNA/lane was electrophoresed on 1% denaturing formaldehyde/agarose gel, electrotransferred, and hybridized with 1.4-kb EcoRI gene fragment corresponding to bovine bFGF cDNA and 1.3-kb PstI* gene fragment corresponding to rat GAPDH cDNA. **Lane A,** *SN12C cells in culture;* **lane B,** *SN12C kidney tumor;* **lane C,** *SN12C SC tumor;* **lane D,** *SN12C intramuscularis tumor;* **lane E,** *SN12PM6 cells in culture;* **lane F,** *SN12PM6 kidney tumor;* **lane G,** *SN12PM6 SC tumor;* **lane H,** *SN12PM6 intramuscularis tumor. Densitometric quantitation was performed as described in Materials and Methods.*

Figure 3 shows that all of the samples analyzed expressed four specific mRNA transcripts for bFGF. Here again, the highly metastatic SN12PM6 cells growing in culture expressed at least three times the amount of bFGF mRNA transcripts as did parental SN12C cells growing in culture. Growth in the kidney resulted in eight and seven times higher expressions of bFGF mRNA in SN12C and SN12PM6 cells, respectively, as compared with their *in vitro* counterparts. HRCC tumors in the subcutis or muscularis of nude mice showed decreased levels of bFGF mRNA transcripts as compared with their counterparts growing in culture. HRCC tumors in the kidney had a 10- to 20-fold increase in bFGF mRNA as compared with HRCC SC tumors. Control, normal skin, and normal kidney of nude mice were also analyzed for mRNA expression of bFGF, and none was detected (data not shown).

Immunohistochemistry studies of HRCC tumors growing SC and in the kidney revealed significant differences in staining intensity. The tumors in the kidney were highly positive for bFGF protein, whereas the SC tumors were not (Figure 4). The staining of human tumor cells was more intense than that of mouse stroma.

We next confirmed that the differential expression of HRCC cells for bFGF mRNA correlated with cellular bFGF protein. SN12PM6 cells growing continuously in culture as well as cells recovered from skin or kidney tumors were analyzed by FACS. Cells growing in the kidney showed three times the amount of cellular bFGF in continuously cultured cells (Figure 5), whereas the HRCC in the subcutis yielded cells with a decreased (by 0.8 times) amount.

We also analyzed the level of human bFGF in the serum and urine of normal nude mice ($n = 10$) or mice with SN12PM6 tumors in the subcutis ($n = 8$) or kidney ($n = 10$). The level of human bFGF in the urine of mice with HRCC growing in the kidney averaged 350 pg/ml, whereas in normal mice or in mice with HRCC growing in the subcutis, we did not detect any human bFGF. The serum level of human bFGF in mice with HRCC in the kidney averaged 284 pg/ml, and in mice with HRCC in the subcutis it averaged 131 pg/ml ($P < 0.01$).

The Expression of bFGF in HRCC Tumors is Transient and Dependent on the Organ Environment

To confirm that the difference in expression of bFGF in HRCC growing in the kidney and skin was caused by adaptation to the organ environment, we harvested the tumors and, following their enzymatic dis-

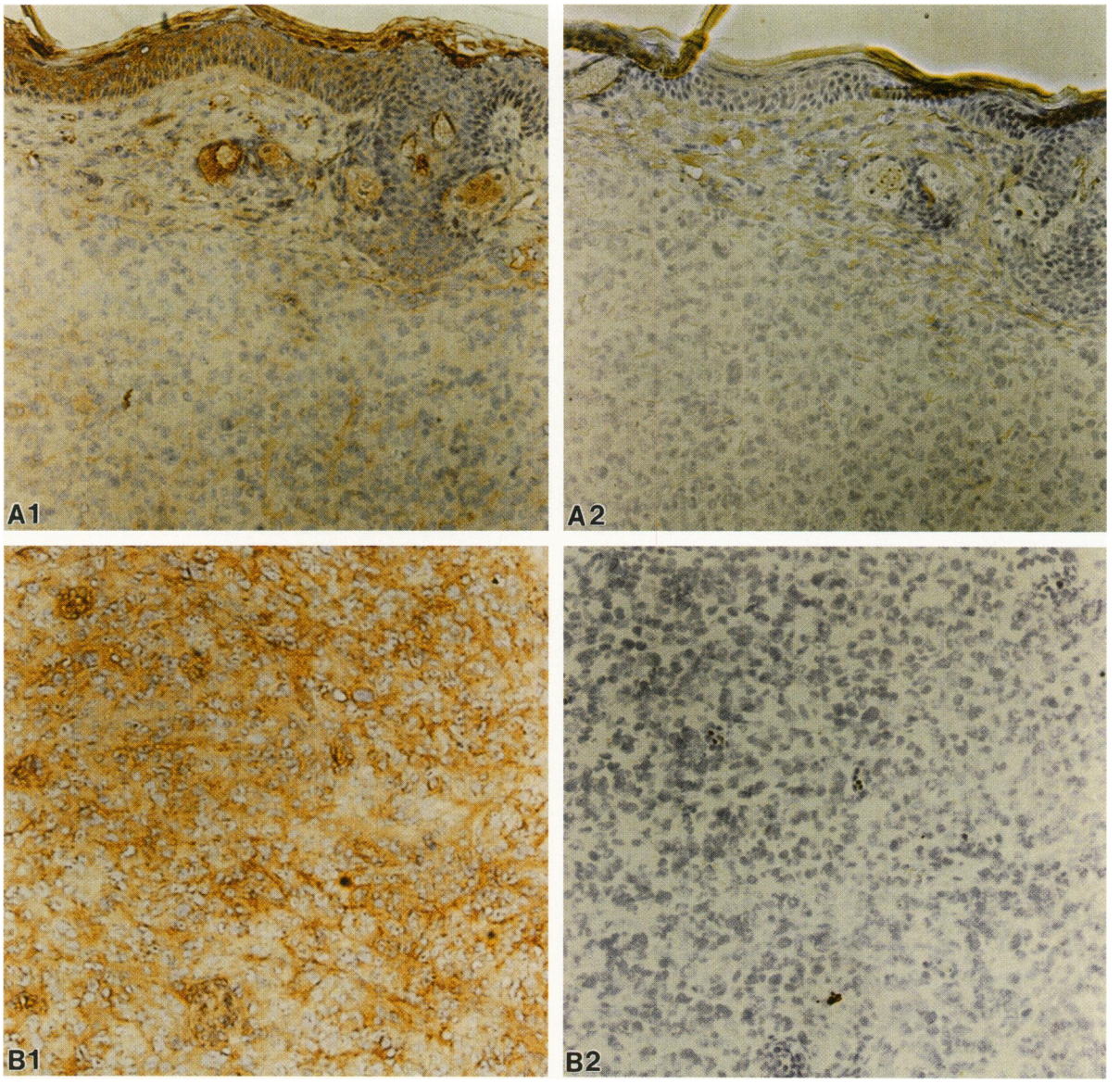

Figure 4. *Immunohistochemical staining of HRCC tumors with anti-bFGF antibodies.* **A1:** *SC tumor 4 weeks after implantation. Note immunoperoxidase staining in dermal blood vessels and absence of reactivity in tumor cells (×200).* **A2:** *Control immunoperoxidase reactivity (×200).* **B1:** *HRCC tumor in the kidney of nude mice 4 weeks after implantation. Note intensive immunoperoxidase staining in tumor cells (×200).* **B2:** *Control immunoperoxidase activity (×200).*

sociation, established them in culture. We then analyzed bFGF at different times. After 2 weeks in culture, the SN12PM6 cells established from kidney tumors showed a decrease in bFGF mRNA (Figure 6), and by 4 weeks the level was equal to that of the continuously cultured SN12PM6 cells. In HRCC tumors established in culture from SC tumors, the expression of bFGF mRNA increased. After 4 weeks, the bFGF mRNA level equaled that of the continuously cultured SN12PM6 cells. This was confirmed at the protein level (Table 2). Cellular bFGF level was high in cell cultures established from HRCC growing in the kidney. The protein level, however, decreased with prolonged culture of HRCC lines established from kidney tumors. As shown in Table 2, after 14 days in culture, it reverted to the level of cultured cells. These findings suggest that the increase or decrease in bFGF levels in HRCC tumors growing in the kidney or SC of nude mice is transient and dependent on the organ environment.

Discussion

The present results demonstrate that the orthotopic implantation of HRCC cells into nude mice yielded highly vascularized tumors that produced high levels

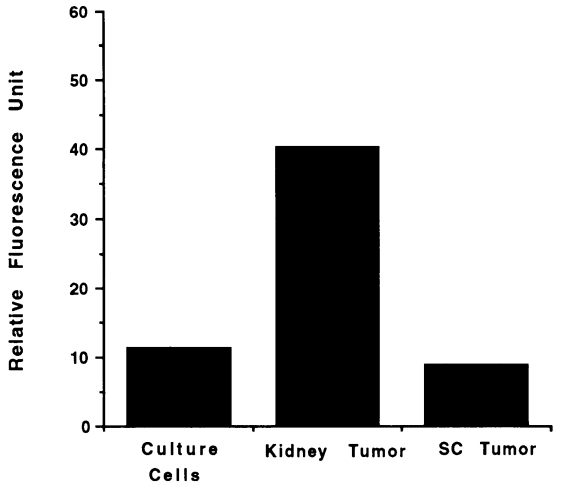

Figure 5. *Cellular bFGF level in cultured HRCC cells, kidney tumors, and SC tumors. HRCC tumors were dissociated with DNAse/collagenase, and cells were processed for FACS analysis. The values are presented as relative fluorescence units. This is one representative experiment of three.*

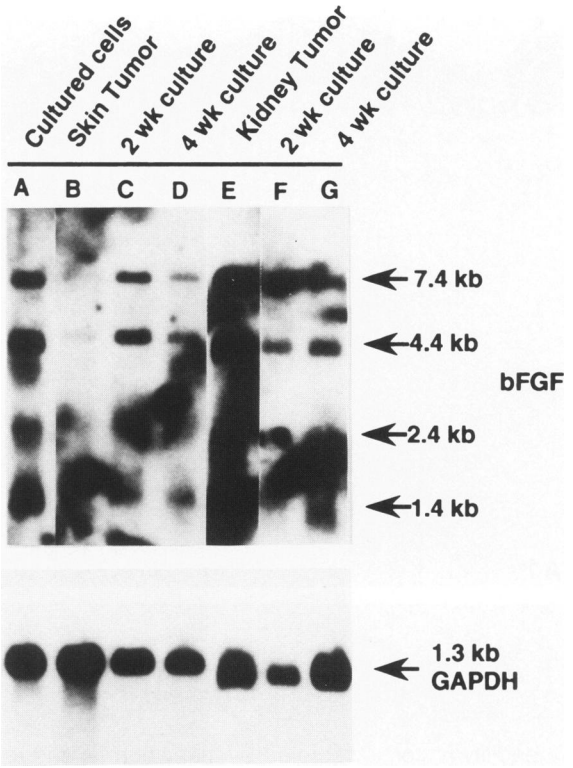

Figure 6. *bFGF mRNA expression level of SN12PM6 kidney or SC tumors adopted for growth in culture. 2.5 μg mRNA/lane was electrophoresed on 1% denaturing formaldehyde agarose gel, electrotransferred, and hybridized with 1.4-kb EcoRI gene fragment corresponding to bovine bFGF and 1.3-kb PstI gene fragment corresponding to rat GAPDH cDNA;* lane A, *continuously cultured SN12PM6 cells used as a baseline control;* lane B, *SC tumor;* lane C, *SC tumor cells in culture for 2 weeks;* lane D, *SC tumor cells in culture for 4 weeks;* lane E, *kidney tumor;* lane F, *kidney tumor cells in culture for 2 weeks;* lane G, *kidney tumor cells in culture for 4 weeks.*

of bFGF mRNA and protein. In contrast, the ectopic implantation of HRCC into the SC of nude mice yielded tumors that were poorly vascularized and produced significantly lower levels of bFGF mRNA and protein. These data, therefore, demonstrate an association between the production of bFGF by tumor cells and vascularization[12,14] and that the expression level of bFGF in HRCC cells is influenced by a specific organ's microenvironment.

The influence of organ microenvironment on the biology of tumor cells has been recognized since Paget's "seed and soil" hypothesis,[2] which suggests that the interaction between tumor cells and target organ determines whether metastasis will occur. Recent evidence supports the role of the microenvironment in regulating tumorigenesis[26] and tumor cell properties, such as production of degradative enzymes,[5,27] sensitivity to cytotoxic drugs,[9,28] and melanin formation.[29,30] Our present study extends these observations by showing that HRCC tumors growing in the kidney (of nude mice) are highly vascularized, whereas HRCC tumors growing SC (in nude mice) are not. This difference in vascularization studied by immunohistochemistry with antibodies against factor VIII[15–17] was associated with differences in level of expression of bFGF. We detected 10 to 20 times the amount of bFGF mRNA in HRCC growing in the kidney as compared with HRCC growing in the subcutis. These differences were confirmed at the protein level.

In our study, HRCC growing in the kidney produced lung metastases, whereas HRCC cells growing in the subcutis did not. The differential expression of bFGF could have contributed to the invasive-metastatic phenotype of the HRCC growing in the kidney, as bFGF can stimulate the activity of proteolytic enzymes such as tissue type and urokinase type plasminogen activator[31] and collagenase type IV,[32] all of which are produced by the HRCC cells.[33,34]

Renal cell carcinomas produce various angiogenic factors that include bFGF.[35–37] Recent reports indicate that the expression of bFGF in primary HRCC

Table 2. *Expression of bFGF Protein in SN12PM6 Tumors Adopted to Culture*

Days in culture	bFGF expression index in cultures established from	
	Kidney tumor	Subcutaneous tumor
3	3.083	.818
7	3.563	.750
14	1.079	.840

Tumors (250 to 400 mg) were rinsed and cut into small pieces, degraded by incubating with collagenase/DNAse for 2 hours, washed twice with HBSS, resuspended in complete medium, and cultured. After 3, 7, or 14 days, cells were harvested and washed, and an equal number of cells were lysed. Cell lysates were analyzed for bFGF protein by ELISA (see Materials and Methods). Expression index was calculated by comparing test samples with SN12PM6 cells incubated in culture for the same duration.

inversely correlates with survival[37] as do elevated levels of bFGF in the urine of patients.[38] The molecular mechanism for the upregulation of bFGF production in HRCC growing in the kidney remains unclear. Many inducers and inhibitors of angiogenesis are present together in tissues, and it is the balance between their relative activities and availability that determines the angiogenic phenotype *in vivo*. The regulation of the production of the angiogenic factors occurs in response to myriad environmental perturbations.[12] Growth of new blood vessels in disorders involving immune/inflammatory reactions such as rheumatoid arthritis or corneal panus is a situation in which multiple cytokines could modulate the vascular response.[39,40] The role of cytokines, however, is complex. Recent reports suggest that interferon-α and interleukin-2 synergistically enhance bFGF synthesis and induce its release in endothelial cells.[41] Yet interferon-α has also been associated with the regression of hemangiomas.[42] Another possible mediator is angiotensin II, which can induce production of bFGF[43] or its release from the interstitial matrix. bFGF is by no means the only angiogenic factor. Vascular endothelial growth factor, also known as vascular permeability factor, is secreted by many normal and tumor cells.[44] Future studies are necessary to determine whether expression of this factor is also modulated by the organ microenvironment.

In conclusion, we have shown that the production of bFGF by HRCC can be modulated by the microenvironment of different organs. A diverse group of compounds has been shown to inhibit neovascularization *in vivo*.[45] A common property of these compounds is that almost all of them can influence the ability of a cell to produce, interact with, or degrade its surrounding matrix. Several inhibitors of angiogenesis, including retinoid A and herbimycin A, lead to reversion of tumorigenic cells to resemble normal cells.[46,47] It might be possible that in the SC environment, normal cells produce inhibitors of angiogenic factors; the identification of such inhibitors is a subject for intensive research.

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