Expression of Angiogenesis-Related Genes Regulates Different Steps in the Process of Tumor Growth and Metastasis in Human Urothelial Cell Carcinoma of the Urinary Bladder

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Key Words
Angiogenesis • Metastasis • Transitional cell carcinoma • Tumor progression • Urothelial cell carcinoma

Abstract
Objective: This study was designed to determine the relative activity of angiogenesis-related genes in the regulation of tumorigenicity and subsequent metastases of urothelial cell carcinomas (UC) of the urinary bladder. Methods: We selected the clones with the highest and lowest expression level of basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF)/vascular permeability factor or interleukin-8 (IL-8) in the highly tumorigenic and metastatic human UC cell line 253J B-V. Tumorigenicity and production of spontaneous lymph node metastases were evaluated 1, 2, 4, 8 and 12 weeks after orthotopic implantation of each specific expression clone into the urinary bladder of athymic nude mice. Moreover, the transitional changes in the expression of angiogenesis-related genes and neovascularization were determined in tumors and metastases. Results: At the early stage of tumor growth following orthotopic implantation, tumorigenicity and metastases were significantly increased in the clones with the highest expression of bFGF and IL-8, while they were significantly inhibited in the clones with the lowest expression of bFGF and IL-8 compared to parental 253J B-V. In the tumors, specific expression of angiogenesis-related genes and intratumor neovascularity of each clone were gradually regulated to the same level as parental 253J B-V. In metastasized tumors of the highest and lowest IL-8-expressing clones, IL-8 expression was consistently high and low, respectively. Conclusions: These findings indicate that at the early stage of tumor growth, bFGF and IL-8 expression play important roles in the regulation of angiogenesis, tumorigenicity and subsequent metastases of human bladder cancer.

Introduction
Induction of blood supply is required in a multistep process such as tumor growth, invasion and subsequent metastases. This process of angiogenesis is mediated in part by the secretion of angiogenesis-related genes, e.g. basic fibroblast growth factor (bFGF) [1, 2], vascular endothelial growth factor (VEGF) [3, 4] and interleukin (IL)-8 [5, 6], by tumor cells and their microenvironment. Angiogenesis plays a critical role in the development and progression of cancer. Overexpression of bFGF and VEGF has been identified in the tissue, serum and urine of patients with bladder cancer and has also been associa-

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ated with disease progression [1–4]. IL-8 is a putative angiogenic factor, and previously IL-8 expression was found to regulate angiogenesis, tumorigenicity and metastases in an orthotopic murine model of human urothelial cancer (UC) [6, 7]. Microvessel density (MVD) has also been shown to predict early progression in muscle-invasive disease [8, 9]. Each of the above-mentioned angiogenesis-related genes (bFGF, VEGF and IL-8) and MVD have previously been demonstrated in bladder cancer, either singly or in combination. Accordingly, we have demonstrated that VEGF overexpression and increased MVD in biopsy specimens prior to therapy and also bFGF overexpression and increased MVD in cystectomy specimens following neoadjuvant chemotherapy could predict a poor prognosis in advanced UC following neoadjuvant chemotherapy and cystectomy [10]. However, bFGF, VEGF and IL-8 are expressed by UC, and their exact involvement in UC progression has not been elucidated.

In the present study, we aimed to explore whether angiogenesis-related genes (bFGF, VEGF and IL-8) differentially regulate distinct steps of angiogenesis in the process of tumor growth and subsequent metastases of human UC in the urinary bladder.

**Materials and Methods**

**Cell Lines and Culture Conditions**

The non-tumorigenic human bladder carcinoma cell line 253J-P and the highly tumorigenic and highly metastatic variant 253J B-V were grown as monolayers in modified Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, nonessential amino acids and penicillin-streptomycin (CMEM) [11]. Cells were maintained at 37°C in a 5% CO₂ environment.

**Selection of Tumor Cells Expressing bFGF, VEGF or IL-8**

The monolayers (60–70% confluent) of tumor cells were selected to single cell by the serial diluted method onto 96-well plates. The CMEM medium was replaced every 3 days until individual colonies were isolated and established in culture as individual lines in a 37°C incubator. All individual cell lines were maintained in CMEM. Using the serial dilution method, we selected the highest (bFGF-H) and the lowest bFGF-expressing 253J B-V clone (bFGF-L), the highest (VEGF-H) and the lowest VEGF-expressing 253J B-V clone (VEGF-L), and the highest (IL-8-H) and the lowest IL-8-expressing 253J B-V clone (IL-8-L), determined by the expression of bFGF, VEGF or IL-8 total RNA and protein using RT-PCR analysis and ELISA, respectively. To avoid clonal variations, the specific expression clones were frozen after one to three in vitro passages for the in vitro and in vivo studies. Individual colonies were established as separate adherent cultures.

**RT-PCR Analysis**

RT-PCR analysis was performed as previously described [12]. Briefly, 1 mg of total cellular RNA was extracted from various cell lines by the acid guanidinium thiocyanate-phenol chloroform method using a TRizol kit (Invitrogen, Carlsbad, Calif., USA). Reverse transcription was performed using the SuperScript first-strand synthesis system for RT-PCR (Invitrogen). Five-microgram samples of total RNA were transcribed in a 20-μl volume according to the manufacturer’s instructions, after which 2 μl of synthesized cDNA was used for the PCR reaction. Two-microliter aliquots were amplified in a 50-μl reaction mixture consisting of 1× PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP mix, 0.2 μM of each primer and 2 units of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, Calif., USA). PCR was performed with predenaturation (94°C for 2 min), 35 cycles of denaturation (94°C for 15 s), annealing (GAPDH, 58°C for 60 s; bFGF, 55°C for 60 s; VEGF, 63°C for 60 s, and IL-8, 55°C for 60 s), extension (71°C for 1 min) and a final extension step at 72°C for 5 min after completion of all cycles. PCR products were run on electrophoresis in 1.5% agarose gel for bFGF, IL-8 and GAPDH and 3% agarose gel for VEGF, and stained with ethidium bromide. The bands of expected sizes were confirmed by sequencing. Primer sequences used were GAPDH-forward, 5’-GGGGGAATTTGTCAGACGCAGAAGGATGGGTAAGACAGA-3’; GAPDH-reverse, 5’-TTCTGGAGATGTTGAGTGTG-3’; bFGF-forward, 5’-ATCGAATTGTTGTCGAGG-3’; bFGF-reverse, 5’-TCCAGGGAGTTGGTAAAGACAG-3’; VEGF-forward, 5’-CCGCCAGCGTGAATGTTTCC-3’; VEGF-reverse, 5’-CCGGCTGTGCACTCTGCAAGTA-3’; IL-8-forward, 5’-CAGTTTTGGCC-AAGGAGTGCTGCTA-3’; and IL-8-reverse, 5’-CCCGTGCAA-TATCTAGGAAATAC-3’.

**ELISA for bFGF, VEGF and IL-8**

Viable 253J-P cells, 253J B-V cells and the specific expression clones were seeded onto a 96-well plate in the conditioned medium. After 24 h, the cells were then washed with 200 μl of Hanks’ balanced salt solution (HBSS), and 200 μl of 10× bovine serum supplemented with fresh MEM were added. Forty-eight hours later, the amounts of VEGF and IL-8 in cell-free culture supernatants and cell-associated bFGF in freeze-thaw cell lysates were determined using the commercial Quantime ELISA kit (R&D Systems, Minneapolis, Minn., USA). The protein concentration for each factor was then determined by comparing the optical density with that of a standard. Results were expressed in terms of cell numbers [6].

**Growth Curve**

Viable cells (1×10⁶) were seeded in a 96-well plate. Conditioned medium was removed after 24 h, and the cells were washed with 200 μl of HBSS; 200 μl of 10× bovine serum supplemented with fresh MEM were added. Every 24 h, the number of viable cells in each cell line was assessed by optical density comparison. The doubling time of each cell line was determined by plotting the optical density on a semilogarithmic axis versus time (Cricket Software, Malvern, Pa., USA). The doubling time of 253J-P and 253J B-V was 67.5 and 50.6 h, respectively.

The doubling time of the bFGF-expressing 253J B-V clones (bFGF-H: 44.9 h, and bFGF-L: 54.3 h), VEGF-expressing 253J B-V clones (VEGF-H: 49.6 h, and VEGF-L: 52.4 h) and also IL-8-expressing 253J B-V clones (IL-8-H: 46.9 h, and IL-8-L: 54.5 h) were similar to those for 253J B-V (fig. 1).
Animals
Male athymic BALB/cA Jc1-nu nude mice were obtained from Clea Japan, Osaka, Japan. The mice were maintained in a laminar-airflow cabinet in pathogen-free conditions and used at 8–12 weeks of age.

Orthotopic Implantation of Tumor Cells
To study the differences in tumorigenicity and metastases by 253J-B-V cells and the specific expression clones, mice were randomly separated into seven groups, and 253J-B-V cells and six clones were orthotopically injected. Thirty mice in the groups with high- or low-expressing clones of each factor and five mice in the 253J-B-V cell group were orthotopically injected with cancer cells at a time. Cultured 253J-B-V cells and the specific expression clones (60–70% confluent) were prepared for injection as previously described [6]. Mice were anesthetized with Nembutal. For orthotopic implantation, a lower midline incision was made, and viable tumor cells (1 × 10^6/0.05 ml in HBSS) were implanted into the bladder wall. The formation of a bulla was a sign of a satisfactory injection. The bladder was returned to the abdominal cavity, and the abdominal wall was closed with a single layer of metal clips.

Tumorigenicity and Metastases of Human Transitional Cell Carcinoma Growing in the Bladders of Athymic Nude Mice
Mice were necropsied approximately 1, 2, 4, 8 and 12 weeks after orthotopic implantation. The primary tumors were removed and weighed, and the presence of spontaneous metastases in the lymph nodes and lungs was determined macro- and microscopically. The bladders were then either quickly frozen in liquid nitrogen for mRNA extraction, fixed in 10% buffered formalin, or mechanically dissociated and put into tissue culture. The lymph nodes were fixed in 10% buffered formalin or mechanically dissociated and put into tissue culture.

In situ mRNA Hybridization Analysis
Specific antisense oligonucleotide DNA probes were designed complementary to the mRNA transcripts based on published reports of the cDNA sequences: bFGF (CGGGAGGGCGCCGT-GCCGCC), 85.7% guanosine cytosine (GC) content [13]; VEGF/vascular permeability factor (TGGTGTGTTGAGCTCTTCA-GTGCCCC), 57.7% GC content [14], and IL-8 (CTCCACCGCTCTGGACCC), 66.0% GC content [9]. The specificity of the oligonucleotide probes was initially determined by a GeneBank European Molecular Biology Library database search with the use of the Genetics Computer Group sequence analysis program (Madison, Wisc., USA) based on the FastA algorithm; these sequences showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each of the sequences was also confirmed by Northern blot analysis [15]. A poly d(T)$_{20}$ oligonucleotide was used to verify the integrity of mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) added to the 3’ end via direct coupling, with the use of standard phosphoramidite chemistry (Research Genetics, Huntsville, Ala., USA). The lyophilized probes were reconstituted to a stock solution at 1 g/l in 10 mmol/l Tris (pH 7.6) and 1 mmol/l EDTA. Immediately before use, the stock solution was diluted with probe dilution (Research Genetics).

In situ mRNA hybridization was performed as described previously, with minor modifications [16, 17]. In situ hybridization (ISH) was carried out using the Microprobe Manual Staining System (Fisher Scientific, Pittsburgh, Pa., USA) [18]. Tissue sections (4 μm) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific [16, 17]). The slides were placed in a Microprobe slide holder, dewaxed and rehydrated with Autodewaxer and Autolcohol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was carried out for 45 min at 45°C, and the samples were then washed with alkaline phosphatase-labeled avidin for 30 min at 45°C, rinsed with 50 mm Tris buffer (pH 7.6) and alkaline phosphatase enhancer for 1 min and incubated with fresh chromogen substrate if necessary to enhance a weak reaction. A positive reaction in this assay appears as a red stain. The control for endogenous alkaline phosphatase included treatment of the sample in the absence of the biotinylated probe and the use of chromogen alone.

Quantification of Color Reaction
Stained sections were examined with a Zeiss photomicroscope (Carl Zeiss, Thornwood, N.Y., USA) equipped with a three-chip, charge-coupled device color camera (model DXC-969 MD; Sony, Tokyo, Japan). The images were analyzed using Optimas image analysis software (version 4.10; Optimas, Bothell, Wash., USA). The slides were prescreened by one of the investigators to determine the range of staining intensities. This range was captured electronically, a color bar (montage) was created, and a threshold value was set in the red, green and blue modes of the color camera. All subsequent images were quantified based on this threshold. The integrated optical density of the selected fields was deter-
Result clones to that of the corresponding 253J B-V defined as the relative ratio of total RNA expression of the specific expression. The expression levels of bFGF, VEGF or IL-8 are shown in figure 2, the bFGF, VEGF and IL-8 PCR products of 394, 95 and 513 bases were detected, respectively. In vitro, the highest and lowest expression clone cells express stable total RNA for bFGF, VEGF or IL-8. The bFGF, VEGF and IL-8 PCR products of 394, 95 and 513 bases were detected, respectively. RT-PCR analysis revealed that 253J-P, 253J B-V and the specific expression clones (bFGF-H, bFGF-L, VEGF-H, VEGF-L, IL-8-H and IL-8-L) are shown in figure 2. As shown in figure 2, the bFGF, VEGF and IL-8 PCR products of 394, 95 and 513 bases were detected, respectively. The level of expression is shown as the ratio of total RNA expression of the specific expression clones to that of the corresponding 253J B-V defined as 1.00.

Fig. 2. Total RNA expression of 253J-P, 253J B-V and the specific expression clone cells by RT-PCR analysis. RT-PCR analysis was performed by the following primer sequences: forward 5'-CGGATTGTTGCTATTGG-3' and reverse 5'-TCCCTGGAGATGGTGATG-3' for GAPDH; forward 5'-ATGCAATTTTGTGGTGCGAGG-3' and reverse 5'-TCCAGGGGATGGGTAAGACAG-3' for bFGF; forward 5'-CCGCAGACGTGTAAATGTTCCT-3' and reverse 5'-CAGTTTTGCCAAGGAGTGTGCTAA-3' for VEGF, and forward 5'-CAGGTTTTCAGCCAGGATGGCTAA-3' and reverse 5'-CCGTGAATATCTAGGAAAATC-3' for IL-8. RT-PCR analysis revealed that 253J-P, 253J B-V and the specific expression clone cells express stable total RNA for bFGF, VEGF or IL-8. The bFGF, VEGF and IL-8 PCR products of 394, 95 and 513 bases were detected. In vitro, the highest and lowest expression clones of bFGF, VEGF or IL-8 demonstrated stable total RNA expression. The expression levels of bFGF, VEGF or IL-8 are shown as the relative ratio of total RNA expression of the specific expression clones to that of the corresponding 253J B-V defined as 1.00.

mined based on its equivalence to the mean log inverse gray value multiplied by the area of the field. The samples were not counterstained, so the optical density was due solely to the product of the ISH reaction. Three different fields in each sample were quantified to derive an average value. The intensity was determined by comparison with the integrated optical density of poly d(T)20. The results for each cell line are presented relative to 253J B-V, which was set to 100 [6].

Immunohistochemistry

For immunohistochemical analysis, frozen tissue sections (8 μm thick) were fixed with cold acetone. Endogenous peroxidases were blocked by incubation in 3% hydrogen peroxide in PBS for 12 min. The samples were washed three times with PBS and incubated for 20 min at room temperature with a protein-blocking solution containing 5% normal horse serum and 1% normal goat serum in PBS (pH 7.5). Excess blocking solution was drained, and the samples were incubated for 18 h at 4 °C with the appropriate dilution (1:100) of rat monoclonal anti-CD31 antibody (Pharmingen, San Diego, Calif., USA) [19]. The samples were then rinsed four times with PBS and incubated for 60 min at room temperature with the appropriate dilution of the secondary antibody, peroxidase-conjugated anti-rat Immunoglobulin G (H + L; Jackson ImmunoResearch Laboratory, West Grove, Pa., USA). The slides were rinsed with PBS and incubated for 5 min with diamnobenzidine (Research Genetics). The sections were then washed three times with PBS, counterstained with Gill’s hematoxylin (Biogenex Laboratories, San Ramon, Calif., USA) and again washed three times with PBS. The slides were mounted with Universal Mount (Research Genetics).

Quantification of MVD

Tumor-induced neovascularization was determined by light microscopy after immunostaining of sections with anti-CD31 antibodies according to the procedure of Weidner et al. [20]. Clusters of stained endothelial cells distinct from adjacent microvessels, tumor cells or other stromal cells were counted as one microvessel. The tissue images were recorded using a cooled CCD Optotronics Tec 470 camera (Optotronics Engineering, Goleta, Calif., USA) linked to a computer and digital printer (Sony). MVD was expressed as the average of the five highest areas identified within a single ×200 field [6].

Statistical Analysis

Statistical differences in the number of vessels and staining intensity for mRNA expression of bFGF, VEGF and IL-8 within the bladder tumors were analyzed with the Mann-Whitney test. The incidence of tumors and metastases was statistically analyzed using the χ² test. p < 0.05 was considered significant.

Results

In vitro Total RNA Expression of bFGF, VEGF and IL-8

RT-PCR analyses for bFGF, VEGF and IL-8 steady-state gene expression by 253J-P, 253J B-V and the specific expression clones (bFGF-H, bFGF-L, VEGF-H, VEGF-L, IL-8-H and IL-8-L) are shown in figure 2. As shown in figure 2, the bFGF, VEGF and IL-8 PCR products of 394, 95 and 513 bases were detected, respectively. The level of expression is shown as the ratio of total RNA expression of the specific expression clones to that of the corresponding 253J B-V. The total RNA expression of bFGF was significantly increased (2.40-fold) by bFGF-H,
and significantly decreased (0.36-fold) by bFGF-L, while there was no change in either VEGF or IL-8 RNA expression compared with 253J B-V. The total RNA expression of VEGF was significantly increased (2.02-fold) by VEGF-H, and significantly decreased (0.39-fold) by VEGF-L, while there was no change in either bFGF or IL-8 total RNA expression compared with 253J B-V. The total RNA expression of IL-8 was significantly increased (3.09-fold) by IL-8-H and significantly decreased (0.51-fold) by IL-8-L, while there was no change in either bFGF or VEGF total RNA expression compared with 253J B-V.

**In vitro Protein Expression of bFGF, VEGF and IL-8**

The protein production of bFGF, VEGF and IL-8 by 253J-P, 253J B-V and the specific expression clones was evaluated by ELISA (fig. 3). The level of expression is shown as the ratio of protein expression of the specific expression clones to that of the corresponding 253J B-V. The specific protein expression level by 253J-P, 253J B-V and the specific expression clones paralleled the specific level seen in total RNA expression.

**Tumorigenicity and Production of Metastasis**

To evaluate whether the expression of angiogenesis-related genes regulates tumorigenicity and metastases of TCC, we implanted 253J B-V and the specific expression clones into the bladders of athymic nude mice and evaluated tumor growth and metastasis 1, 2, 4, 8 and 12 weeks after orthotopic implantation (table 1). Of all 210 animals, 9 animals with operational death, 9 animals with microscopical bladder cancer with mostly infectious lesions and 6 animals with cannibalization were excluded.

**Fig. 3.** Protein expression of 253J-P, 253J B-V and the specific expression clone cells by ELISA. 48 h after seeding of viable 253J-P, 253J B-V and the specific expression clone cells, the amounts of VEGF and IL-8 in cell-free culture supernatants and cell-associated bFGF in freeze-thaw cell lysates were determined using ELISA. The protein concentration for each factor was then determined by comparing the optical density with that of a standard. In vitro, the clones with the highest and lowest expression of bFGF, VEGF or IL-8 demonstrated stable protein expression. The expression levels of bFGF, VEGF or IL-8 are shown as the relative ratio of protein expression of the specific expression clones to that of the corresponding 253J B-V defined as 1.00. Protein expression by 253J-P, 253J B-V and the specific expression clones paralleled the specific level seen in total RNA expression. *p < 0.05, **p < 0.001, vs. 253J B-V (χ² test).
from the evaluation of tumorigenicity and production of metastases.

One week after orthotopic implantation, 5 of 6 animals implanted with parental 253J B-V developed tumors with a median weight of 42.2 mg (range: 25–58 mg). Two of 6 animals implanted with bFGF-L developed tumors with a median weight of 32.0 mg (range: 21–60 mg), whereas all 6 animals implanted with bFGF-H developed tumors with a median weight of 64.7 mg (range: 49–111 mg, p = 0.025 vs. 253J B-V). All 6 animals implanted with VEGF-L or VEGF-H developed tumors with a median weight of 42.3 (range: 28–52 mg) or 44.8 mg (range: 30–63 mg), respectively. There was no significant difference compared with 253J B-V. Three of 6 animals implanted with IL-8-L developed tumors with a median weight of 34.2 mg (range: 22–57 mg), whereas all 6 animals implanted with IL-8-H developed tumors with a median weight of 61.0 mg (range: 53–75 mg; p = 0.005 vs. 253J B-V).

Two weeks after orthotopic implantation, all 6 animals implanted with parental 253J B-V developed tumors with a median weight of 84.2 mg (range: 42–119 mg). Two of 6

Table 1. Transitional change in tumorigenicity and production of spontaneous lymph node metastases after orthotopic implantation of 253J B-V and the individual clone cells in the bladder wall of athymic nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Incidence</th>
<th>1 week</th>
<th>2 weeks</th>
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<th>8 weeks</th>
<th>12 weeks</th>
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<tr>
<td></td>
<td>bladder</td>
<td>lymph</td>
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<tr>
<td>253J B-V</td>
<td>5/6</td>
<td>1/6</td>
<td>6/6</td>
<td>0/6</td>
<td>6/6</td>
<td>0/6</td>
<td>6/6</td>
</tr>
<tr>
<td>bFGF-L</td>
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<td>0/6</td>
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<td>0/6</td>
<td>2/4</td>
<td>0/4</td>
<td>4/6</td>
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<tr>
<td>bFGF-H</td>
<td>6/6</td>
<td>0/6</td>
<td>6/6</td>
<td>0/6</td>
<td>5/5</td>
<td>1/5</td>
<td>6/6</td>
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<tr>
<td>VEGF-L</td>
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<td>0/6</td>
<td>6/6</td>
<td>0/6</td>
<td>5/5</td>
<td>0/5</td>
<td>4/6</td>
</tr>
<tr>
<td>VEGF-H</td>
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<td>0/6</td>
<td>6/6</td>
<td>0/6</td>
<td>4/4</td>
<td>0/4</td>
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<tr>
<td>IL-8-L</td>
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<td>5/5</td>
<td>2/5</td>
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* Operational death. **p < 0.025; ***p < 0.05; ****p < 0.0001; *****p < 0.005, vs. 253J B-V (x² test).

Table 2. Transitional changes in mRNA expression and MVD in the tumors after orthotopic implantation of 253J B-V and the individual clone cells in the bladder wall of athymic nude mice

<table>
<thead>
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<th>Cell line</th>
<th>1 week</th>
<th>2 weeks</th>
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<td>MVD</td>
<td>mRNA expression index</td>
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<tr>
<td>bFGF</td>
<td>VEGF</td>
<td>IL-8</td>
<td>per × 200 field</td>
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<td>253J B-V</td>
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<td>100.0</td>
<td>100.0</td>
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<tr>
<td>bFGF-L</td>
<td>60.4*</td>
<td>97.3</td>
<td>98.9</td>
</tr>
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<td>98.9</td>
<td>95.4</td>
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<td>IL-8-H</td>
<td>100.8</td>
<td>98.9</td>
<td>164.6***</td>
</tr>
</tbody>
</table>

* The intensity of the cytoplasmic color reaction was quantitated by an image analyzer and compared with the maximal intensity of the poly d(T) color reaction in each sample. The results were presented as the number of each cell line compared with 253J B-V defined as 100.
animals (p < 0.025 vs. 6/6 of 253J B-V) implanted with bFGF-L developed tumors with a median weight of 38.3 mg (range: 22–74 mg), whereas all 6 animals implanted with bFGF-H developed tumors with a median weight of 98.5 mg (range: 52–131 mg). All 6 animals implanted with VEGF-L or VEGF-H developed tumors with a median weight of 76.8 (range: 51–101 mg) or 85.8 mg (range: 40–132 mg), respectively (nonsignificant compared with 253J B-V). Three of 6 animals (p < 0.05 vs. 6/6 of 253J B-V) implanted with IL-8-L developed tumors with a median weight of 42.8 mg (range: 22–74 mg), whereas all 6 animals implanted with IL-8-H developed tumors with a median weight of 91.0 mg (range: 59–128 mg).

Four, 8 and 12 weeks after orthotopic implantation, although all highest-expressing clone cells demonstrated no significant enhancement in tumorigenicity, the tumorigenicity of the lowest bFGF- and the lowest IL-8-expressing clone cells was still reduced, but there was no statistical significance.

Spontaneous lymph node metastases of bFGF-H and IL-8-H tumors were more often detected at an earlier stage (4 weeks after orthotopic implantation), while the incidence of metastases of bFGF-L and IL-8-L was less frequent (nonsignificant vs. parental 253J B-V; table 1).

**Tumor Angiogenesis**

MVD was determined by immunohistochemistry using anti-CD31 antibodies (table 2, fig. 4). The numbers of CD31+ microvessels counted per ×200 field in the 253J B-V tumors 1, 2, 4, 8 and 12 weeks after orthotopic implantation were 26.1, 51.3, 90.7, 102.3 and 117.7, respectively. The MVD was significantly reduced from 26.1 and 51.3 per ×200 field in the 253J B-V tumors to 16.7 and 31.5 per ×200 field in the bFGF-L tumors, 1 and 2 weeks after orthotopic implantation (p < 0.05, p < 0.05, respectively). The MVD in the tumor 1 week after orthotopic implantation of bFGF-H and IL-8-H was 36.7 and 35.0 per ×200 field (p < 0.05 and p = 0.025 vs. 253J B-V, respectively).

**Expression of bFGF, VEGF and IL-8**

The in vivo mRNA expression of bFGF, VEGF and IL-8 in tumors (table 2) and also metastasized lymph nodes (table 3) was evaluated by ISH and correlated directly with the in vitro expression of these factors. The results were presented as a number of each clone compared with 253J B-V defined as 100.

The bFGF mRNA expression in bFGF-L tumors and the VEGF mRNA expression in VEGF-L tumors were significantly down-regulated 1 week after orthotopic implantation. The bFGF mRNA expression in bFGF-H tumors, the VEGF mRNA expression in VEGF-H tumors as well as the IL-8 mRNA expression in IL-8-H tumors were significantly up-regulated 1 week after orthotopic implantation. However, the expression level of bFGF, VEGF and IL-8 in the tumor of each specific expression clone was gradually regulated to the same expression level as parental 253J B-V cell lines within 4 weeks.

In metastasized lymph nodes of the highest and the lowest IL-8 expression clones, IL-8 mRNA was consis-

<table>
<thead>
<tr>
<th>mRNA expression indexa</th>
<th>MVD</th>
<th>mRNA expression indexa</th>
<th>MVD</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 weeks</td>
<td>12 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>bFGF</td>
<td>bFGF</td>
<td>VEGF</td>
<td>IL-8</td>
</tr>
<tr>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
<td>102.3</td>
</tr>
<tr>
<td>90.1</td>
<td>97.0</td>
<td>94.4</td>
<td>88.2</td>
</tr>
<tr>
<td>110.3</td>
<td>94.2</td>
<td>93.7</td>
<td>106.3</td>
</tr>
<tr>
<td>93.8</td>
<td>102.5</td>
<td>93.8</td>
<td>94.5</td>
</tr>
<tr>
<td>94.1</td>
<td>104.5</td>
<td>100.5</td>
<td>104.8</td>
</tr>
<tr>
<td>96.5</td>
<td>112.8</td>
<td>95.5</td>
<td>94.0</td>
</tr>
<tr>
<td>95.1</td>
<td>119.6</td>
<td>109.7</td>
<td>101.0</td>
</tr>
</tbody>
</table>

MVD was expressed as the average number of the five highest areas identified within a single ×200 field. * p < 0.025; ** p = 0.025; *** p = 0.01; **** p < 0.05, vs. 253J B-V (Mann-Whitney test).
tently expressed at high and low level, respectively, at a later stage (8 and 16 weeks after orthotopic implantation). Although bFGF mRNA expression levels were consistently high and low in metastasized lymph nodes of the highest and the lowest bFGF expression clones, respectively, 8 weeks after orthotopic implantation, the bFGF expression level was gradually regulated to the same expression level as parental 253J B-V cell lines within 12 weeks.

**Discussion**

Recently, a novel approach to anti-cancer therapy has been to target molecular pathways, especially angiogenesis. To enhance anti-tumoral and anti-metastatic effects of anti-angiogenic therapy, we evaluated the relative activity of angiogenesis-related genes in the control of tumorigenicity and metastasis of UC of the urinary bladder. At the early stage of tumor growth, bFGF and IL-8 expression regulated angiogenesis, tumorigenicity and subsequent lymph node metastases of human UC in the urinary bladder of athymic nude mice. These findings evidenced that a therapy targeting angiogenesis pathways, e.g. bFGF and IL-8 expression, especially at early stages of tumor growth, could enhance anti-tumoral as well as anti-metastatic effects of the urinary bladder on UC. bFGF [1, 2], VEGF [3, 4] and IL-8 [5, 6], which are secreted by tumors and their relevant microenvironment, are major factors significantly associated with multistep processes such as angiogenesis, tumor growth, invasion and subsequent metastases. Accordingly, Kumar et al. [21] demonstrated that small tumors express more bFGF and IL-8 than large tumors, whereas more VEGF is expressed in large tumors. Angiogenic factors were heterogeneously distributed within large tumors, and the expression of bFGF and IL-8 was highest in the tumor periphery, where cell division is greatest, whereas VEGF expression is higher in the center of the tumor. Our present study also supports the findings of the distribution patterns of bFGF, IL-8 and VEGF. Moreover, Izawa et al. [22] investigated mRNA expression of several progression-related genes, including bFGF, VEGF and IL-8, in 77 patients with transitional cell carcinoma of the bladder. They showed that bFGF, VEGF and IL-8 expression was higher in muscle-invasive than in superficial papillary UC (p < 0.05) [22]. Particularly bFGF and IL-8 appear to be up-regulated in early precursor lesions, whereas VEGF appears to be up-regulated at later stages in the development of muscle-invasive UC [22]. Our results also supported the expression of bFGF and IL-8 especially at an ‘early stage’ (1–2 weeks) of tumor growth in human UC. However, at a ‘later stage’ (after 4 weeks), bFGF, VEGF and IL-8 expression in the tumor of all specific expression clones gradually approached the expression level of parental 253J B-V cell lines. bFGF, VEGF and IL-8 are not only secreted by neoplastic cells but also by inflammatory and interstitial cells in the microenvironment of a

### Table 3. Transitional changes in mRNA expression in spontaneous lymph node metastases after orthotopic implantation of 253J B-V and the individual clone cells in the bladder wall of athymic nude mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>4 weeks</th>
<th>8 weeks</th>
<th>12 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mRNA expression index&lt;sup&gt;a&lt;/sup&gt;</td>
<td>mRNA expression index&lt;sup&gt;a&lt;/sup&gt;</td>
<td>mRNA expression index&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>bFGF</td>
<td>VEGF</td>
<td>IL-8</td>
</tr>
<tr>
<td>253J B-V</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>bFGF-L</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>bFGF-H</td>
<td>99.5</td>
<td>77.7</td>
<td>80.0</td>
</tr>
<tr>
<td>VEGF-L</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VEGF-H</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IL-8-L</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>IL-8-H</td>
<td>69.3</td>
<td>79.4</td>
<td>121.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> The intensity of the cytoplasmic color reaction was quantitated by an image analyzer and compared with the maximal intensity of the poly d(T) color reaction in each sample. The results were presented as the number of each cell line compared with 253J B-V defined as 100. 
* p < 0.05; ** p < 0.01, vs. 253J B-V 8 weeks after orthotopic implantation; *** p < 0.05 vs. 253J B-V 12 weeks after orthotopic implantation (Mann-Whitney test).
Fig. 4. MVD by immunohistochemistry using anti-CD31 antibodies. The number of CD31+ microvessels was expressed as the average of the five highest areas identified within a single ×200 field. One and 2 weeks after orthotopic implantation of bFGF-H and IL-8-H, the tumor expression level of MVD was increased compared to that of bFGF-L, IL-8-L and also 253J B-V. However, there was no difference in the MVD in the tumors of all specific expression clones 12 weeks after orthotopic implantation.
neoplasm. Therefore, it may be caused by the interaction between cancer cells and their microenvironment. Supposing VEGF regulates tumor growth especially at a later stage, it is understandable that the same expression level of VEGF expressed at a later stage of tumor progression by each specific expression clone could not differentially affect tumor growth.

Moreover, in a previous study, therapy targeting bFGF or IL-8 of human non-established UC in an athymic nude mice [6, 23–25]. Therapy with the adenoviral-mediated antisense bFGF gene [23] or angiogenic inhibitor TNP-470 (AGM-1470, O-chloracetyl-carbamoyl fumagillol) [24] induced the regression of human non-established UC in athymic nude mice, which was mediated in part by the down-regulation of bFGF expression. In addition, following orthotopic implantation into the bladders of athymic nude mice, the transfection with the full-sequence antisense cDNA for IL-8 significantly inhibited tumorigenicity and production of spontaneous metastases in 253J B-V [6]. Therapy with an adenoviral-mediated antisense gene targeting IL-8 expression also inhibited tumor growth of human non-established UC growing ectopically in the subcutis of athymic nude mice [25]. In agreement with previous results, the down-regulation of bFGF and IL-8, especially at an early stage of tumor progression, was important in inhibiting tumor growth and subsequent spontaneous lymph node metastases of human UC. Nevertheless, further experiments are needed to elucidate the precise reasons why bFGF, VEGF and IL-8 expression levels in the tumors of all specific expression clones approached those of parental 253J B-V cell lines at a ‘later stage’ (after 4 weeks).

In summary, we demonstrated that bFGF and IL-8 expression, especially at an early stage of tumor growth, regulated tumor growth and subsequent spontaneous lymph node metastases of human UC in the bladder of athymic nude mice, and provided the benefit of enhancing therapeutic effects which result from different activity of angiogenesis-related genes. These studies indicate that bFGF and IL-8 expression may be a promising target for therapy of advanced bladder cancer.

References


