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Expression of Vascular Endothelial Growth Factor Gene and Its Receptor (flt-1) Gene in Urinary Bladder Cancer

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Sato, K., Sasaki, R., Ogura, Y., Shimoda, N., Togashi, H., Terada, K., Sugiyama, T., Kakinuma, H., Ogawa, O. and Kato, T. *Expression of Vascular Endothelial Growth Factor Gene and Its Receptor (flt-1) Gene in Urinary Bladder Cancer.* Tohoku J. Exp. Med., 1998, **185** (3), 173-184 —— We investigated expression of the vascular endothelial growth factor (VEGF) gene and its receptor gene (flt-1) in 30 patients with transitional cell carcinoma (TCC) of the urinary bladder by Northern blot hybridization analysis. The VEGF transcript was observed in all of the tumors and the normal mucosae. Of the 20 tumors in which a comparative study was done, eight (40.0%) overexpressed the gene with a tumor versus normal ratio of equal to and greater than 3.0. Invasive TCCs expressed significantly more VEGF gene than superficial TCCs. Cytoplasm of tumor cells was positively stained by immunohistochemistry with an anti-VEGF monoclonal antibody, while the intratumoral endothelial cells and the vascular smooth muscle cells were weakly positive for the staining. TCCs, normal mucosae and human umbilical endothelial cells expressed flt-1 gene, while leucocytes from peripheral blood did not. The expression level of flt-1 gene significantly correlated with that of the VEGF gene in the tumor. These results indicate that the VEGF gene is frequently overexpressed in TCC of the urinary bladder, especially in muscle invasive tumors, and that a paracrine system including VEGF and flt-1 exists between the TCC cells and the adjacent endothelial cells so as to regulate the angiogenesis in this tumor. ———— vascular endothelial growth factor; flt-1; transitional cell carcinoma; urinary bladder; angiogenesis

Recent investigations have revealed that tumor angiogenesis, the generation and development of new capillary blood vessels, is induced by a variety of angiogenic factors produced by the tumor cells (Risau 1997). Among the an-

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giogenic factors, vascular endothelial growth factor (VEGF) (Folkman and Klagsbrun 1987; Ferrara and Henzel 1989), also known as vascular permeability factor (Senger et al. 1983; Keck et al. 1989), has been considered the most relevant to the tumor angiogenesis.

VEGF binds its specific receptors which are specifically expressed on the cell membrane of vascular endothelial cells and exerts its tyrosine kinase activity (Shibuya et al. 1990; De Vries et al. 1992; Terman et al. 1992). Shalaby et al. (1995) demonstrated that mice deficient in the VEGF receptor failed to form blood-islands and vasculogenesis. These mice died in their embryonal period. These results indicated that the interaction of VEGF and its receptors is indispensable in proliferation and differentiation of vascular endothelial cells.

The VEGF gene and its receptor genes have proved to be expressed in transitional cell carcinoma (TCC) (Brown et al. 1993; O'Brien et al. 1995). Thus, it is predictable that VEGF and its receptors should control angiogenesis of TCC of the urinary bladder, and that their interaction should influence malignant potential of this tumor. However, the expression pattern and its exact role in the TCCs have not been elucidated yet. To clarify this issues, we investigated the expression of VEGF and its receptor flt-1 genes in this tumor and compared them with pathological findings.

Materials and Methods

Study subjects

Bladder tumors were obtained from the 30 patients, who underwent transurethral resection (TUR) or total cystectomy (TCX) for the tumor at our institutes and its affiliated hospitals from 1992 to 1995, and subjected to the present study.

Tissue specimens of superficial TCCs (pT0-1) were obtained from bladder tissues removed by TUR or TCX. Those of invasive TCCs (pT2-4) were obtained only from TCX specimens so that the necrotic area and smooth muscle layer were carefully excluded from tissue sampling. Bladder mucosae were thinly dissected from the TCX specimens where preoperative random mucosal biopsy had proved negative for carcinoma, and were used as normal control. A part of each tissue sample was frozen in liquid nitrogen immediately after the surgical removal and stored at $-80^{\circ}C$ for molecular biological analysis. The remaining samples were fixed in formaldehyde and embedded in paraffin for histopathological study. For the other controls, white blood cells (WBC) isolated from peripheral blood of the patients and human umbilical vein endothelial cell line (HUVEC-1; Riken Cell Bank, Tsukuba) were used. The cell line was cultured in a medium containing 10% fetal calf serum, and harvested after washing with phosphate buffered saline after 7 day's culture.

The histopathological findings and tumor stages, assessed in accordance with the classification of Japanese Urological Association and The Japanese Pathological Society (1993), are listed in the Table 1. Because the numbers of nodular

TCCs and G1 TCCs were small, nodular and papillonodular tumors were combined together as nodular TCCs, as were the G1 and G2 tumors as G1-2 TCCs, for comparative analyses.

Northern blot hybridization

Each VEGF receptor, KDR and flt-1 gene, has a distinct expression pattern in human fetal endothelial cells (Kaipainen et al. 1993), but not in either the adult normal bladder tissue or TCCs (Brown et al. 1993). We therefore investigated flt-1 gene as a representative of the VEGF receptors. DNA probes identical to the human VEGF gene (Tischer et al. 1991) and flt-1 gene (Shibuya et al. 1990) were prepared by reverse transcribed polymerase chain reaction (RT-PCR). Briefly, cDNA, synthesized from total RNA extracted from the human normal kidney with Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco BRL, Gaitherburg, MD, USA), was subjected to 35 rounds of amplification by polymerase chain reaction (PCR). Cycles were 5 seconds at 94°C, 10 seconds at 55°C and 15 seconds at 72°C in a thermal cycler (Air Thermo-Cycler 1605, Idaho Technology Co., Ltd., Idaho fall, ID, USA). The oligonucleotide primers for PCR were as follows; sense strand, 5′-GAGGAGTCCACCATCACCATGCAG-3′ (VEGFUP) and antisense strand, 5′-GGCTCACCGCCTCGGCTTGTAACA-3′ (VEGFDW) for the VEGF gene, and sense strand, 5′-TGTTAGAGAAATCCTTCCTA-3′ (FLTUP) and antisense strand, 5′-TGTCCATCTGCTCCTGGCTG-3′ (FLTDW) for flt-1 gene. The PCR using VEGFUP and VEGFDW yielded three sizes of products spanning 156, 288 and 360 base pair (bp) of coding region, corresponding to $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ subunits (Tischer et al. 1991), respectively. Of these, a product of 288 bp was isolated from an agarose gel, purified and cloned into pMOSBlue vector by using an A-tailing kit and pMOSBlue T-vector kit (Amersham Life Science, Little Chalfont, Buckinghamshire, UK). A product of expected size of 413 bp yielded by the PCR using FLTUP and FLTDW was cloned into pMOSBlue by the same method as the VEGF gene and provided for flt-1 gene probe. Identification of the probes was confirmed by the direct sequencing technique using a Sequi Therm™ cycle sequencing kit (Epicentre Technologies, Madison, WI, USA) (data not shown). A rat β-actin probe, which we originally cloned, was used for the internal control for signal alignment. Denatured 10 μg of total RNAs extracted from tissue samples by guanidium acid phenol method were electrophoretically fractionated on a 0.8% agarose gel and transferred onto a nylon membrane (Gene Screen Plus Filter, NEN, London, UK). The membrane was hybridized with 50 ng of ^{32}P-labeled VEGF gene probe described above (specific activity, at least 10^8 cpm/μgDNA), washed (Maniatis et al. 1989) and subjected to measurement of gene expression by using a FUJI Bio Imaging Analyzer (BAS1000, Fuji Film Co., Ltd., Tokyo). After the quantitative measurement, we also carried out autoradiography on the membrane for photoprints. The VEGF gene probe was then stripped off by boiling the mem-

brane in 15 mM Tris HCl, pH 7.0/1% sodium dodecyl sulfate/1 mM EDTA for 15 minutes. Repeating the steps, the membrane was hybridized, image analyzed and autoradiographed for all the DNA probes described above. The degree of gene expression was adjusted by β-actin gene expression to eliminate the difference in the amount of RNA loaded on each lane. RNA pairs of the normal mucosa and tumor from one case (Case 10) were loaded on each nylon membrane to calculate relative intensities of gene expression. A relative intensity of gene expression in the tumor versus the corresponding normal kidney was designated as a T/N ratio. A T/N ratio of equal to or greater than 3 was defined as overexpression.

Immunohistochemical analysis

To investigate the localization of VEGF protein in the TCC, an immunohistochemical study was done on 4 superficial TCCs using the Avidin-Biotin complex method. The paraffin-embedded tissue samples were sliced in 4 μm thick and deparaffinized. The tissue sections were subjected to the avidin-biotin peroxidase staining, utilizing a mouse anti-human VEGF antibody (IBL, Fujioka) for detection of VEGF peptide. Negative control was included by omitting the primary antibody and replacing it with non-immune mouse serum. After the immmunostaining, the tissue sections were counter-stained with Myer's hematoxylin.

Statistical analysis

Chi-square test was applied to evaluate differences between 2 groups with regard to gene expression. Linear regression and Student's t-test were used to evaluate the correlation between expression of VEGF and flt-1 genes. All tests of statistical significance were two sided. A level of $p<0.05$ was taken as significant.

Results

Immunohistochemistry

Cytoplasm of tumor cells were positively stained with the anti-VEGF antibody (Fig. 1). The intensity of VEGF staining was homogeneous within the tumor, with no detectable "hot spots." Normal epithelial cells and infiltrating leukocytes were negative for VEGF staining. Weak staining was detected on the endothelial cells adjacent to the tumor nest and the vascular smooth muscle cells. A few fibroblasts stained weakly scattered sparsely.

Northern blot hybridization

In the present study, the 3.7 kilobases (kb) VEGF transcript was observed in all of the tumors and normal mucosae examined. WBCs from the patients and HUVEC-1 cells did not express VEGF gene. The 4.4 and 5.5 kb VEGF transcripts were observed in the tumors having overexpression of 3.7 kb transcript

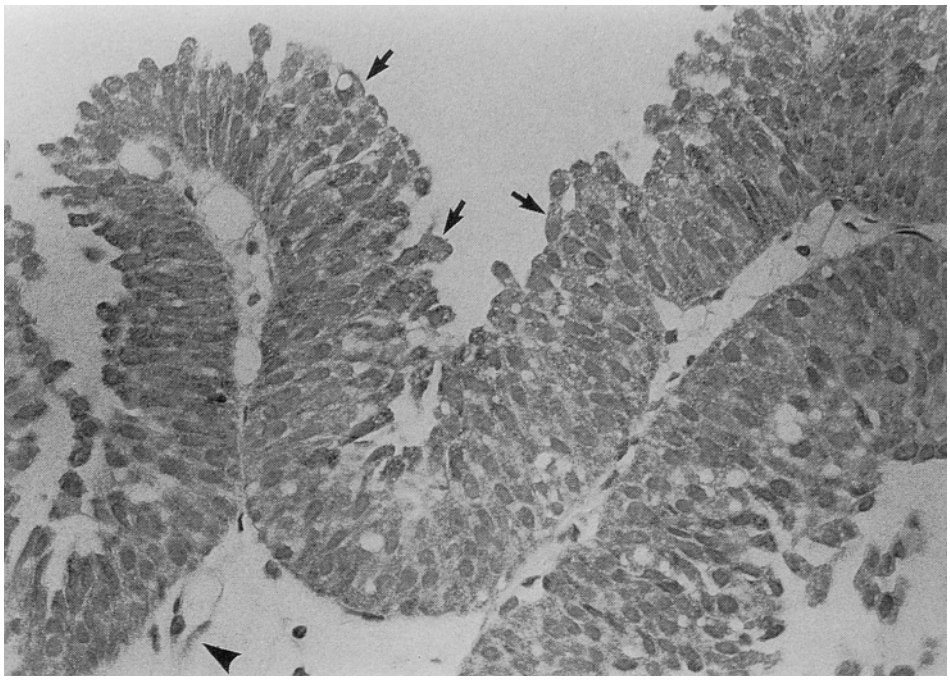

Fig. 1. Immunohistochemistry of VEGF in transitional cell carcinoma. Cytoplasm of the tumor cells was homogeneously stained (→). The microvessel endothelial cells (▶) in the tumor stroma reacted very weakly.

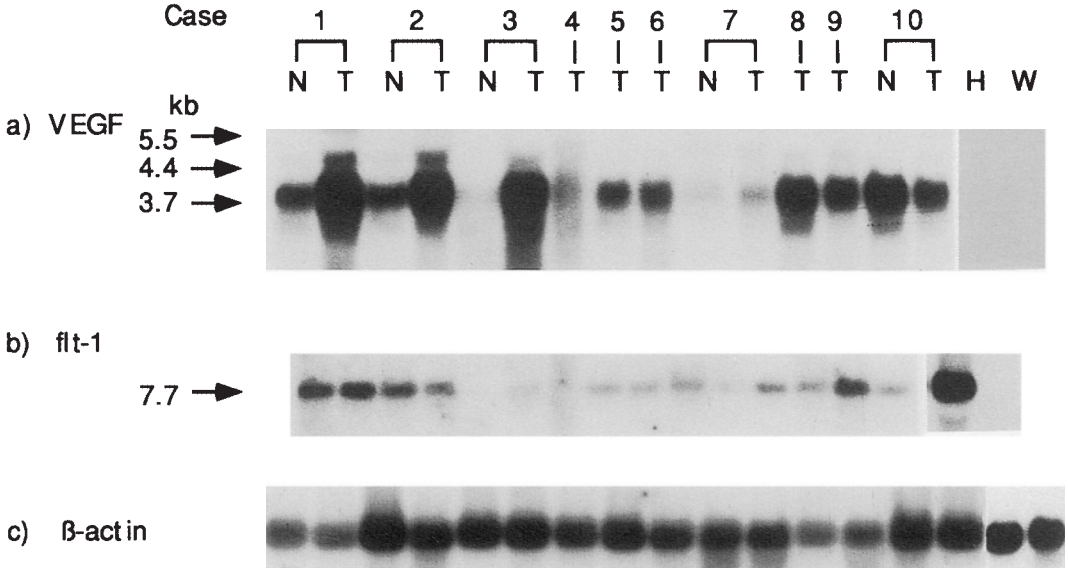

Fig. 2. Gene expression detected by Northern blot hybridization. Ten micrograms of total RNA extracted from normal bladder mucosa, bladder carcinoma, HUVEC-1 and peripheral blood leukocytes were analyzed. After the quantitative measurement of gene expression, autoradiography was carried out. a) VEGF gene: The VEGF gene transcript of 3.7 kb was detected in all bladder samples examined, but not in WBC and HUVEC-1. N; normal mucosa, T; tumor, W; WBC, H; HUVEC-1. b) flt-1 gene: All bladder samples as well as HUVEC-1 expressed flt-1 gene. However, no signal was detected on the lane of WBC. c) β-actin: As a control for the amount of RNA loaded.

(Fig. 2A-a).

Eight (40.0%) of the 20 tumors to which normal mucosa was available for comparison overexpressed the VEGF gene with a T/N ratio ranging from 3.0 to 8.1 (Table 1). Mean expression level of VEGF gene of TCCs (1.36 ± 0.77) was significantly higher than that of normal mucosa (0.66 ± 0.37) ($p < 0.001$). Furthermore, mean expression level of the VEGF gene of 17 invasive TCCs

TABLE 1. *Pertinent data of 30 urinary bladder carcinomas*

No	Patient	Shape	Grade	Stage	Gene Expression				
					VEGF			flt-1	
					TCC	Normal	T/N	TCC	Normal
1	MA-74-F	nodular	G3	pT4	2.47	0.41	6.02	1.50	1.31
2	KT-72-F	nodular	G3	pT3	1.91	0.46	4.17	0.90	0.82
3	NS-72-M	papillary	G2	pT2	2.75	0.56	4.90	0.75	0.45
4	NW-79-F	papillary	G2	pT1	0.65			0.87	
5	YM-28-M	papillary	G1	pT1	0.24			0.26	
6	KH-55-M	papillary	G2	pT3b	1.05			1.24	
7	HH-65-M	nodular	G3	pT1	0.65	0.32	2.02	0.81	0.93
8	TT-68-M	nodular	G3	pT2	2.28			1.48	
9	FO-63-M	nodular	G3	pT3b	1.27			1.18	
10	KK-60-M	nodular	G2	pT1	1.00	0.99	1.01	1.00	1.06
11	YI-70-M	nodular	G2	pT2	1.81	0.85	2.13	0.97	0.99
12	SK-69-M	papillary	G2	pT2	1.35	0.48	2.81	2.03	1.11
13	SK-78-M	nodular	G3	pT4	2.34	0.29	8.07	1.49	0.27
14	YK-70-M	papillary	G1	pTa	1.04			1.78	
15	HT-62-M	nodular	G3	pT2	1.72	0.44	3.90	0.83	1.64
16	SS-69-M	papillary	G3	pT1	0.61	0.28	2.18	1.02	0.96
17	HS-63-M	nodular	G2	pT2	1.85	1.02	1.81	2.08	1.72
18	ZS-60-M	papillary	G2	pT1	2.13			2.38	
19	YS-63-M	nodular	G3	pT3a	0.67	0.97	0.69	0.62	1.62
20	SY-71-M	papillary	G1	pT1	0.48			0.56	
21	AS-62-M	papillary	G3	pT1	2.12	0.52	4.09	1.38	1.62
22	NS-75-M	papillary	G2	pT2	3.04	0.68	4.45	3.12	3.02
23	KA-68-M	nodular	G3	pT3	0.38	0.69	0.55	0.92	1.22
24	SI-72-M	papillary	G2	pTa	0.69			1.12	
25	RO-65-M	nodular	G2	pT3	0.92	1.52	0.61	0.99	1.91
26	HO-62-M	papillary	G2	pT1	1.46			0.84	
27	JT-68-M	papillary	G3	pT1	0.67	0.45	1.49	0.90	0.88
28	SF-69-M	nodular	G2	pT3	0.52	0.28	1.86	1.66	0.98
29	YR-67-M	papillary	G2	pT2	1.63	0.47	3.47	2.07	1.36
30	YS-79-M	papillary	G2	pT1	1.20	1.49	0.80	2.07	1.41
	Mean				1.36	0.66	2.85	1.29	1.26

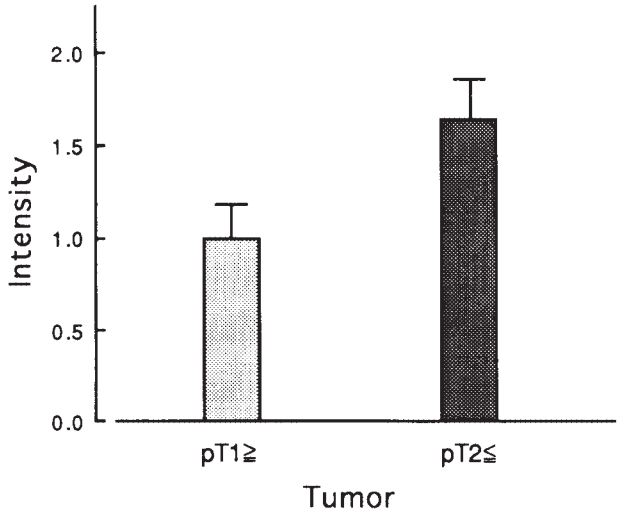

Fig. 3. Expression of the VEGF gene in transitional cell carcinoma.
The VEGF gene was expressed more highly in the muscle invasive tumor (pT2≦; $n=17$) than in the superficial tumor (pT1≧; $n=13$) ($p<0.05$).

(1.65 ± 0.78) was significantly higher than that of 13 superficial TCCs (1.00 ± 0.59) ($p<0.05$) (Fig. 3). The mean expression levels of the VEGF gene of 18 G1-2 TCCs and 12 G3 TCCs were 1.32 ± 0.77 and 1.42 ± 0.80, respectively, and showed no significant difference ($p>0.8$). As shown in the Table, the mean expression level of the VEGF gene of 16 papillary TCCs was not different from that of 14 nodular TCCs.

Flt-1 gene expression

All of the tumors as well as the normal mucosae and the HUVEC-1 cells expressed flt-1 gene as demonstrated at an expected signal size of 7.7 kb, while WBCs of patients did not (Fig. 2A-b). Mean expression levels of flt-1 gene in

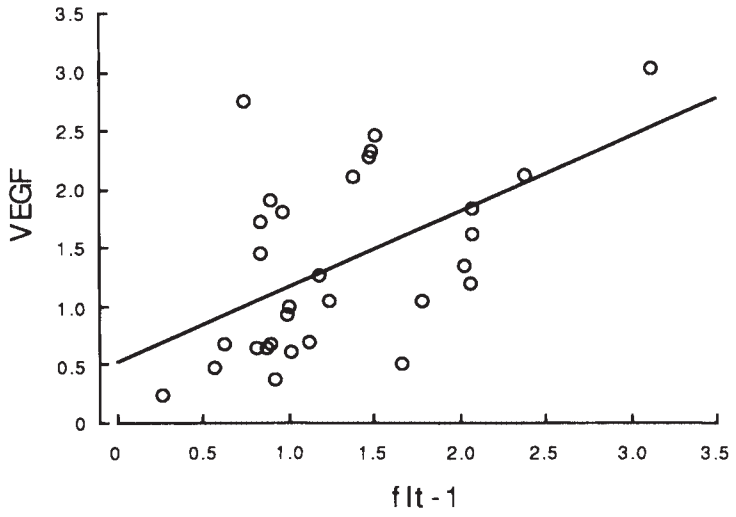

Fig. 4. Correlation of expression levels of the VEGF gene and the flt-1 gene in transitional cell carcinoma.
$y=0.53+0.65x$, $r=0.53$, $p<0.003$

TCCs and normal mucosae were 1.29 ± 0.63 and 1.26 ± 0.68, respectively, and showed no significant difference between the 2 groups. Compared among the TCCs categorized in terms of stage, grade or growth pattern, expression levels of flt-1 gene yielded no significant difference in each category (Table 1).

The gene expressions of VEGF and flt-1 in the TCCs were analyzed by linear regression. As shown in Fig. 4, the higher the expression level of the VEGF gene was, the more the flt-1 gene was expressed. The flt-1 gene expression was thus significantly correlated with the VEGF gene expression ($r = 0.53$, $p < 0.003$) (Fig. 4).

Discussion

In this study, we found that the VEGF gene was expressed in all of the bladder samples but not at all in the WBCs from the patients and the HUVEC-1 cells. Eight (40.0%) of 20 TCCs overexpressed the VEGF gene with a T/N ratio of $\geqq 3$. In addition, immunohistochemical analysis revealed that positive staining for VEGF was found in cytoplasm of the tumor cells and very weakly on the adjacent endothelial cells and the vascular smooth muscle cells, but not on the normal urothelial cells, as indicated in our previous report (Ogura et al. 1998). These results suggest that the tumor cells are a major producer of VEGF in TCCs.

O'Brien et al. (1995) reported that an average expression level of the VEGF gene in superficial TCCs of the bladder was 4 times greater than that in invasive TCC and 10 times greater than that in normal tissue. However, our study demonstrated that invasive TCCs expressed more VEGF gene than superficial TCCs. There, thus, was a controversy in the status of the VEGF gene expression between the two studies, though both studies recognized higher expression level of the VEGF gene in the tumor tissue than in the normal. Superficial TCCs are usually protruding intraluminally and can be obtained by TUR without contamination of surrounding normal mucosa and smooth muscle. On the other hand, for invasive TCCs, it is difficult to obtain their samples without contamination of those normal tissues by TUR. The expression of VEGF gene in the smooth muscle cells and the fibroblasts has been proved to be much lower than that in the epithelial cells (Berse et al. 1992). Thus, a tissue sample contaminated with the smooth muscle possibly shows lower intensity of the VEGF gene expression than its actual intensity. Such possibility could not be ruled out in the results by O'Brien et al. (1995), because they obtained all samples by TUR. In the present study, we obtained normal bladder tissue and invasive TCCs from surgical specimens of TCX under direct vision in order to exclude underlying or surrounding smooth muscle layer. We thus believe that our results reflected the actual intensity of the VEGF gene expression of each tissue sample.

It has been generally accepted that after a solid tumor has attained a small size of a few millimeters in diameter (about 10^6 cells), further expansion of the tumor-cell population requires the induction of new capillary blood vessels (Folk-

man 1990). VEGF may be up-regulated and play an important role in such a hypoxic condition to induce the new vessels (Plate et al. 1992). VEGF also acts on endothelial cells to increase capillary permeability, resulting in the extravasation of plasma protein into the perivascular space where the tumor cells migrate to create infiltrating foci (Senger et al. 1983; Brown et al. 1993). In addition, the VEGF gene is up-regulated under pathological conditions other than hypoxia such as EGF overexpression (Goldman et al. 1993), mutation of *ras* oncogene (Rak et al. 1995) and genomic instability associated with *p53* mutation (Kieser et al. 1994). These pathological conditions are frequently found in invasive TCCs but not in superficial TCCs (Wright et al. 1991). In our study, the expression level of the VEGF gene correlated with the tumor stage, but not with the grade and the growth pattern of the TCCs. Crew et al. (1997) reported that VEGF was associated with progression to a more invasive phenotype and with a earlier relapse. Thus, the expression level of the VEGF gene well reflects the invasiveness of the tumor and may be a marker of malignant potential.

VEGF receptors, flt-1 as well as KDR, have been found on the vascular endothelial cells in the bladder tissue (Brown et al. 1993), but no quantitative study has been done. The present study revealed that all of the TCCs and the normal mucosae expressed the flt-1 gene. The flt-1 gene was highly expressed in HUVEC-1 vascular endothelial cells, but not at all in peripheral WBCs. These findings imply that the flt-1 gene detected in the tumor samples possibly came from the endothelial cells in the tumor stroma, reflecting the angiogenic activity in the tissue. The expression level of the flt-1 gene of the TCCs was neither higher than that of the normal mucosae, nor correlated with the pathological parameters such as the stage, the grade and the growth pattern, suggesting poor angiogenesis in this tumor. This is in line with the pathological findings that the TCC is less vascularized than the renal cell carcinoma and the breast carcinoma in which VEGF receptor genes are frequently overexpressed (Sato et al. 1994; Takahashi et al. 1994; Toi et al. 1996). However, the expression level of flt-1 gene was significantly correlated with that of the VEGF gene in the tumor. Thus these results suggest that a paracrine system exists between the TCC cells and the adjacent endothelial cells, by which VEGF interacts with flt-1, and regulates the angiogenesis in the TCC.

We could not find any difference in expression level of VEGF and flt-1 genes between papillary and nodular tumors. Growth pattern of bladder tumor provides clinically important information, i.e., nodular tumor is more aggressive than papillary tumor. From the histopathological view point, the papillary tumor grows around a thin vascular stalk to make a tumor frond, while the nodular tumor grows in a network of capillary blood vessels. In other words, the growth pattern may be attributed to the difference of vascular architecture among the tumor tissues. Thus, VEGF and its receptors seem, at least in part, to contribute to the difference in the tumor architecture, although our study failed to demon-

strate it. Experimental evidence suggested that bFGF acts synergistically with VEGF in angiogenesis (Goto et al. 1993). Expression of bFGF has been demonstrated in the bladder tumor (Jouanneau et al. 1997). Thus, bFGF could be involved in this process together with VEGF. Campbell et al. (1998) reported that secretion of thrombospondin-1, one of antiangiogenic factor, was reduced in bladder carcinoma when compared to normal mucosa, and suggested that this factor plays a important role in the switching from a poorly angiogenic phenotype to an angiogenic phenotype. To clarify the molecular mechanism responsible for a particular vascular architecture, further investigations, including other angiogenic factors and antiangiogenic factors, are needed.

Our study has demonstrated that the VEGF gene is frequently overexpressed in transitional cell carcinoma of the bladder, especially in muscle invasive tumors, and that a paracrine system exists between the TCC cells and the adjacent endothelial cells, by which VEGF interacts with flt-1, for regulating the angiogenesis in this tumor.

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