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Markedly Increased Amounts of Messenger RNAs for Vascular Endothelial Growth Factor and Placenta Growth Factor in Renal Cell Carcinoma Associated with Angiogenesis[1]

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ABSTRACT

The presence of mRNAs for vascular endothelial growth factor (VEGF) and a VEGF-related protein, placenta growth factor (PlGF) was examined in 29 cases of renal cell carcinoma tissues and adjacent normal kidney tissues and in 4 human renal cell carcinoma cell lines. Northern blot analysis showed that 26 of 27 hypervascular renal cell carcinoma tissues (96%) exhibited a markedly elevated level (3–13 fold) of VEGF mRNA compared to the adjacent normal kidney tissues. Even tumors of small size, whenever they were hypervascular, overexpressed VEGF mRNA. We also demonstrated that mRNA for PlGF was expressed in 21 of 23 hypervascular renal cell carcinoma tissues (91%) but was not detected in the adjacent normal kidney tissues. Two hypovascular carcinoma tissues neither overexpressed VEGF mRNA nor had PlGF mRNA. VEGF mRNA was detected in four human renal cell carcinoma cell lines, while PlGF mRNA was not. There was no difference in the level of basic fibroblast growth factor mRNA between tumor tissues and normal kidney tissue, although our previous study demonstrated elevated basic fibroblast growth factor protein in the serum of renal cell carcinoma patients (K. Fujimoto *et al.*, Biochem. Biophys. Res. Commun., *180:* 386–392, 1991). Taken together, these results suggest that VEGF, PlGF, and basic fibroblast growth factor are cooperatively working to increase the angiogenesis in renal cell carcinoma *in vivo*.

INTRODUCTION

Many observations show that angiogenesis plays an important role in the growth, progression, and metastasis of solid tumor (1–3). Several angiogenic factors have been identified including acidic fibroblast growth factor (4), bFGF (4),[4] epidermal growth factor (4), transforming growth factor α and β (4), angiogenin (4), tumor necrosis factor-α (4), hst-1[5] and hst-2/FGF-6 (5). A notable feature of most renal cell carcinomas is hypervascularity. Therefore, renal cell carcinoma cells may produce angiogenic factors. We have reported previously that only bFGF is expressed in renal cell carcinoma tissues among the heparin-binding growth factor family including acidic fibroblast growth factor, bFGF, int-2, hst-1, FGF-5, hst-2/FGF-6, and keratinocyte growth factor and that the level of bFGF protein in serum is elevated significantly in more than one-half of renal cell carcinoma patients (6). In four of six cases, bFGF mRNA is expressed 2- or 3-fold higher in renal cell carcinoma tissues than in surrounding normal tissues (7). These findings suggest that bFGF is one of the factors involved in the angiogenesis of renal cell carcinoma.

Recently, a new family of two secreted angiogenic factors, both structurally related to PDGF, was identified. The first is VEGF, also known as vascular permeability factor, which has been purified from various types of cells (8–10). VEGF stimulates endothelial cell proliferation *in vitro* and has angiogenic activity *in vivo* (8–10). The VEGF gene is encoded in a disulfide-linked dimeric glycoprotein. By alternative splicing of mRNA, four different molecular species with 121, 165, 189, and 206 amino acids are determined (11, 12). VEGF has some features different from bFGF: (*a*) VEGF is a specific mitogen for endothelial cells; (*b*) VEGF, especially two shorter forms ($VEGF_{121}$ and $VEGF_{165}$) are secreted proteins, although two longer forms ($VEGF_{189}$ and $VEGF_{206}$) are bound to heparin-containing proteoglycans in the cell surface or in the basement membrane (13); (*c*) VEGF/vascular permeability factor induces vascular permeability which may result in extravasation of protein such as fibrinogen to form an extravascular fibrin gel that provides an optimal substratum for both tumor and endothelial cell growth. VEGF mRNA is recently reported to be overexpressed in glioblastoma cells of highly vascularized lesions, suggesting that VEGF is another candidate for tumor angiogenic factors (14, 15).

The second secreted angiogenic factor is PlGF, which was isolated from human placenta and choriocarcinoma cells (16, 17). It has been demonstrated that PlGF, like VEGF, is a secreted, dimeric, glycosylated protein which can stimulate the growth of endothelial cells *in vitro*. PlGF has 53% homology with VEGF in the cysteine-rich domain of 94 amino acids, which contains the PDGF-like domain of VEGF. The role of PlGF in tumor angiogenesis has not been studied.

These properties of VEGF and PlGF prompted us to investigate whether these factors may also be responsible for angiogenesis of renal cell carcinoma. We report here the results of studies on the expression of VEGF, PlGF, and bFGF genes in human renal cell carcinoma tissue, surrounding normal kidney tissue, and human renal cell carcinoma cell lines.

MATERIALS AND METHODS

Cell Lines and Tissue Samples. Four human renal cell carcinoma cell lines (SMKT-R-1, SMKT-R-2, SMKT-R-3, and SMKT-R-4) were maintained in a minimal essential medium with D-valine modification medium supplemented with 10% fetal calf serum at 37°C under a humidified atmosphere of 5% CO_2 (18, 19). Human glioblastoma mulitiforme cell line, U-251MG, was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Tumor and adjacent normal kidney tissues were obtained after resection from 29 patients who had undergone surgery at the National Cancer Center Hospital, Tokyo. All of the tissues were frozen quickly and stored at −80°C until used. All the tumor specimens were staged and graded histologically based on TNM Classification of Malignant Tumors by Union International Contre Cancer (20).

Northern Blot Analysis and RT-PCR. Total RNA was extracted from the tissues and cell lines using ISOGEN kit (Nippon Gene, Toyama, Japan) according to the recommended procedure by the supplier. Poly(A)$^+$ RNA from the U-251MG cell was obtained by oligodeoxythymidylate cellulose column

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[4] The abbreviations used are: bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor; PlGF, placenta growth factor; RT-PCR, reverse transcription polymerase chain reaction; poly(A)$^+$, polyadenylated; cDNA, complementary DNA.

[5] T. Yoshida, unpublished data.

chromatography (21). Poly(A)$^+$ RNA from the human placenta was purchased from Clontech (Palo Alto, CA). For Northern blot analysis, RNA samples were electrophoresed through 1.0% agarose-formaldehyde gel and transferred to the NitroPlus membrane (Micron Separations, Inc., Westboro, MA) as described by Sambrook *et al.* (21). Hybridization was carried out in 50% formamide, 5X standard saline citrate (1X standard saline citrate = 0.15 M NaCl, 0.015 M sodium citrate), 5X Denhard's solution, 5 mM EDTA, 0.1% sodium dodecyl sulfate, 10% dextran sulfate, and 100 μg/ml denatured salmon sperm DNA at 42°C for 14–16 h. The filters were washed twice with 0.1X standard saline citrate and 0.1% sodium dodecyl sulfate at room temperature for 10 min each and then washed twice at 65°C for 30 min each. All DNA probes were labeled with [α-^{32}p]dCTP (Amersham, Tokyo, Japan) using a random primer labeling system (Boehringer-Mannheim, Mannheim, Germany). After hybridization, all blots were exposed to Kodak XAR film with an intensifying screen at −80°C. The relative expression level was determined using the Bio-image-Analyzer (BAS2000; Fujix, Kanagawa, Japan). cDNA probes specific for VEGF and PlGF were obtained by the following method. Randomly primed cDNAs were synthesized from 0.1 μg of poly(A)$^+$ RNA of U-251MG for VEGF and human placenta for PlGF as a template, respectively. Then PCR was carried out with the following primers: forward primer 5′-TCGGGCCTCCGAAACCATGA-3′ and reverse primer 5′-CCTGGTGAGAGATCTGGTTC-3′ for VEGF (22); forward primer 5′-ATGCCGGTCATGAGGCTGTT-3′ and reverse primer 5′-GGTAATAAATACACGAGCCG-3′ for PlGF (16) at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles. The PCR product with expected size was purified, cloned into LamdaZAPII (Stratagene, La Jolla, CA), and then confirmed with sequencing by the dideoxynucleotide chain termination method using a 7-diaza GTP Sequenase version 2.0 kit (United States Biochemicals, Cleveland, OH). bFGF cDNA (23) was kindly provided by Takara Chemical Industry. The rat β-actin cDNA we originally cloned was used as an internal control probe. For human renal cell carcinoma cell lines, RT-PCR was performed under the same conditions as described above.

RESULTS

Expression of VEGF mRNA, PlGF mRNA, and bFGF mRNA in Renal Cell Carcinoma Tissues and Normal Kidney Tissues. The expression of VEGF mRNA was examined in renal cell carcinoma tissues and the adjacent normal kidney tissues from 29 patients.

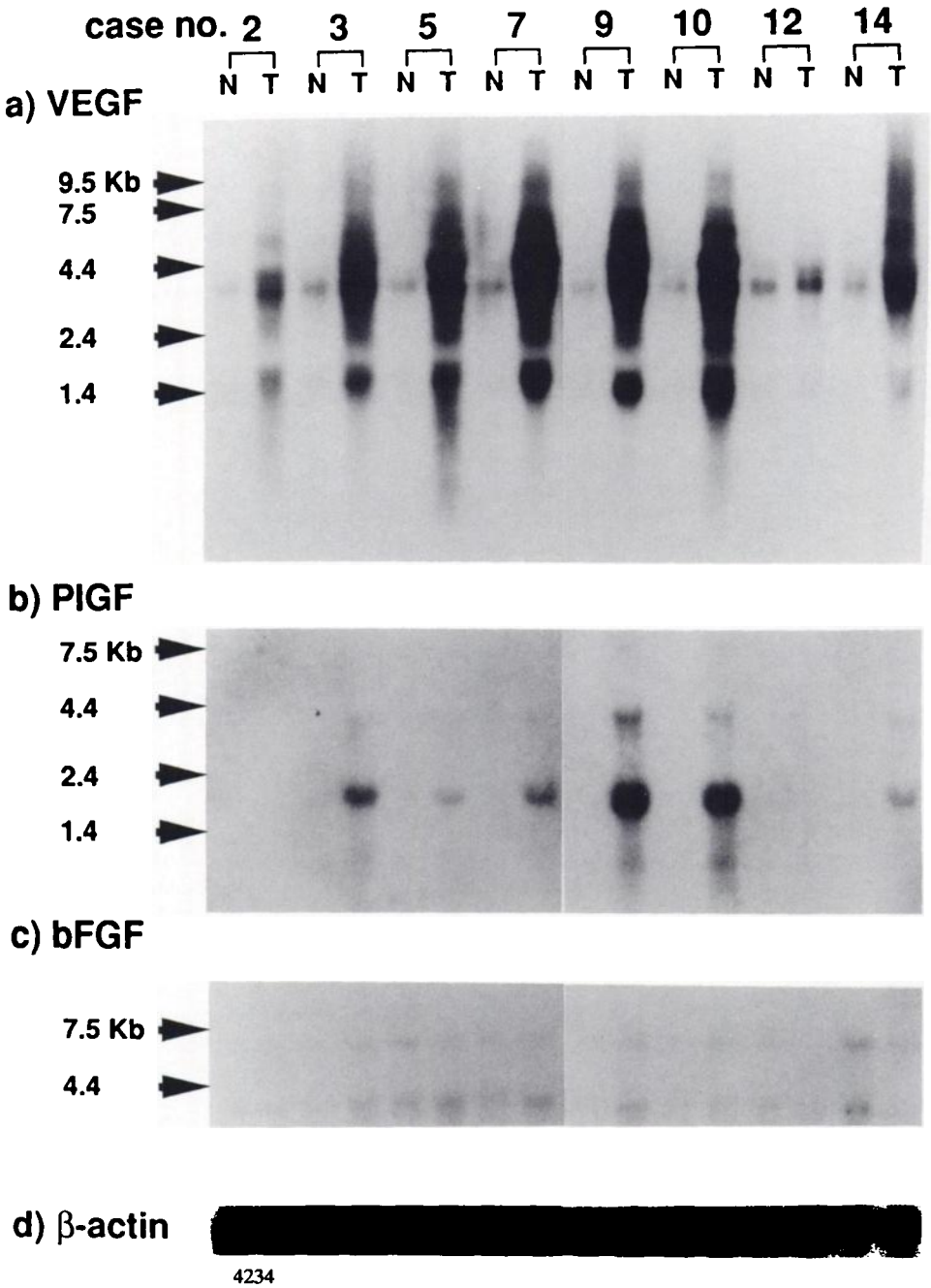

Fig. 1. Northern blot analysis of VEGF, PlGF, and bFGF mRNA in surgical specimens of renal cell carcinoma tissues. Total RNA from renal cell carcinoma tissues (*T*) and adjacent normal kidney tissues (*N*) were extracted, and 20 μg of total RNA in each lane were electrophoresed, transferred to nitrocellulose filters, hybridized with the ^{32}P-labeled 662 base pairs of $VEGF_{165}$ fragment (*a*), 565 base pairs of $PlGF_{170}$ fragment (*b*), bFGF probe of 450 base pairs *Bgl*II-*Eco*RI fragment of pTB669 (*c*), and β-actin probe (*d*), respectively. Case numbers, corresponding to those in Table 1, are given at the *top* of the blots. The size of the transcripts were estimated by coelectrophoresis of 0.24–9.5 Kb RNA Ladder (GIBCO BRL, Gaithersburg, MD).

Representative results of Northern blot analysis are shown in Fig. 1*a*. Two major transcripts of 4.3 and 3.9 kilobases, corresponding to the VEGF transcripts reported previously (15, 22), were observed in both normal and tumor tissues. Although RNAs from tumor tissues showed a smear on Northern blots, the shorter exposure time revealed discrete bands corresponding to 4.3 and 3.9 kilobases. In addition, several other bands of 6.0 and 1.6 kilobases were also detected after a prolonged exposure of the film. By estimations of the VEGF mRNA level in both normal and tumor tissues, we observed a substantially elevated level (3–13 fold) of VEGF mRNA in 26 of 27 (96%) renal cell carcinoma tissues which demonstrated hypervascularity in the angiography and/or computed tomography (Table 1). In two cases (cases 11 and 19) having hypovascular findings, the level of VEGF mRNA in the tumor was equivalent or lower than in the matched normal kidney (Table 1). Overexpression of the VEGF gene in tumor tissues was associated with hypervascularity of renal cell carcinoma except in one case (case 20). Even tumors of small size overexpressed VEGF mRNA.

A Northern blot analysis was also performed using PlGF cDNA probe to demonstrate PlGF mRNA expression. Variable levels of PlGF transcript of 1.7-kilobases (17) were detected in 21 of 23 (91%) hypervascular renal cell carcinoma tissues examined (Fig. 1*b*). The band corresponding to approximately 4.2 kilobases was also seen in tumor tissues. Two tumor samples (cases 2 and 20) with no expression of PlGF mRNA contained VEGF mRNA, although comparatively lower than those of other tumor tissues (tumor:normal ratio, 3.8 and 1.8, respectively; Table 1). On the other hand, in neither of two hypovascular renal cell carcinoma tissues (cases 11 and 19) could PlGF mRNA be detected. Although small amounts of VEGF mRNA were detected in all adjacent normal kidney tissues, none of them had PlGF mRNA.

The same RNA samples from human renal tumors and normal kidneys were examined for the presence of bFGF mRNA. As shown in Fig. 1*c*, two major transcripts of 7.0 and 3.7 kilobases corresponding to the bFGF transcripts reported previously (24), were detected in both tissues. However, the amounts of bFGF mRNA were small in both tumor tissues and the adjacent normal tissues.

Expression of VEGF mRNA and PlGF mRNA in Human Renal Cell Carcinoma Cell Lines. Primary renal cell carcinoma tissue is known to contain a large number of mesenchymal portions including tumor infiltrating lymphocyte and macrophage. Therefore, four human renal cell carcinoma cell lines were examined for the presence of VEGF mRNA. As shown in Fig. 2, the two major transcripts as those shown in surgical specimens were detected in all the cell lines, SMKT- R-1, SMKT-R-2, SMKT-R-3, and SMKT-R-4. All of the cell lines had expressed VEGF mRNA at a level that was comparable to or higher than a glioblastoma mulitiforme cell line, U-251MG, which was reported previously to contain high levels of VEGF mRNA (25). We performed RT-PCR to investigate the form of the VEGF mRNA. Oligonucleotide primers corresponding to the 5′ noncoding region and 3′ noncoding region of the gene were used to amplify the whole coding region of all known splicing forms of the VEGF mRNA (22). By RT-PCR analysis in all four human renal cell carcinoma cell lines as well as U-251MG, two major products (530 and 662 base pairs) corresponding to VEGF121 and VEGF165, which were known to secrete efficiently, were detected (Fig. 3). The PCR products were identified as human VEGF by sequencing analysis (data not shown). This observation indicates that mRNAs of the two shorter forms (VEGF121 and VEGF165) were predominantly expressed in human renal cell carcinoma cells. The presence of mRNAs for *flt-1* and KDR gene, encoding high affinity receptors for VEGF (26, 27), were examined in human renal cell carcinoma cell lines. Neither *flt-1* mRNA nor KDR mRNA could be detected in the four cell lines by Northern blot analysis and by RT-PCR (data not shown). The pres-

Table 1 *Summary of VEGF, PlGF, and bFGF mRNA expression in 29 renal cell carcinomas*

Case no.	Stage[a]	Grade[a]	Cell type	Tumor size (cm)	Relative expression (T:N)[b]		
					VEGF	PlGF	bFGF
1	pT3bN0M1	2	Granular	8.0	9.6	+[c]	1.4
2	pT3bN0M0	3	Clear	14.0	3.8	−	1.2
3	pT2N0M0	2	Clear	6.0	4.0	+	1.2
4	pT2N0M0	2	Clear	7.0	7.4	+	1.5
5	pT2N0M0	2	Clear	7.0	3.5	+	0.7
6	pT2N0M0	2	Mixed	9.0	9.4	+	0.8
7	pT3bN0M0	3	Clear	5.7	7.8	+	1.2
8	pT3bN0M0	3	Clear	5.0	4.2	+	1.0
9	pT3bN0M1	3	Clear	4.7	8.2	+	0.7
10	pT3bN0M1	2	Clear	7.0	13.5	+	1.3
11[d]	pT3aN0M0	3	Granular	11.2	1.0	−	0.7
12	pT2N0M0	3	Clear	4.4	9.2	+	0.6
13	pT2N0M0	2	Clear	4.5	4.3	+	0.5
14	pT3bN1M1	3	Clear	8.5	9.4	+	1.0
15	pT3aN0M0	2	Clear	3.5	5.0	+	0.9
16	pT2N0M0	2	Clear	3.5	6.9	+	1.0
17	pT3bNXM0	3	Clear	6.5	5.0	+	1.1
18	pT3bNXM0	2	Clear	6.0	5.2	+	ND[e]
19[d]	pT3bNXM0	3	Spindle	9.5	0.4	−	ND
20	pT4NXM1	2	Clear	5.0	1.8	−	ND
21	pT2N0M0	2	Clear	6.0	12.8	+	ND
22	pT3bN0M0	3	Granular	12.5	8.9	ND	0.9
23	pT2NXM1	2	Mixed	11.0	8.9	ND	1.0
24	pT2N0M0	2	Clear	7.0	5.9	ND	1.0
25	pT3aN0M0	1	Clear	6.2	4.9	ND	0.9
26	pT1N0M0	1	Clear	2.4	12.5	+	1.2
27	pT2N0M0	2	Clear	5.5	4.9	+	1.0
28	pT2N0M0	2	Clear	7.0	4.7	+	1.1
29	pT2N0M0	1	Clear	7.0	4.0	+	1.4

[a] Pathological stage and grade were determined according to TNM Classification of Malignant Tumors by Union International Contre Cancer.
[b] Relative amounts (T:N) were determined by the ratio of the intensities of the bands for the tumors (T) to those for adjacent normal kidneys (N) and then was corrected for those with β-actin mRNAs.
[c] +, mRNA is only detectable in tumor tissue; −, no detectable mRNA in tumor tissue and adjacent normal kidney tissue.
[d] The angiographic study showed a hypovascular finding.
[e] ND, not determined.

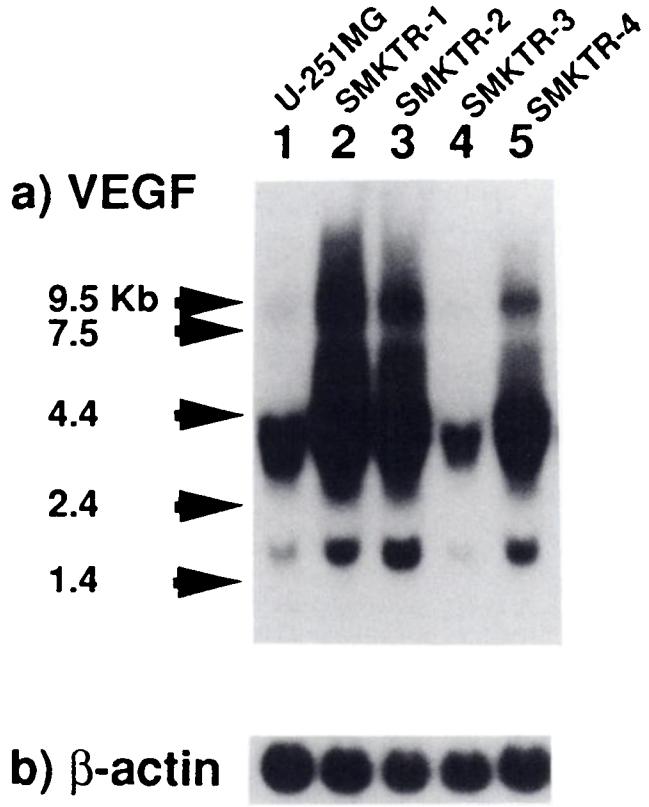

Fig. 2. Northern blot analysis for VEGF mRNAs in renal cell carcinoma cell line. Twenty μg of total RNA were used in each lane and were hybridized with VEGF probe (*a*) or β-actin probe (*b*). *Lane 1,* U-251MG; *Lane 2,* SMKT-R-1; *Lane 3,* SMKT-R-2; *Lane 4,* SMKT-R-3; *Lane 5,* SMKT-R-4. The size of the transcripts were estimated by coelectrophoresis of 0.24–9.5 Kb RNA Ladder (GIBCO BRL, Gaithersburg, MD).

ence of PlGF mRNA in four human renal cell carcinoma cell lines was examined by Northern blot and RT-PCR analyses. Only by analysis on RT-PCR products of 1 μg of total RNAs were faint bands of 501 and 565 base pairs corresponding to the mRNA encoding $PlGF_{149}$ and $PlGF_{170}$ detected, which were generated by alternative splicing of the PlGF gene (17). By Northern blot analysis on 20 μg of total RNAs, the PlGF mRNA was not detected in all of the cell lines, even after exposure for 1 week (data not shown).

DISCUSSION

One of the characteristics of renal cell carcinoma is its hypervascularity. In the present studies, we examined the presence of VEGF mRNA and PlGF mRNA in renal cell carcinoma tissues in order to investigate whether these recently identified angiogenic factors were responsible for the hypervascularity of renal cell carcinoma. To our knowledge, there has been no report on the presence of VEGF mRNA in a significant number of human renal cell carcinomas except one report in a small set of specimens. Berse *et al.* (28) reported that a high level of VEGF mRNA was observed in one of two renal cell carcinomas. We demonstrated here that the VEGF mRNA levels in the carcinoma tissues were significantly much higher than those in adjacent normal tissues in 26 of 27 (96%) of hypervascular renal cell carcinomas including small-sized tumors. The other two hypovascular renal cell carcinomas did not have increased amounts of VEGF mRNA. These results suggest that elevated VEGF expression is involved in the hypervascularity of renal cell carcinoma. A consistent correlation was not found between the level of VEGF mRNA and the tumor size, the stage, or grade of carcinoma. It is most likely that renal cell carcinoma cells themselves express high levels of VEGF mRNAs, since all the four human renal cell carcinoma cell lines contained large amounts of VEGF mRNA.

Despite the fact that VEGF is known to be a specific mitogen for endothelial cells, VEGF receptors, *flt-1,* and KDR proteins (26, 27) have been observed on not only endothelial cells but also on nonvascular endothelial cells (29). It was demonstrated that, in human melanoma cells but not normal melanocytes, VEGF receptors are expressed, suggesting the possibility that aberrant expression of VEGF receptors may be involved in malignant transformation (30). The fact that we failed to detect *flt-1* and KDR mRNA in human renal cell carcinoma cell lines suggested that VEGF might not participate in the development of renal cell carcinoma by generating autocrine loops.

The mRNA for a recently identified angiogenic factor PlGF was reported to be abundantly expressed in placenta tissue but very weakly or not expressed among other tissues including kidney tissue (17). Moreover, it was reported that the PlGF mRNA was detected in the hepatoma cell line and the cervical carcinoma cell line (17). But an investigation in tumor tissues has not yet been performed. We found PlGF mRNA expression in 21 of 23 (91%) hypervascular renal cell carcinoma tissues. Two hypovascular carcinomas which did not contain elevated levels of VEGF mRNAs also did not have detectable levels of PlGF mRNAs. In contrast to VEGF, PlGF mRNAs could not be detected in all of the four renal cell carcinoma cell lines by Northern blot analysis. These findings suggest that PlGF as well as VEGF is involved in the angiogenesis of renal cell carcinoma, but PlGF is not excreted from the carcinoma cells themselves, whereas VEGF is. Alternatively, PlGF gene expression is shut down when the cells are cultured *in vitro*. Further studies are required to demonstrate

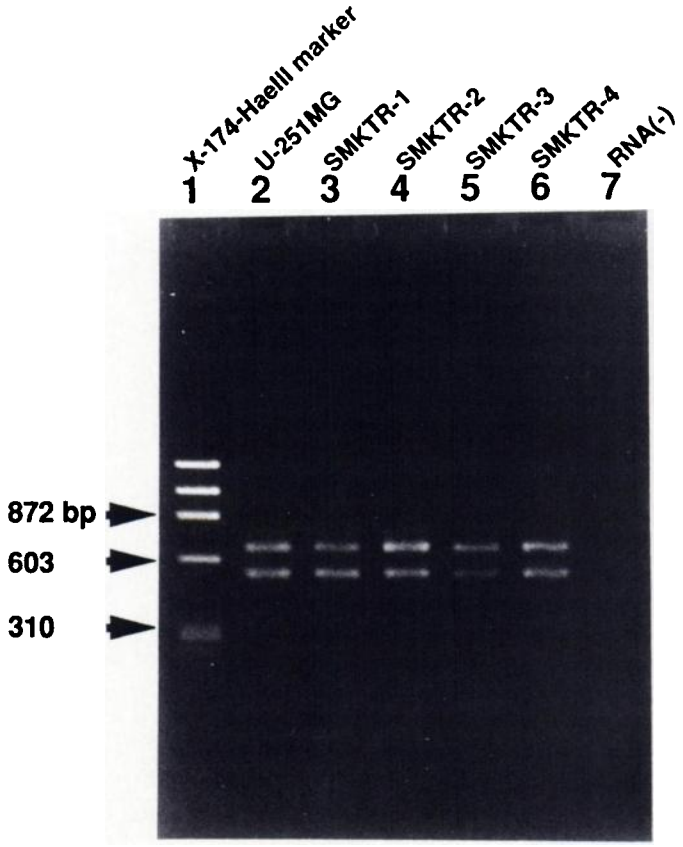

Fig. 3. RT-PCR analysis of VEGF transcript in renal cell carcinoma cell lines. cDNA synthesized from 1 μg of total RNA was amplified by PCR and analyzed on 1.5% agarose gel. *Lane 1,* x-174-*Hae*III digest marker fragment; *Lane 2,* U-251MG; *Lane 3,* SMKT-R-1; *Lane 4,* SMKT-R-2; *Lane 5,* SMKT-R-3; *Lane 6,* SMKT-R-4; *Lane 7,* RT-PCR was carried out in the absence of RNA.

the origins of the cells for the presence of VEGF and PlGF in renal cell carcinoma *in vivo*.

An immunohistochemical study revealed strong staining for VEGF and bFGF within the same populations of cells in human glioma tissues (31). Additionally, Pepper *et al.* (32) and Goto *et al.* (33) demonstrated a potent synergism between VEGF and bFGF in the induction of angiogenesis *in vitro*. These findings suggest that VEGF may act in conjunction with bFGF, or it may complement the action of bFGF. Our earlier results showed that bFGF is elevated highly in serum in over 50% of renal cell carcinoma patients (6). Therefore, we also examined the expression of bFGF mRNA in the same specimens. Elevated expression of bFGF mRNA in tumor tissues was not observed. Kandel *et al.* (34) demonstrated that, in a transgenic mouse model of multistep tumorigenesis, a switch from cell-associated to exported bFGF correlates neovascularization *in vivo* and tumorigenesis, despite no difference in the mRNA expression level between normal fibroblast and fibrosarcoma. Therefore, bFGF may be secreted efficiently in carcinoma tissues.

These results suggest that VEGF, PlGF, and bFGF are cooperatively working to increase the angiogenesis in renal cell carcinoma and indicate the possibility that VEGF and PlGF can be used as a tumor marker for renal cell carcinoma, especially in the early stage of the disease. We are currently investigating whether VEGF is detected in the serum of renal cell carcinoma patients.

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