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INCREASED SERUM LEVELS OF BASIC FIBROBLAST GROWTH FACTOR IN PATIENTS WITH RENAL CELL CARCINOMA

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Summary The serum level and urinary output of basic and acidic fibroblast growth factors (FGFs) were measured by sandwich enzyme immunoassay (EIA) in patients with renal cell carcinoma. In over fifty percent (16/31) of renal cell carcinoma patients, basic FGF was elevated (>30 pg/ml) in their sera. There is relatively good correlation between serum levels of basic FGF and tumor stage or grade, while urinary daily output of basic FGF did not correlate with increased malignancy. The present results indicate that serum basic FGF level of patients with renal cell carcinoma is a useful diagnostic and prognostic marker for renal cell carcinoma. On the other hand, acidic FGF was not detectable in all sera and urine.

Recent developments in diagnostic methods, using ultrasonography, computed tomography scanning and magnetic resonance imaging, make it possible to detect a relatively small tumor in the kidney. However, there have been no available diagnostic or prognostic serum tumor markers for renal cell carcinoma. One of the characteristics of renal cell carcinoma is hypervascularity. It is likely that renal cell carcinoma produces an angiogenic growth factor which is necessary for this malignant tumor to exhibit aggressiveness including local invasion and distant

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Abbreviations: FGF, fibroblast growth factor; KGF, keratinocyte growth factor; HRP, horseradish peroxidase; EIA, enzyme immunoassay.

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metastases. It was also reported that malignant conversion of benign tumors was accompanied by angiogenesis (1,2). Heparin-binding growth factors include acidic fibroblast growth factor (FGF) and basic FGF, *int-2* (3), *hst-1* (4,5), FGF-5 (6), *hst-2*/FGF-6 (7,8), and keratinocyte growth factor (KGF) (9). These heparin-binding growth factors are likely to play important roles in angiogenesis (10). It was also reported that a heparin-binding growth factor, similar to basic FGF in molecular weight, and in mitogenic and angiogenic activity, was isolated and purified from renal cell carcinoma (11). Another study showed increased level of basic FGF-like activity in urine derived from patients with renal cell carcinoma (12).

In this study, we measured the basic and acidic FGFs levels in serum and urine of patients with renal cell carcinoma by a newly established enzyme immunoassay (EIA) system and investigated whether basic FGF could be a novel marker for renal cell carcinoma.

MATERIALS AND METHODS

Northern blot hybridization analysis Renal cell carcinomas and the corresponding normal tissues were obtained at the time of surgery. RNA was extracted, and poly(A)$^+$RNA was prepared as described (13). Northern blot hybridization was performed as described previously (13) using probes for basic FGF (14), acidic FGF (unpublished data), *int-2* (3), *hst-1* (5), FGF-5 (6), *hst-2* /FGF-6 (unpublished data) and KGF (9).

Antibodies The characteristics of antibodies used in the experiments were described previously in detail (15,16). MAb52, MAb98 and MAb3H3 were used as monoclonal antibodies against basic FGF. MAb52 and MAb98 were used to coat the wells of microtiter plates, whereas Fab' fragments of MAb3H3 were labeled with HRP (horseradish peroxidase) (15). These two antibodies for well-coating recognize different sites of human basic FGF. Recently it has been demonstrated that MAb3H3 can neutralize the biological activity of human basic FGF. A mixture of AF1-81, AF1-114 and IC10 was employed as solid phase antibodies. These three antibodies recognize different epitopes of human acidic FGF. AF1-52 was processed with HRP as a labeled antibody (16). None of the four antibodies could neutralize the biological activity of human acidic FGF.

Serum and Urine The blood of patients with renal cell carcinoma was obtained before breakfast and the serum was collected by centrifugation of clotted blood for 10 minutes at 1100g and stored at -80°C. The urine was collected and stored at -80°C after centrifugation for 10 minutes at 650g to remove debris or blood cells.

Enzyme Immunoassay (EIA) Acidic and basic FGFs were measured by the two-site sandwich EIA technique using antibodies for coating and labeling with HRP. These EIA systems are applied because of the ability to detect a very small quantity of both FGFs, specifically and sensitively, without intervention by heparin. The biological activity of basic FGF was not inactivated in its assay. The system of this assay is described as follows. A mixture of two antibodies, MAb52 and MAb98, was used for coating wells of microtiter plates. This mixture was adjusted at 10μg/ml in 0.1M carbonate buffer (pH9.6). 100μl of the mixture was incubated in the wells of a 96-well microtiter plate at 4°C overnight. The plate was washed in PBS (0.02M phosphate buffer, pH7.2 with 0.15M NaCl) and each well was incubated with 300μl of Buffer A (PBS containing 25% Block Ace; Snow Brand Milk Products Co., Japan) at 4°C overnight. After being washed in PBS, 100μl of each sample, 3-fold diluted in equal volume of Buffer B (Buffer A with 100μg/ml heparin) and equal

volume of Buffer C (Buffer A containing 1.5M NaCl, 100μg/ml heparin and 30μg/ml mouse IgG) was added to each well. After incubation for 24 hours at 4°C, the plate was washed in PBS. 100μl of MAb3H3 labeled with HRP, 200-fold diluted in Buffer A with 10μg/ml mouse IgG, was added to each well and incubated in the wells for two hours at 25°C. Then the plate was washed and the bound peroxidase activities were measured with o-phenylenediamine. Acidic FGF in serum or urine was also measured in similar procedures as described for basic FGF. In these enzyme immunoassay systems, the detection limit of basic FGF in serum or urine was 30pg/ml and that of acidic FGF was 75pg/ml.

Others All the serum and urine samples examined were obtained from patients who had neither chemotherapy nor radiation therapy prior to collecting these materials, and were diagnosed pathologically as renal cell carcinoma later. All the resected tumor specimens were staged and graded histologically by two pathologists based on TNM Classification of Malignant Tumours by UICC (17) with a minor modification (18).

RESULTS AND DISCUSSION

Northern blot analyses of mRNA from several renal cell carcinomas were performed with basic FGF, acidic FGF, *int-2*, *hst-1*, FGF-5, *hst-2*/FGF-6 and KGF probes. Only basic FGF probe gave a clear signal of bands (data not shown). Based on these experimental results, we measured basic FGF levels in sera of 31 patients with renal cell carcinoma. In addition, acidic FGF levels in sera from these patients were measured. The concentration of serum basic FGF was elevated significantly over 30pg/ml within the range from 41pg/ml to 540pg/ml (Fig.1) in 16 of 31 patients (52%) with renal cell carcinoma.

All the tumors were histologically classified into stages pT1, pT2, pT3 and pT4 based on the degree of the anatomical extension of the primary tumor. Tumors in pT1 are surrounded by renal parenchyma without enlargement of the kidney. Stage pT2 was divided into two substages, pT2a and pT2b (18). Although tumors in pT2a are confined to renal parenchyma with evidence of a large tumor with deformity and/or enlargement of the kidney or calyceal or pelvic involvement, the continuity of the parenchyma is preserved. Tumors in pT2b lose the continuity of the subcapsular parenchyma with intact renal capsule. In pT3, the renal capsule is broken through and perinephric fat, peri-pelvic fat and hilar renal vessels are involved, but Gerota's fascia is intact. Tumors in pT4 extend into neighboring organs and peritoneum. Among the patients tested for the serum basic FGF levels, we had no patients in stage pT1. Although in two pT2a patients the serum basic FGF levels were less than 30pg/ml, five (38%) of thirteen pT2b patients showed increased levels of basic FGF in serum. Eleven (73%) of fifteen patients with advanced stage pT3 carcinoma showed increased levels of serum basic FGF (Table 1). The remaining one pT4 carcinoma did not show increased serum level of basic FGF. The level of serum basic FGF in patients with renal cell carcinoma in pT3 was much higher than in patients with renal cell carcinoma in pT2 (Fig. 1). This difference in the level of serum basic FGF between pT2 and pT3 might be

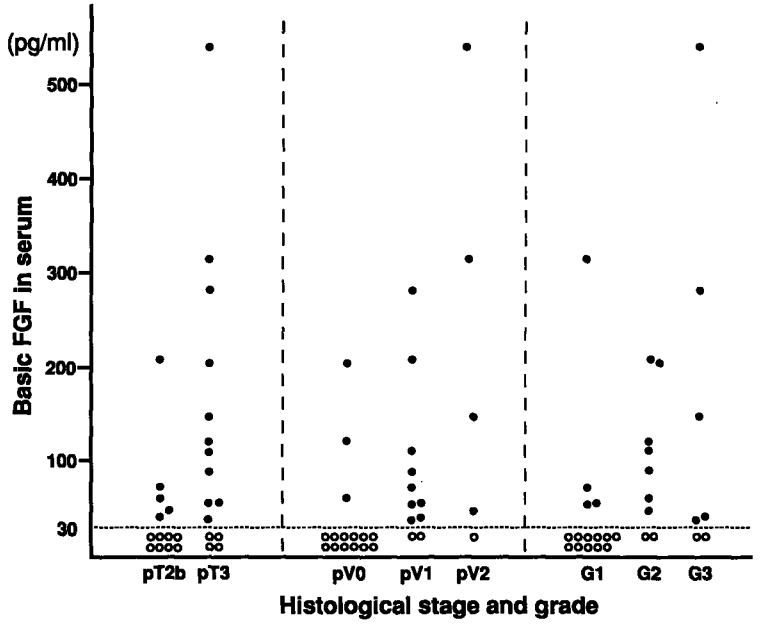

Figure 1. **Increased serum levels of basic FGF in patients with renal cell carcinoma of various stages and grades.** Patients with renal cell carcinoma were classified according to the histological stage (T categories and V categories) and grade. Closed circles in each column indicate the concentration of serum basic FGF in each patient with renal cell carcinoma of various stages and grades. Open circles represent patients with serum levels of basic FGF being less than 30pg/ml. The definition of each stage and grade is described in the text.

Table 1. Serum Basic FGF and Histological Stage and Grade in Patients with Renal Cell Carcinoma

Histological diagnosis[a]			No. of positive patients / No. of total patients	
Stage	pT2	pT2a	0/2	(0%)
		pT2b	5/13	(38%)
	pT3		11/15	(73%)
	pVo		3/15	(20%)
	pV1		9/11	(82%)
	pV2		4/5	(80%)
Grade	G1		4/15	(27%)
	G2		7/9	(78%)
	G3		5/7	(71%)

a) The definition of each classification of stage and grade is described in the text.

dependent on the tumor size, and on aggressiveness of the advanced tumor invading out of the renal capsule. Moreover, all of three patients with lymph node metastasis and three of four patients with distant metastasis showed highly increased levels of serum basic FGF.

The degree of invasion of renal cell carcinoma to renal vein or vena cava is one of the important factors to predict the prognosis of the patients. The increased levels of serum basic FGF were found in patients with a high degree of venous invasion (Table 1). In patients with renal cell carcinoma without venous invasion (pV0), basic FGF was detected in only three (20%) of fifteen patients. Nine (82%) out of eleven patients with invasion to renal vein (pV1) and four (80%) out of five patients with invasion to vena cava inferior (pV2) showed increased levels of serum basic FGF, respectively. The level of basic FGF among patients with pV2 was higher than that among patients with pV0 (Fig. 1).

All the tumors were graded according to the degree of differentiation of the tumors. Grade1 (G1) and grade2 (G2) are high and medium degrees of differentiation, respectively. Grade 3 (G3) is low degree of differentiation or undifferentiation. Grade of cancer cells is also one of the important prognostic factors for patients with renal cell carcinoma. The increased level of serum basic FGF was quite often seen in patients with increased grade of renal cell carcinoma. Seven (78%) of nine patients in grade 2 and five (71%) of seven in grade 3 were found to have high levels of serum basic FGF, whereas only four (27%) of fifteen patients in grade 1 showed increased levels of serum basic FGF (Table 1, Fig. 1).

Renal cell carcinoma represents a variety of histological patterns that are composed of two subtypes, clear and granular cells (18). Among the 31 patients with renal cell carcinoma, there is no significant difference in the percent of patients with elevated serum basic FGF between clear cell type and granular cell type; the percent of clear cell type and that of granular cell type with increased levels of serum basic FGF were 47% (9/19) and 67% (4/6), respectively. The remaining six cases were mixed type and half of them showed increased levels of serum basic FGF. Renal cell carcinoma can be also classified histologically into solid, tubular, alveolar, papillary and cystic patterns (18). Six (86%) out of seven patients with solid pattern and all of four patients with tubular pattern showed increased levels of serum basic FGF. Five (36%) out of fourteen patients with alveolar pattern carcinoma showed increased levels of serum basic FGF. A patient with a solid pattern carcinoma showed a high level of serum basic FGF, 540pg/ml. Conversely, serum basic FGF levels were less than 30pg/ml in four patients with papillary carcinoma or two with cystic pattern carcinoma. It should be noted that three of five renal cell carcinomas with multiple primary lesions showed high levels of serum basic FGF. Two of three patients with multiple renal cell carcinomas could be diagnosed as the multiple form by the histological examination, but not by any of the imaging instruments. Detection of multiple renal cell carcinomas in a patient

makes it easy to select a reasonable surgical treatment, chemotherapy and/or immunotherapy.

The results presented here showed that increased levels of serum basic FGF were frequently detected in patients with renal cell carcinoma with advanced stage in the extent of growth and spread and with increased grade of malignancy.

In comparison with patients with renal cell carcinoma, all of seven patients with advanced stomach cancer showed levels of serum basic FGF less than 30pg/ml. Acidic FGF was less than 75pg/ml in all the sera of eight patients with renal cell carcinoma. Basic and acidic FGFs in urine of eight patients with renal cell carcinoma were investigated. None of these renal cell carcinoma patients showed elevated levels of both basic and acidic FGFs in urine.

Basic FGF or acidic FGF has been observed as an angiogenic factor that stimulates the proliferation of endothelial cells and induces neovascularization *in vivo* (19). In mouse embryonic kidney, FGF stimulates renal angiogenesis (20). The results of the present studies suggest that basic FGF is produced by renal cell carcinoma and might be responsible for the development of this hypervascular tumor. Alternatively, basic FGF is produced in endothelial cells as a consequence of increasesd vascularization caused by development of renal cell carcinoma. Further study is required whether the basic FGF is produced in tumor cells or in endothelial cells. It is also necessary to study other types of tumors with increased angiogenesis and malignancy. In any event, the present results showed that the serum basic FGF level of patients with renal cell carcinoma is a useful diagnostic and prognostic marker for screening and treatment for renal cell carcinoma, since there are no suitable diagnostic or prognostic tumor markers for this type of carcinoma.

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