Introduction of Basic Fibroblast Growth Factor Gene into Mouse Renal Cell Carcinoma Cell Line Enhances Its Metastatic Potential

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ABSTRACT

To clarify the role of basic fibroblast growth factor (FGF-2) in the malignant progression of renal cell carcinoma, we transfected the FGF-2 gene, which lacks the typical signal sequence, into RenCa, a mouse renal cell carcinoma cell line that does not express FGF-2 mRNA. In an in vitro tumor cell invasion assay, the FGF-2-transfected cell lines (RenCa/F) exhibited 3- to 4-fold higher invasive potential than either the parental RenCa (RenCa/P) or the vector-only transfected cell line (RenCa/C). Zymography showed a marked increase in matrix metalloproteinase 2 (MMP-2) production in the culture supernatants of RenCa/F.

Furthermore, when injected i.v. or into the renal subcapsule in syngeneic mice, metastases to the liver and mesenteric lymph nodes were observed only after the injection of RenCa/F into the renal subcapsule. In contrast, there was no significant difference in either cell proliferation in vitro or tumor growth in vivo among RenCa sublines. These results suggest that if it is overexpressed, endogenous native FGF-2 plays an important role in the invasion and metastasis of renal cell carcinoma, probably through the production of MMP-2.

INTRODUCTION

The growth of solid tumors and their ability to metastasize are, at least in part, dependent upon angiogenesis, the induction of which is thought to be mediated by angiogenic factors (1). Among these factors, FGF-2 is one of the most potent angiogenic inducers in vivo. FGF-2 also stimulates cell growth and modulates differentiation in a variety of cells.

Human renal cell carcinoma, a malignant tumor derived from the renal tubular epithelium, is characterized by hypervascularity, high frequency of metastasis, and poor prognosis. Recent evidence suggests a close relationship between FGF-2 and renal cell carcinoma. Serum FGF-2-like activity was increased in patients with renal cell carcinomas. Treatment with FGF-2 monoclonal antibody inhibited cell growth of a renal cell carcinoma cell line in vitro. The expression of FGF-2 in primary human renal cell carcinoma was significantly correlated with poor prognosis. Consistent with previous reports, our preliminary study showed that FGF-2 mRNA was detected in 11 of 11 human renal cell carcinoma cell lines.

All of these findings suggest that increased FGF-2 synthesis plays a crucial role in the malignant progression of renal cell carcinoma; however, its precise mechanism remains largely unknown. To clarify the role of FGF-2 in the malignant progression of renal cell carcinoma, we transfected FGF-2 cDNA into RenCa, a mouse renal cell carcinoma cell line that does not express FGF-2 mRNA. We then examined the malignant potential of the FGF-2-expressing RenCa cell lines both in vitro and in vivo.

MATERIALS AND METHODS

Tumor Cell Lines. RenCa, a mouse renal cell carcinoma of BALB/c origin (kindly provided by Dr. Ko Okumura, Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan), was maintained in modified Eagle’s medium supplemented with 5% fetal bovine serum.

Expression Plasmid and Transfection to Tumor Cells. The 0.8-kb cDNA fragment encoding rat FGF-2 (8) was inserted at the EcoRI site of the expression vector, pcD-SRα, which contains SRα promoter composed of the SV40 early promoter and the R-U5 segment of human T-cell leukemia virus type 1 long terminal repeat (9). The pcD-SRα/FGF-2 construction (Fig. 1) was transfected to RenCa cells by a standard calcium phosphate method (10).

In Vitro Tumor Cell Invasion Assay. Tumor cell invasion was measured in a modified Boyden chamber assay. Two micromolar of basement membrane matrigel (Becton Dickinson Labware, Lincoln Park, NJ) was coated on the upper compartment of the chamber. Briefly, we used polycarbonate filters with a pore size of 8 μm, coated with varying amounts of basement membrane matrigel (Becton Dickinson Labware, Lincoln Park, NJ). The coated filters were placed in Boyden chambers, in the upper compartment of which 1 × 10^5 cells of each cell line were seeded in each well of 12-well plates (3.8 cm^2/well), and the number of cells in each cell line was counted daily by triplicate. To examine the effect of exogenous FGF-2 on the cell proliferation of RenCa/F in vitro, we incubated the cells with 0.1, 1, 10, and 20 ng/ml of bovine FGF-2 (Wako, Osaka, Japan) for 24 h and counted the number of cells.

In Vitro Tumor Cell Invasion Assay. Tumor cell invasion was measured with a membrane invasion culture system (13) in a minor modification. Briefly, we used polycarbonate filters with a pore size of 8 μm, coated with varying amounts of basement membrane matrigel (Becton Dickinson Labware, Lincoln Park, NJ). The coated filters were placed in Boyden chambers, in the upper compartment of which 1 × 10^5 cells of each cell line were suspended in serum-free conditioned medium (DMEM/F-12) and in the lower compartment of which fibronectin (25 μg/ml) diluted with DMEM/F-12 was added as a chemotaxtractant. After a 48-h incubation at 37°C in 5% CO₂, the cells attached
study, RenCa/C1 and RenCa/C2 showed the almost same results in the following experiments; therefore, we subsequently give the data of only RenCa/C1.

Total RNA was extracted from each of the cell lines that contained parental RenCa (RenCa/P). All of the RenCa sublines were initially screened by reverse transcription-PCR. FGF-2 mRNA was detected in all of the FGF-2-transfected cell lines, but was not detected in either RenCa/P or RenCa/C1 (Fig. 2). The cell lysates and culture supernatants of RenCa sublines were then analyzed by sandwich EIA to examine the expression of FGF-2 protein. Abundant immunoreactive FGF-2 protein was detected in the cell lysates of all the FGF-2-transfected cell lines, and a little was detected in the culture supernatants. In contrast, neither the cell lysates nor the culture supernatants of RenCa/P and RenCa/C1 contained any FGF-2 protein (Table 1). Two sublines, RenCa/F5 and RenCa/F6, which expressed a large amount of FGF-2 protein in the cell lysates, were selected for further analysis.

In Vitro Studies of RenCa Sublines. There was no significant difference in cell proliferation in vitro among RenCa/P, RenCa/C1, RenCa/F5, and RenCa/F6 (Fig. 3). The doubling time of these cell lines was estimated to be 32 h. On the other hand, the doubling time of RenCa/P treated with exogenous FGF-2 at concentrations of 1, 10, and 20 ng/ml was significantly shortened to 20 h irrespective of the concentrations, but was not shortened at a concentration of 0.1 ng/ml. The effects of exogenous FGF-2 on the proliferation of RenCa/F5 and RenCa/F6 cells in vitro were similar to its effects on RenCa/P cells (data not shown).

The invasive potential of the RenCa sublines was examined with an in vitro tumor cell invasion assay. The invasive potential of RenCa/F5 and RenCa/F6 was increased approximately 3- to 4-fold compared with RenCa/P and RenCa/C1 (Table 2). MMPs also secreted by RenCa sublines were identified by zymography. Small amounts of MMP-9 (Mr 92,000 gelatinase) were secreted by all the RenCa sub-
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Fig. 3. In vitro proliferation of RenCa and transfected clones of RenCa. O, RenCa/P cells, the RenCa parental cell line; △, RenCa/C1 cells transfected with control vector; ●, RenCa/F5 cells transfected with the FGF-2 gene (these cells expressed high levels of FGF-2 protein); •, RenCa/F6 cells transfected with the FGF-2 gene (these cells expressed nearly same levels of FGF-2 as RenCa/F5). Five thousand cells of each cell line were seeded in 12-well plates (3.8 cm²/well). The cells were counted daily in triplicate. Bars, SD.

Table 2. Comparison of in vitro invasive potential of RenCa sublines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Migrated cell number/field (×200)²</th>
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<tbody>
<tr>
<td>RenCa/P</td>
<td>25.3 ± 5.2²</td>
</tr>
<tr>
<td>RenCa/C1</td>
<td>22.3 ± 8.4³</td>
</tr>
<tr>
<td>RenCa/F5</td>
<td>91.5 ± 17.3³</td>
</tr>
<tr>
<td>RenCa/F6</td>
<td>86.7 ± 15.2²</td>
</tr>
</tbody>
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*Cells (1 × 10⁶) were seeded in the upper chamber. The cells were cultured for 48 h.
²The number of migrated cells/field (×200) was determined.
³Mean ± SD.
⁴Mean number of migrated cells was significantly different from that of RenCa/P and RenCa/C1 at P < 0.01 (Student’s t test).

DISCUSSION

FGF-2 is a potent mitogen for a variety of cell types and affects a number of additional cellular functions, such as protease production, surface integrin levels, cell migration, and angiogenesis (2, 3). Renal cell carcinomas are characterized by hypervascularity, high frequency of metastasis, resistance to chemotherapeutic agents, and poor prognosis (4). Recent studies have revealed that FGF-2 plays an important role in the malignant progression of renal cell carcinoma. The presence of FGF-2 was demonstrated in renal cell carcinoma (6, 7). Furthermore, FGF-like activity was shown to be increased in the serum of patients with renal cell carcinoma (5). Although these findings suggest a close relationship of FGF-2 and renal cell carci-

In Vivo Studies of RenCa Sublines. To examine the in vivo effect of FGF-2 expression on tumor growth, 1 × 10⁶ cells of each cell line were injected s.c. in the right flank of syngeneic +/+ BALB/c mice. There was no significant difference in tumor growth in vivo among RenCa/P, RenCa/C1, RenCa/F5, and RenCa/F6 (Fig. 5).

To study the effect of FGF-2 expression on metastatic potential, we injected 5 × 10⁵ cells of each cell line into the tail vein or renal subcapsule. The mice were sacrificed 2 weeks later, at which time we found that RenCa/F5 and RenCa/F6 had formed more than 10 times as many metastatic nodules in lungs as had RenCa/P and RenCa/C1 after i.v. injection (P < 0.01; Table 3). Similarly, more metastatic nodules were observed in the lungs (Fig. 6) after the renal subcapsular injection of RenCa/F5 and RenCa/F6 (P < 0.01; Table 4). Only the mice injected with RenCa/F5 or RenCa/F6 cells showed metastatic nodules in the liver and mesenteric lymph nodes (Fig. 6). In the mice given RenCa/F5 or RenCa/F6, liver metastasis was observed at the rate of 20 or 40%, and mesenteric lymph node metastasis at the rate of 40 or 60%, respectively (Table 4). However, there was no significant difference in the size of the primary tumors formed by the RenCa sublines when these were transplanted into the renal subcapsule (data not shown).

DISCUSSION

FGF-2 is a potent mitogen for a variety of cell types and affects a number of additional cellular functions, such as protease production, surface integrin levels, cell migration, and angiogenesis (2, 3). Renal cell carcinomas are characterized by hypervascularity, high frequency of metastasis, resistance to chemotherapeutic agents, and poor prognosis (4). Recent studies have revealed that FGF-2 plays an important role in the malignant progression of renal cell carcinoma. The presence of FGF-2 was demonstrated in renal cell carcinoma (6, 7). Furthermore, FGF-like activity was shown to be increased in the serum of patients with renal cell carcinoma (5). Although these findings suggest a close relationship of FGF-2 and renal cell carci-
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Fig. 5. Tumor growth in BALB/c mice of the RenCa parental cell line (RenCa/P), the control vector-transfected cell line (RenCa/C1), and clones of RenCa-transfected FGF-2 (RenCa/F5 and RenCa/F6). Mice were s.c. given 1 × 10⁶ cells in the right flank on day 0. Tumor size was measured as the product of the greatest diameter multiplied by the perpendicular diameter. Bars, SD of tumor size; five mice/group.

Table 3 Production of metastases by RenCa sublines injected into the tail vein of mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>No. of lung metastases</th>
</tr>
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<tbody>
<tr>
<td>RenCa/P</td>
<td>24.0 ± 26.7</td>
</tr>
<tr>
<td>RenCa/C1</td>
<td>21.8 ± 20.2</td>
</tr>
<tr>
<td>RenCa/F5</td>
<td>340.0 ± 41.5</td>
</tr>
<tr>
<td>RenCa/F6</td>
<td>290.7 ± 52.3</td>
</tr>
</tbody>
</table>

* Cells (5 × 10⁵) were injected into the tail vein of mice. The mice were killed 2 weeks after injection.

* The number of surface metastatic nodules in the lung was determined.

* The mean number of metastases was significantly different from that of RenCa/P and RenCa/C1 at P < 0.01 (Student's t test).

Fig. 6. Metastatic nodules in the lungs of BALB/c mice whose renal subcapsules were injected with RenCa/P (1), RenCa/C1 (2), RenCa/F5 (3), and RenCa/F6 (4). Metastatic nodules in the liver (5) and mesenteric lymph nodes (6) of BALB/c mice given injections of RenCa/F5 into the renal subcapsule.

noma, the significance of FGF-2 expression in renal cell carcinoma is obscure.

In this study, we examined the role of native FGF-2 in renal cell carcinoma by introducing the FGF-2 gene without a typical signal sequence into RenCa, a mouse renal cell carcinoma cell line that originally does not express FGF-2 mRNA. Using a sandwich EIA, we found high FGF-2 protein levels in the cell lysates of FGF-2-transfected cell lines, but extremely low levels in the culture supernatants of these cell lines. We did not observe significant differences in cell proliferation among RenCa sublines either in vitro or in vivo. However, with an in vitro tumor cell invasion assay, we demonstrated that the FGF-2-transfected cell lines were significantly more invasive than the control. Also, we observed a marked increase in MMP-2 production in the culture supernatants of transfectants by zymography. Furthermore, in the in vivo study, we demonstrated that FGF-2-transfected cell lines had greater potential for metastasis than did the control. In the FGF family, it has been reported that both a FGF-1-transfected rat bladder cancer cell line and a FGF-4-transfected human mammary cancer cell line had more metastatic potential than each parental cell line (16, 17). However, the reported changes in metastasis in the transfected do not seem to be as remarkable as ours. It has also been reported that NIH 3T3 cells transfected with FGF-2 fused to a signal sequence were highly tumorigenic and metastatic, whereas NIH 3T3 cells transfected with native FGF-2 were nontumorigenic in syngeneic
mice (18). In this study, we demonstrated for the first time that the introduction of the native FGF-2 gene to cells enhanced metastatic potential in a syngeneic animal model. We believe that this model will be useful for investigating the mechanism of metastasis, the functional role of FGF-2 in vivo, and the development of new ant metastatic agents.

The mechanism by which FGF-2 transfection induces high metastatic potential is of interest. In this study, we suggest that FGF-2 transfection may increase the metastatic potential, in part, through oversecretion of MMP-2. Although several reports have already shown exogenous FGF-2 stimulated the production of MMPs by tumor cells (19-21), to our knowledge, this is the first report to show that the production of MMP-2 was markedly changed by the transfection of FGF-2. A good correlation has been reported between the content of MMP-2 and the metastatic potential of various tumors (22, 23). The phenomenon of the enhanced production of MMP-2 may not be entirely responsible for the high metastatic potential of the FGF-2-transfected cells, but there is no doubt that MMP-2 is involved in promoting this metastatic potential.

Another point of interest is the mechanism whereby FGF-2 induces MMP-2. Some studies have suggested that FGF-2 acts as an autocrine and/or paracrine growth factor, because a monoclonal antibody against FGF-2 inhibited the cell growth of various tumors (24, 25). On the other hand, another study showed that NIH 3T3 cells transfected with the native FGF-2 gene exhibited increased rates of cell locomotion, and these increases were not significantly modified by the presence of a signal sequence for secretion (26). This finding suggests that FGF-2 stimulates cell locomotion through a mechanism other than an autocrine and/or paracrine pathway. Such a different mechanism for the action of FGF-2 (i.e., a mechanism differing from autocrine and paracrine pathways) has been reported by others (27-29). In the present study, although the addition of exogenous FGF-2 at the same concentration as that in the culture supernatants of FGF-2-transfected RenCa failed to stimulate either the cell proliferation or the production of MMP-2, the introduction of the FGF-2 gene to the cells increased the production of MMP-2. Furthermore, the monoclonal antibody against FGF-2 (24, 25) had no effects on the production of MMP-2 by FGF-2-transfected cell lines. Taken together, our findings indicate that the endogenous expression of FGF-2 in renal cell carcinoma stimulates MMP-2 production rather than cell proliferation, with the mechanism of MMP-2 activation by FGF-2 probably being exerted through an intracrine rather than an autocrine and/or paracrine pathway.

In conclusion, we have demonstrated for the first time that the overexpression of FGF-2 causes an increase in MMP-2 production, resulting in the enhanced metastatic potential of a renal cell carcinoma cell line, RenCa. Our findings suggest that this model will be useful for investigating the mechanism of metastasis and the functional role of FGF-2 in renal cell carcinoma.

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