Relative Expression of Matrix Metalloproteinase-2 and Tissue Inhibitor of Metalloproteinase-2 in Mouse Renal Cell Carcinoma Cells Regulates Their Metastatic Potential

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ABSTRACT

To clarify the significance of the balance between matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinase-2 (TIMP-2) in the progression of renal cell carcinoma, we transfected both the MMP-2 and TIMP-2 genes simultaneously into RenCa, a mouse renal cell carcinoma cell line that does not express detectable levels of either MMP-2 or TIMP-2 mRNAs, and established several clones with various MMP-2:TIMP-2 expression ratios. On the basis of the quantitative evaluation of the MMP-2:TIMP-2 mRNA expression ratio by Northern blot analysis, we selected a clone overexpressing MMP-2 alone (RenCa/M), a clone overexpressing TIMP-2 alone (RenCa/T), and two kinds of clones overexpressing both, i.e., one with a high (RenCa/MTh) and one with a low (RenCa/MTl) MMP-2:TIMP-2 ratio, to compare the tumor cell phenotypes. In an in vitro tumor cell invasion assay, the MMP-2:TIMP-2 ratios of the RenCa sublines were directly correlated with their invasive potential. The invasive abilities of the parental RenCa cells induced by conditioned media from RenCa sublines were also correlated with the MMP-2:TIMP-2 ratios of the sublines. The cell adhesion assay showed the inverse correlation between the MMP-2 expression levels in the sublines and their cell adhesion to several extracellular matrix components. Furthermore, when injected i.v. or into the renal subcapsule in syngenic mice, RenCa sublines formed metastatic nodules in the lungs, and the number of nodules was correlated with the MMP-2:TIMP-2 ratio of each clone. In contrast, despite the growth-inhibitory effects of TIMP-2 overexpression, MMP-2 overexpression had no effect on either proliferation in vitro of RenCa sublines or on their growth as tumors in vivo. These results suggest that the MMP-2:TIMP-2 expression ratio is a critical factor in the invasion and metastasis of renal cell carcinoma.

INTRODUCTION

Metastasis, a major cause of mortality in cancer patients, consists of a series of events during which cancer cells detach from the primary tumor by invading the surrounding tissues, enter the circulatory system by penetrating blood and/or lymphatic vessels, and exit the vessels at distant organs to form secondary tumors (1). Proteolytic degradation of ECM is an essential part of this process, and several enzyme systems have been shown to be involved (2). Among them, MMPs, a family of Zn²⁺-dependent endopeptidases with a broad spectrum of proteolytic activity for several components of the ECM, are some of the most important enzymes involved in invasion and metastasis of tumor cells (3). Recent studies have demonstrated that, in particular, the expression level of MMP-2 (gelatinase A or M₇₂, 000 type IV collagenase) in cancer cells is closely associated with their aggressiveness (4, 5). On the other hand, TIMP-2 inhibits the protease activity of MMP-2 by forming a specific complex with MMP-2 and has been shown to consequently suppress growth, invasion and metastasis of human and rodent tumors (6, 7). Accordingly, the balance of MMP-2 and TIMP-2 may be a critical factor affecting tumor invasion and metastasis.

Human RCC, a malignant tumor derived from renal tubular epithelium, is characterized by hypervascularity, high frequency of metastasis, and poor prognosis (8). Several investigators have reported a close relationship between RCC progression and the MMP/TIMP system. For example, Kugler et al. (9) showed by a PCR-based assay that the MMP/TIMP ratio is significantly increased in RCC compared with normal kidney tissue. Walther et al. (10) reported an inverse correlation of MMP-2 expression in metastatic RCC with survival. Consistent with these studies, we also demonstrated the involvement of MMP-2 activity in the metastatic phenotype of mouse RCC cells (11). Collectively, these findings suggest the importance of the MMP/TIMP ratio in the malignant progression of RCC; however, direct evidence supporting this hypothesis has not yet been obtained.

In this study, we transfected MMP-2 and TIMP-2 cDNAs simultaneously into RenCa, a mouse renal cell carcinoma cell line that does not express detectable levels of MMP-2 or TIMP-2 mRNAs, to generate several clones with various MMP-2:TIMP-2 ratios and characterize the changes of their pheno-
types, focusing on invasive and metastatic potential both in vitro and in vivo.

MATERIALS AND METHODS

Tumor Cell Line. 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, 200 mM glutamine, 100 μg/ml penicillin, and 0.1 mg/ml streptomycin.

Expression Plasmid and Transfection into Tumor Cells. The human MMP-2 and TIMP-2 expression plasmids used in this study were generated by subcloning human MMP-2 or TIMP-2 cDNA into the mammalian expression vector pBK-CMV (Stratagene, La Jolla, CA). The expression vectors were transfected into RenCa cells by the liposome-mediated gene transfer method (12). Briefly, 2 × 10⁷ RenCa cells were plated in a 6-cm dish 1 day before transfection. Two and a half μg of purified MMP-2 cloned into pBK-CMV (pBK-CMV/MMP-2) or TIMP-2 (pBK-CMV/TIMP-2) or 5 μg pBK-CMV alone (as a control) were added to RenCa cells after preincubation for 30 min with 5 μg of lipofectamine reagent and 3 ml of serum-free OPTI-MEM (Life Technologies, Inc., Gaithersburg). Drug selection in 1 mg/ml Genticin (Sigma Chemical Co., St. Louis, MO) was begun 3 days after transfection. Two weeks after the drug selection, colonies were harvested with cloning cylinders and expanded to cell lines.

Northern Blot Analysis. Total RNA was isolated from RenCa sublines by the acid-guanidium thiocyanate-phenol-chloroform method. Twenty μg of total RNA from each sample was subjected to electrophoresis on 1.2% agarose-formaldehyde gels and transferred to nylon membranes (Amersham Life Science, Arlington Heights, IL) overnight according to standard procedures (13). The RNA blots were hybridized with a human MMP-2 cDNA probe labeled with 32P-deoxycytidinetriphosphate by random primer labeling. The membranes were subsequently stripped and rehybridized with 32P-labeled human TIMP-2 cDNA probe and further rehybridized with 32P-labeled human GAPDH cDNA probe. Densities of bands for MMP-2 and TIMP-2 were normalized against that of GAPDH by densitometric analysis.

Western Blot Analysis. The expression levels of MMP-2 and/or TIMP-2 protein in RenCa sublines were determined by Western blot analysis as described previously (12). Briefly, samples containing equal amounts of protein (25 μg) from lysates of the RenCa sublines were electrophoresed on 12% SDS-polyacrylamide gels and transferred to nitrocellulose filters with a constant current of 140 mA for 2 h. The filters were blocked in PBS containing 10% nonfat milk powder at 4°C overnight and then incubated for 1 h with a 1:200-diluted antihuman MMP-2 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:200-diluted antihuman TIMP-2 monoclonal antibody (Santa Cruz Biotechnology, Inc.) or 1:10,000-diluted antirat β-tubulin mouse monoclonal antibody (Chemicon International, Inc., Tumecula, CA), which reacts with β-tubulin from all species (plant to human), in PBS containing 0.03% Tween 20. After washing with PBS containing 0.3% Tween 20, the filters were incubated for 30 min with horseradish peroxidase-conjugated antimouse IgG antibody (Amersham Life Science), and specific proteins were detected using an enhanced chemiluminescence Western blotting analysis system (Amersham Life Science).

Zymography. The activities of the matrix metalloproteinases (MMPs) secreted by the RenCa sublines were analyzed by zymography, as described previously (11). Briefly, the serum-free culture medium (DMEM/F-12) of each cell line was collected after incubation for 24 h and sequentially centrifuged at 800 × g and 18,000 × g. Aliquots of the supernatants were concentrated with Centriplus 3 concentrators (Amicon, Inc., Beverly, MA) and mixed with SDS sample buffer. Electrophoresis was carried out at 4°C in a 7.5% polyacrylamide gel containing 1.0 mg/ml gelatin. The gel was then rinsed twice with 2.5% Triton X-100 in 50 mM Tris-HCl buffer (pH 7.5) and incubated at 37°C for 16 h in 0.15 mM NaCl, 10 mM CaCl₂, and 50 mM Tris-HCl buffer (pH 7.5). The gel was stained with 0.05% Coomassie blue, 10% isopropanol, and 10% acetic acid in deionized water and then destained with 10% isopropanol and 10% acetic acid in deionized water. MMPs were detected as transparent bands on the blue background of the Coomassie blue-stained slab gel.

Cell Proliferation Assay. To compare the in vitro proliferation of RenCa sublines, 5 × 10⁵ cells of each cell line were seeded in each well of 12-well plates (3.8 cm²/well), and the number of cells in each cell line was counted daily in triplicate samples.

In Vitro Tumor Cell Invasion Assay. Tumor cell invasion was measured with a membrane invasion culture system with a minor modification (11). 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 × 10⁵ 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 chemoattractant. After a 48-h incubation at 37°C, the cells attached on the upper surface of the filter were completely removed by wiping with a cotton swab. The filters were fixed in methanol and stained with Giemsa solution. The number of cells that had migrated to the lower surface of the filters was counted manually.

The effects of treatment of parental RenCa cells with conditioned medium from RenCa sublines on their invasive potential was then assessed by using the same system described above. In this experiment, the lower chamber contained DMEM/F-12 collected from RenCa sublines after incubation for 24 h as a chemoattractant. Similarly, cell motility was also assessed by using the Boyden chambers without Matrigel. Each assay was performed in triplicate.

Cell Adhesion Assay. The ability of RenCa sublines to bind ECM molecules was evaluated by the adhesion assay as described previously (14). Briefly, 96-well flat-bottomed plates were coated with fibronectin, laminin, type IV collagen, or vitronectin (Sigma Chemical Co.) in PBS overnight at 4°C. The plates were washed with PBS, and nonspecific sites were blocked with 10 mg/ml BSA in PBS for 2 h at 37°C. Cells were collected from plates by 10 mM EDTA in PBS, and 5 × 10⁵ cells
were added to each well. Adhesion was allowed to proceed for 3 h at 37°C. The plates were inverted and centrifuged at 150 × g for 5 min, and unadherent cells were aspirated. One hundred μl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Chemical Co.) were added to each well, followed by incubation for 2 h at 37°C. The absorbance was determined with a microculture plate reader (Becton Dickinson Labware) at 540 nm. Absorbance values were normalized to the values obtained for the cells initially screened. Each assay was performed in triplicate.

**Animal Studies.** BALB/c +/+ female mice, ages 6–8 weeks, were purchased from CLEA Japan, Inc. (Tokyo, Japan) and housed in a controlled environment at 22°C on a 12-h light/12-h dark cycle. Each experimental group consisted of 10 mice. The tumor cells were trypsinized, washed twice with PBS, and injected s.c. with 1 × 10⁶ cells in the flank, i.v. with 5 × 10⁶ cells in the tail vein, or directly administered with 5 × 10⁶ cells under the renal subcapsule, as described previously (11). Briefly, each mouse was anesthetized with pentobarbital, and a small incision was made in the left flank. The kidney was lifted out of the peritoneum, and a 27-gauge needle was inserted into the lower pole until its point reached just below the renal subcapsule. Visible bulla formation between the renal parenchyma and capsule was the criterion for a successful injection.

Tumor volume was measured twice weekly and calculated by the formula: length × width × depth × 0.5236 (13). Two weeks after the injection of tumor cells in the tail veins or renal subcapsules, the mice were sacrificed, and the presence of metastasis was macroscopically examined in all abdominal and thoracic internal organs. The organs with macroscopic metastases were removed, and the number of surface metastatic nodules was counted.

**RESULTS**

**MMP-2 and/or TIMP-2 Expression in RenCa Sublines.** RenCa cells were transfected with pBK-CMV/MMP-2 and/or pBK-CMV/TIMP-2 or with pBK-CMV alone as a control. After drug selection, a number of Geneticin-resistant stable transfectants were developed and then analyzed for expression of MMP-2 and TIMP-2 mRNAs by Northern blotting. As shown in Fig. 1A, variable levels of the MMP-2 and/or TIMP-2 mRNAs were detected in six independent transfectants, whereas no detectable MMP-2 or TIMP-2 mRNAs were expressed in either the parental RenCa (RenCa/P) or the controlvector-transfected cell line (RenCa/C). On the basis of the results of densitometric analysis, a clone overexpressing MMP-2 alone (RenCa/M), a clone overexpressing TIMP-2 alone (RenCa/T), and two kinds of clones overexpressing both, i.e., one with a high (RenCa/MTh) and one with a low (RenCa/MTI) MMP-2/TIMP-2 ratio, were selected for further analysis.

Western blot analysis was used to measure MMP-2 and TIMP-2 protein expression in the RenCa sublines. As expected, MMP-2 protein was detected in RenCa/M, RenCa/MTh, and RenCa/MTI, whereas TIMP-2 was detected in RenCa/MTh, RenCa/MTI, and RenCa/T (Fig. 1B). In addition, the MMP-2:TIMP-2 ratios of the RenCa sublines at protein level were consistent with those at the mRNA level.

The MMP-2 activity in conditioned media from RenCa sublines was analyzed by zymography. As shown in Fig. 1C, MMP-2 (Mₙ = 72,000) was secreted by RenCa/M, RenCa/MTh, and RenCa/MTI, and the amount of secreted MMP-2 reflected the MMP-2:TIMP-2 ratio of each clone.

**In Vitro Studies of RenCa Sublines.** The cell proliferation assay revealed that overexpression of TIMP-2 reduced cell growth in vitro, despite the lack of effect by MMP-2 overex-
expression. As shown in Fig. 2, there was no significant difference in cell proliferation among RenCa/P, RenCa/C, and RenCa/M; however, RenCa/MTh, RenCa/MTl, and RenCa/T showed decreased growth rates in proportion to the level of TIMP-2 expression.

The invasive potential of the RenCa sublines was examined with an in vitro tumor cell invasion assay. As shown in Fig. 3A, the invasive ability was enhanced by MMP-2 expression but inhibited by TIMP-2 expression, with the highest and lowest invasive potential exhibited by RenCa/M and RenCa/T, respectively, indicating a direct correlation with the differences between MMP-2 and TIMP-2 expression levels (correlation coefficient \( r > 0.9 \)). Furthermore, supernatants from RenCa/M significantly enhanced the invasive potential of RenCa/P; in contrast, the invasive potential of RenCa/P was reduced by supernatants from RenCa/T (Fig. 3B). However, there were no significant differences in cell motility among RenCa sublines (data not shown).

The ability of RenCa sublines to bind ECM components was analyzed by the cell adhesion assay. As shown in Fig. 4, adhesion to RenCa/M and RenCa/MTh cells was decreased by 40–60% and 25–35%, respectively, on fibronectin, laminin, type IV collagen, and vitronectin, compared with RenCa/P, RenCa/C, RenCa/MTl, and RenCa/T.

**In Vivo Studies of RenCa Sublines.** To examine the in vivo effects of MMP-2 and/or TIMP-2 expression on tumor growth, \( 1 \times 10^6 \) cells of each cell line were injected s.c. in syngeneic mice. There was no significant difference in tumor growth in vivo among RenCa/P, RenCa/C, and RenCa/M; however, tumors formed by RenCa/MTh, RenCa/MTl, and RenCa/T were significantly smaller than those formed by the other clones (Fig. 5).

To study the effects of MMP-2 and/or TIMP-2 expression on metastatic potential, we injected \( 5 \times 10^5 \) 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/M and RenCa/MTh had formed more than five and two times as many metastatic nodules in lungs, respectively, as had RenCa/P and RenCa/C after i.v. injection. In contrast, the mice i.v. injected with RenCa/T had no metastatic nodules in their lungs (Table 1). Similarly, in comparison with RenCa/P and RenCa/C, 6-fold and 3-fold more metastatic nodules were observed in the lungs after the renal subcapsular injection of RenCa/M and RenCa/MTh, respectively, whereas the renal subcapsule injection of RenCa/MTl and RenCa/T resulted in 30 and 80% reduction of metastatic nodules in the lungs, respectively. In addition, liver and mesenteric lymph node metastasis was observed only in mice injected with RenCa/M, at the rates of 20 and 40%, respectively (Table 2).
DISCUSSION

MMP-2 degrades the components of the basement membrane, such as type IV collagen, and is involved in cancer metastasis (3). A number of clinical and experimental studies have demonstrated the close relationship between metastatic potential of cancer cells and their secretion of MMP-2 (4, 5). On the other hand, TIMP-2 has been shown to effectively inhibit the protease activity of MMP-2, resulting in the suppression of growth, invasion, and metastasis of cancer cells (6, 7). Therefore, the balance of MMP-2 and TIMP-2 may critically affect the metastasis of cancer cells. In fact, some investigators have reported the usefulness of the MMP-2:TIMP-2 ratio as a marker reflecting malignant potential. For example, Nuovo et al. (15) detected a strong association between the prognosis of patients with cervical carcinoma and their MMP:TIMP ratio. Kugler et al. (9) found that the MMP-2:TIMP-2 ratio in renal cell carcinoma is significantly higher than that in normal kidney. We also reported that serum MMP-2:TIMP-2 ratio in patients with urothelial cancer could be used as a prognostic marker of recurrence (16). However, to date, there has been no direct evidence demonstrating the effect of the MMP-2:TIMP-2 ratio in cancer cells on their invasive and metastatic potentials.

In this study, to generate several kinds of cell lines with various MMP-2:TIMP-2 ratios, we introduced the MMP-2 and TIMP-2 genes simultaneously into RenCa, a mouse renal cell carcinoma cell line that does not express detectable levels of either MMP-2 or TIMP-2 mRNAs. After screening for Northern and Western blotting, we obtained a clone overexpressing MMP-2 alone, a clone overexpressing TIMP-2 alone, and two kinds of clones overexpressing both, one with a high and one with a low MMP-2:TIMP-2 ratio. Despite the lack of effect of MMP-2 overexpression, the growth of RenCa cells was inhibited by TIMP-2 overexpression both in vitro and in vivo. The in vitro invasive and in vivo metastatic potentials of RenCa sublines were well correlated with their MMP-2:TIMP-2 ratios. Furthermore, cell adhesion assay demonstrated the inverse correlation between MMP-2 expression levels in RenCa sublines and their adhesion to various ECM components. The differential adhesion among RenCa sublines could be due to the altered expression of ECM receptors, such as integrin. In fact, several recent studies showed that integrin and MMPs cooperatively regulate their function, which is consistent with the idea that increased MMP-2:TIMP-2 ratio and loss of stable adhesion were linked to invasion and metastasis in various types of malignancies (17, 18). Collectively, these findings are the first evidence supporting the hypothesis that a higher MMP-2:TIMP-2 ratio in cancer cells renders them phenotypically more aggressive through enhancement of their invasive and metastatic potentials.

The mechanism by which the changes of the MMP-2:TIMP-2 ratios of the RenCa sublines influence their metastatic potentials is of interest because the in vivo effects of interaction between MMP-2 and TIMP-2 remain controversial. For example, Koop et al. (19) reported that the decreased metastatic ability of TIMP-transfected melanoma cells is attributable to the inhibition of tumor growth at the metastatic site, but that TIMP up-regulation had no effect on tumor cell extravasation. Valente et al. (20) showed that reduction of MMP activity by TIMP-2 is

Table 1  Production of metastases by RenCa sublines injected into the tail veins of mice

<table>
<thead>
<tr>
<th>Cell line*</th>
<th>No. of lung metastasesb</th>
</tr>
</thead>
<tbody>
<tr>
<td>RenCa/P</td>
<td>33.2 ± 22.4</td>
</tr>
<tr>
<td>RenCa/C</td>
<td>31.3 ± 18.7</td>
</tr>
<tr>
<td>RenCa/M</td>
<td>171.5 ± 69.4</td>
</tr>
<tr>
<td>RenCa/MTh</td>
<td>68.2 ± 33.4</td>
</tr>
<tr>
<td>RenCa/MTl</td>
<td>30.6 ± 27.8</td>
</tr>
<tr>
<td>RenCa/T</td>
<td>0a</td>
</tr>
</tbody>
</table>

* Cells (5 × 10⁶) were injected into the tail veins of mice. The mice were sacrificed 2 weeks after injection.

b The number of surface metastatic nodules in the lungs was determined.

c Mean ± SD.

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

Fig. 4  Cell adhesion assay of RenCa sublines. Each cell line was examined for adhesion to extracellular matrix proteins: fibronectin (1 μg/ml), laminin (25 μg/ml), type IV collagen (1 mg/ml), and vitronectin (25 μg/ml). Columns, means; bars, SD. # and ##, differ from RenCa/P and RenCa/C (P < 0.01 and P < 0.05, respectively) by Student’s t test. □, RenCa/P; □, RenCa/C; □, RenCa/M; □, RenCa/MTh; □, RenCa/MTl; □, RenCa/T.

Fig. 5  Tumor growth of RenCa sublines in nude mice. Nude mice were s.c. injected with 1 × 10⁶ cells in the right flank on day 0. Tumor volume was measured twice weekly and calculated by the formula: length × width × depth × 0.5236. Bars, SD of tumor size. *, differs from RenCa/P and RenCa/C (P < 0.01) by Student’s t test.
not sufficient to change the metastatic phenotype of cancer cells. In contrast, Kawamata et al. (21) demonstrated marked inhibitory effects of TIMP overexpression on extravasation and metastasis of cancer cells. The present results suggest the importance of both proliferation and extravasation of renal cell carcinoma cells for development of metastasis, i.e., despite the lack of effect of MMP-2 overexpression on growth rate, MMP-2 enhanced invasive potential in vitro and metastatic potential in vivo, and the changes of metastatic potential among RenCa sublines were more substantial in the experimental metastasis model (i.e., i.v. injection model) than in the spontaneous metastasis model (i.e., renal subcapsule injection model).

In conclusion, our results provide clear evidence that the imbalance of MMP-2 and TIMP-2 expression is a critical factor in the invasion and metastasis of an RCC cell line, RenCa. Therefore, modification of the MMP-2:TIMP-2 ratio may be a useful target for prevention of RCC progression.

REFERENCES


Table 2  Production of metastases by RenCa sublines injected into the renal subcapsules of mice

<table>
<thead>
<tr>
<th>Cell line&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Lung metastasis</th>
<th>Liver metastasis</th>
<th>Mesenteric lymph node metastasis</th>
<th>No. of lung metastases&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RenCa/P</td>
<td>10/10 (100)</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
<td>43.2 ± 38.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>RenCa/C</td>
<td>10/10 (100)</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
<td>39.7 ± 28.6</td>
</tr>
<tr>
<td>RenCa/M</td>
<td>10/10 (100)</td>
<td>2/10 (20)</td>
<td>4/10 (40)</td>
<td>273.5 ± 98.6&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>RenCa/MTh</td>
<td>10/10 (100)</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
<td>138.7 ± 78.3&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>RenCa/MTl</td>
<td>10/10 (100)</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
<td>27.7 ± 22.4</td>
</tr>
<tr>
<td>RenCa/T</td>
<td>8/10 (80)</td>
<td>0/10 (0)</td>
<td>0/10 (0)</td>
<td>8.2 ± 11.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of mice with tumor/number of injected mice.
<sup>b</sup> Cells (5 × 10<sup>5</sup>) were injected into the renal subcapsule. The mice were sacrificed 2 weeks after injection.
<sup>c</sup> The number of surface metastatic nodules in the lung was determined.
<sup>d</sup> Mean ± SD.
<sup>e</sup> The mean number of metastases was significantly different from that of RenCa/P and RenCa/C at P < 0.01 (Student’s t test).

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