Renal Cell Carcinoma and Natural Killer Cells: Studies in a Novel Rat Model in Vitro and in Vivo

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ABSTRACT

The transplantable rat kidney carcinoma (RKC) provides an excellent experimental model for immunological and therapeutic studies of renal cell carcinoma. In this report, we define the biological characteristics of RKC and explore the interactions between RKC and natural killer (NK) cells. RKC, a transplantable tumor of spontaneous origin, grows progressively over a 12-week period and metastasizes to the lung when implanted orthotopically in the kidneys of female Lewis rats. Rats bearing RKC survived for an average of 10.5 ± 1.5 (SD) weeks postimplantation. Lung metastases were visible between 7.5 and 8.5 weeks postimplantation, and by 9 to 10 weeks the incidence of metastases reached approximately 67%. Injection of the NK cell-specific monoclonal antibody 3.2.3 depleted Lewis rats of their NK activity for up to 14 days. Adherent lymphokine-activated killer cells generated from the spleens of 3.2.3-injected rats were significantly less lytic than those from control rats and contained a significantly lower percentage of 3.2.3 reactive NK cells. The activity of adherent lymphokine-activated killer cells from tumor-bearing rats was lower than that from normal rats, but not significantly. Cultured RKC cells were killed by both splenic NK cells and adherent lymphokine-activated killer cells. These data demonstrate that RKC is NK sensitive and that tumor growth does not abrogate NK activity. The RKC tumor provides a model system for the analysis of immunological factors in renal cell carcinoma growth and presents opportunities for testing therapeutic interventions in a system that closely mimics the human disease.

INTRODUCTION

Patients with RCC have a poor prognosis; those with metastatic disease at the time of diagnosis have a 3-year survival rate of only 4% (1, 2). Aside from resection, metastatic RCC is particularly difficult to treat because it is generally unresponsive to radiation therapy, hormonal therapy, and chemotherapy (3). Recently, complete response rates of up to 11% and partial response rates of up to 24% in patients with advanced RCC have been reported using biological response modifiers such as interleukin 2 and interferons, as well as the adoptive transfer of LAK cells (21); has been shown to deplete Fisher rats of their NK activity against 51Cr-labeled YAC-1 targets over the course of RKC progression. The activity of adherent lymphokine-activated killer cells from tumor-bearing rats was lower than that from normal rats, but not significantly. Cultured RKC cells were killed by both splenic NK cells and adherent lymphokine-activated killer cells. These data demonstrate that RKC is NK sensitive and that tumor growth does not abrogate NK activity. The RKC tumor provides a model system for the analysis of immunological factors in renal cell carcinoma growth and presents opportunities for testing therapeutic interventions in a system that closely mimics the human disease.

MATERIALS AND METHODS

Animals. Female Lewis rats (100–125 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and housed in a modified specific pathogen-free animal facility in the Department of Comparative Medicine at the University of Washington for at least 7 days before use.

Tumor Maintenance. The rat renal adenocarcinoma was maintained by in vivo passage. Donor rats were anesthetized with 68.2 mg/kg ketamine (Aveco Co., Inc., Fort Dodge, IA) and 4.4 mg/kg xylazine (Lloyd...
Laboratories, Shenandoah, IA) in 1 ml phosphate-buffered saline (Gibco, Life Technologies, Inc., Grand Island, NY), i.p. Nonneoplastic tumor tissue was excised from the donor and minced into ~1-mm³ chunks in sterile RPMI 1640 (Flow Laboratories, ICN Biomedicals, Inc., Costa Mesa, CA). An incision was made in the left flank of anesthetized recipient rats. The left kidney was exteriorized, and a small nick was made in the renal capsule. One chunk (approximately 10⁶ cells) of tumor was placed under the renal capsule, the kidney was irrigated with sterile RPMI 1640 and placed back into the peritoneal cavity, and the skin incision was closed with 9-mm autoclips (Clay Adams-Becton Dickinson and Company, Parsippany, NJ). Tumor incidence was 100% in recipient animals after tumor implant surgery.

Euthanasia and Dissection. Rats were euthanized with an overdose of ketamine/xylazine i.p. The primary renal tumor was excised and weighed. The lungs were excised and examined both visually and by palpation for the presence of gross metastases. Tissues to be examined microscopically were fixed in formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

Preparation of Lymphoid Cells. Spleens from both normal and tumor-bearing rats were aseptically removed. Single cell suspensions were prepared in Hanks' medium (Hanks' balanced salt solution; Gibco) with 5% FCS. Peripheral blood was obtained from control and tumor-bearing rats by cardiac or retrobulbar puncture. Mononuclear cells were obtained after centrifugation on Ficoll-Hypaque (density, 1.077) (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) or Lympholyte-Rat (Accurate Chemical and Scientific Corp., Westbury, NY) at a speed of 300 x g for 20 min at room temperature. The PB and splenic lymphoid cells then were washed twice in sterile WM (Irvine Scientific, Santa Ana, CA), counted, and used directly or passed over a nylon wool (Robbins Scientific Corp., Sunnyvale, CA) column to remove monocytes/macrophages and B-cells (25), as noted below in the results. The nylon wool nonadherent mononuclear cells were collected, washed twice, tested immediately for NK activity, and stained for flow cytometric analysis using various monoclonal antibodies or put into A-LAK cultures as described below (data obtained from these cells are referred to as being from "day 0 cells").

Generation of A-LAK Cells. The culture medium and methods used for the generation of LAK and A-LAK cells have been reported previously (26). Briefly, 30 x 10⁶ nylon wool nonadherent mononuclear cells were cultured at 37°C in 5% CO₂/95% air in T-75 flasks (Corning Glass Works, Corning, NY) at an optimal density of 2 x 10⁶/ml in 15 ml of LAK medium, which contains RPMI 1640, 10% FCS, 5 x 10⁻⁵ M 2-mercaptoethanol, 0.1 M nonessential amino acids, 1.0 mM sodium pyruvate, 2 mM l-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Gibco), and 500 units/ml IL-2 (generously provided by Hoffmann-La Roche through the Biological Resources Branch of the National Cancer Institute). After 48 h, nonadherent cells were decanted, and the adherent cells were washed twice with WM. The adherent cells were fed with a 1:1 mixture of fresh LAK medium and conditioned medium filtered through a 0.45-μm filter (Millipore Products Division, Bedford, MA). After an additional 3 days of culture, the adherent cells were harvested with 10 ml of 0.025% EDTA (J. T. Baker, Inc., Phillipsburg, NJ). The flasks were shaken for 5 min to dislodge cells, and detached A-LAK cells were removed, washed, counted, and used (data obtained from these cells are referred to as being from "day 5 cells").

Target Cells. The NK-sensitive Moloney virus-induced mouse T-cell lymphoma YAC-1 was maintained as a cell suspension in RPMI 1640 with 8% FCS with 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. RK cultures were established by mincing fresh tumor in sterile WM. Approximately 20 tumor pieces, 1-2 mm³, were placed in T-75 flasks (Corning) and then cultured in Waymouth's medium (Gibco) with 10% FCS and 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, and 0.1% Mito* serum extender (Collaborative Research, Inc., Bedford, MA). Adherent tumor cells grew out from the tumor pieces. After 2-3 days, nonadherent chunks were removed, and the cultures were respun with Waymouth's medium. RK cells were subcultured when confluent (about once a week) until used. Cells were removed from the flask with 0.025% EDTA, washed once, and labeled as targets for the cytotoxicity assay.

Cytotoxicity Assay. Cytotoxicity was measured in a standard 4-h 5¹Cr release assay using 96-well, V-bottomed plates (Costar, Cambridge, MA). YAC-1 target cells were labeled with 200 μCi of Na₂¹⁵CrO₄ (Amersham Corporation, Arlington Heights, IL)/3.5-7.0 x 10⁶ cells; cultured RK cells were labeled with 100 μCi of Na₂¹⁵CrO₄/3.5-7.0 x 10⁶ cells. Target cells were washed and plated at 5 x 10³ cells/well, and effector cells were added to triplicate wells at effector:target ratios of 100:1 to 12.5:1. Plates were incubated at 37°C for 4 h, and 100 μl of supernatant were harvested from each well and analyzed using a gamma counter (Beckman Gamma 5500) to determine experimental release. Spontaneous release was determined from wells containing only target cells and medium, and total release from wells with target cells and 2% Triton X-100 (Sigma, St. Louis, MO). Percentage cytotoxicity was calculated as follows:

\[
\text{% cytotoxicity} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100
\]

Lytic units per 10⁶ cells were calculated by linear regression for various percentages of killing of 5 x 10⁵ YAC-1 target cells by splenic and PB NK cells and A-LAK cells, as specified in "Results." Antibodies. The monoclonal antibody 3.2.3 is a mouse IgG₁ specific for rat NK cells and IL-2-stimulated rat A-LAK cells. 3.2.3-producing hybridoma cells were obtained from Dr. John Hiserodt, and ascites fluid, produced as described previously (21, 22), was used for the in vivo depletion of NK activity in rats. Purified FITC-conjugated 3.2.3 was used for cell surface staining. NK1.1, used as a control in in vivo studies, is a mouse IgG₂a antibody specific for mouse NK cells, produced in Balb/c mice from the hybridoma PK 136 (American Type Culture Collection, Rockville, MD). Ox-19 (27), obtained from Dr. Craig Reynolds, produces a MAb specific for the rat T-cell equivalent of CD 5. Ascites fluids from the hybridomas PK 136 and Ox-19 were produced as described for 3.2.3.

Flow Cytometric Analysis. FITC-conjugated 3.2.3 was used at an optimal dilution of 1:1000, based on preliminary titrations. Unlabeled Ox-19 ascites was used at a dilution of 1:20,000 with a secondary phycoerythrin-conjugated goat anti-mouse Ig antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) at a dilution of 1:400. Cells were stained and analyzed as described previously (28). For analysis, samples were gated on the lymphocyte population, and the data presented represent the cells in that region.

Statistical Analysis. Significant values were determined by using Student's t test. A P value of ≤0.05 was considered significant.

RESULTS

Tumor Growth and Metastasis. To characterize the biology of the RK tumor, we measured its in vivo growth rate and survival curve and the incidence of grossly palpable lung metastases in rats implanted with RK. Sixty-one rats were implanted with RK and then euthanized 2 to 10 weeks after tumor implantation, and the weight of the tumor was determined (Fig. 1). Primary tumor weight increased slowly through the first 6 weeks; the most statistically significant rise in tumor weight occurred between 6 and 7 weeks (P ≤ 0.008). The tumor grew less rapidly after 7 weeks.

In order to define a survival curve for rats bearing RK, 16 rats were implanted with RK, received no further treatment, and were euthanized when they became moribund. The times of euthanasia, recorded to the nearest 0.5 week after implantation, were used to calculate the survival rate for tumor-bearing rats. The mean survival time was 10.5 ± 1.5 (SD) weeks (Fig. 2).

To determine the incidence of lung metastases in RK-bearing rats, the lungs of 52 tumor-bearing rats were examined for the presence of grossly palpable metastases. The incidence of
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Fig. 1. RKC growth curve. Sixty-one rats were implanted with RKC and euthanized at various times following implant. Weights of primary tumors are shown as the mean of several animals. Bars, SD.

gross metastasis rose rapidly after 7 weeks and reached a maximum of approximately 67% by 9 weeks (Fig. 3). Grossly palpable metastases were not observed elsewhere (e.g., liver, contralateral kidney).

Photomicrographs of representative fields of hematoxylin and eosin-stained sections of primary tumor and of lung tissue containing metastases are shown in Fig. 4. The primary tumor consists of large, frequently multinucleated cells with relatively clear cytoplasm. Lung metastases appeared to be histologically identical to the primary tissue.

Effect of RKC Growth on NK Activity. To determine whether the growth of RKC affected the lytic activity of splenic and PB NK cells, we implanted 25 rats with RKC and tested their NK activity at different times after implantation. Spleen cells and PB cells from normal rats were also tested. The results, reported in lytic units, are summarized in Fig. 5. Although fluctuations were observed, neither splenic nor PB NK activity was diminished significantly from control values in rats bearing tumors. Peripheral blood NK activity tended to decrease in tumor-bearing rats, but activity was still present even in late-stage tumor bearers.

Effect of RKC Growth on A-LAK Precursors. We ascertained the effects of tumor growth on the ability to generate A-LAK cells from the tumor-bearing rats' spleens. A-LAK cultures from normal and tumor-bearing rats were harvested after 5 days and tested for lytic activity. Although A-LAK activity dropped from 400 to 80 lytic units between 0 and 8 weeks after tumor implantation (Fig. 6), the difference between A-LAK activity from normal spleens and that from any of the tumor-bearing spleens was not statistically significant.

In Vivo Depletion of NK Cells in Lewis Rats. To determine the dose of 3.2.3 necessary to deplete NK activity in Lewis rats and the duration of the NK depletion, 8 normal rats were injected with different concentrations of 3.2.3 ascites fluid in

Fig. 2. Survival curve of rats implanted with RKC. Sixteen rats were implanted with RKC and euthanized when they became moribund. Results are reported as a percentage of animals still living at each time point after implantation.

Fig. 3. Incidence of lung metastases. Fifty-two rats were implanted with RKC under the renal capsule and euthanized at various time points. Lungs were excised and examined for the presence of visible or grossly palpable metastases. The percentage of rats euthanized at each time point which were determined to have palpable lung metastases is displayed on the graph.

Fig. 4. Photomicrographs of paraffin tissue sections of primary RKC (× 500) (A) and lung with palpable metastases (× times 160) (B). H&E.
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NK cells, we depleted normal rats of their NK cells in vivo and tested the ability of NK-depleted or control SCs to generate A-LAK cells. Spleen cells were obtained 2 days after the administration of 3.2.3 or NK1.1 MAb. Spleenic NK activity against YAC targets was tested on days 0 and 5 of A-LAK culture. 3.2.3 effectively depleted NK activity from fresh SCs (Fig. 1A), and these cells generated significantly less A-LAK activity than did those from control rats (Fig. 1B). Day 0 and day 5 cells were also tested for their ability to lyse cultured RKC cell targets. Both day 0 (Fig. 7A) and day 5 (Fig. 7B) cells were able to lyse the cultured RKC targets, demonstrating that RKC

Table 1 Titration of MAb 3.2.3-induced depletion of NK cells in Lewis rats

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<td>0.25</td>
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a Microliters of 3.2.3 ascites in 1 ml phosphate-buffered saline.

*b Day 0 timepoint measured before 3.2.3 injection.

sterile phosphate-buffered saline. Peripheral blood was obtained via retroorbital puncture, and lymphoid cells were tested for NK activity against YAC-1 targets in a cytotoxicity assay. Rats were tested before 3.2.3 injection, and on days 2, 5, 8, 11, 14, and 26 following injection. Because an i.p. injection of 50 µl 3.2.3 ascites in 1 ml phosphate-buffered saline depleted NK activity for over 14 days (Table 1), this dose was used in subsequent depletion experiments.

Effect of NK Depletion on A-LAK Precursors. To confirm that the effector cells in the A-LAK cultures were derived from...
cells are susceptible to lysis by NK and A-LAK cells. NK cell depletion with 3.2.3 ablated day 0 NK cell activity and reduced the day 5 A-LAK cell lytic activity against cultured RKC targets.

Day 0 SCs and day 5 A-LAK cells from 3.2.3- and NK1.1-injected rats were stained for flow cytometric analysis with MAb 3.2.3 and OX-19 (Table 2). 3.2.3-injected rats' spleens contained significantly (P < 0.01) fewer 3.2.3 bright* cells than those of the control rats on day 0, which correlated with the decreased cytotoxicity of spleen cells from 3.2.3-injected rats. We noted a concomitant increase in the percentage of OX-19+ T-cells from the 3.2.3-injected rats.

Day 5 A-LAK cells from 3.2.3-injected rats also contained a significantly lower (P < 0.02) percentage of 3.2.3+ cells than those from control rats. As shown in Fig. 8, the distribution of 3.2.3* cells in the A-LAK cultures was unimodal rather than bimodal. This lower percentage of cells again corresponded to less cytotoxicity of spleen cells from 3.2.3-injected rats.

Effect of NK Depletion on RKC Growth. Because NK activity is maintained in RKC-bearing rats, and because RKC cells are susceptible to NK cell lysis in vitro, we tested whether depletion of NK cells in vivo would significantly affect the growth of the tumor. We first examined the effect of weekly 3.2.3 and NK1.1 injections on NK activity in tumor-bearing rats. Rats received injections of 3.2.3 or NK1.1, were implanted with RKC, and received injections of antibody thereafter. Three rats from each group were bled 2 days after each injection, and their PB cells were tested for NK activity against YAC-1 targets (Table 3). The lytic activity of PB cells from 3.2.3-injected rats was considerably reduced compared to that of the NK1.1-injected rats through week 8 after the first set of injections, although activity in 3.2.3-injected rats became more variable after week 5. The lytic activity of PB cells from the control group of rats dropped after 7 weeks, which coincided with rapid tumor growth (see Fig. 1).

We then tested the hypothesis that a depletion of NK cells would allow increased growth of RKC. Eleven rats were injected with 3.2.3, and eleven with NK1.1 as controls. Peripheral blood lymphocytes from these rats in each group were tested for their NK activity against YAC-1 targets 1–2 days after injection. Those rats receiving 3.2.3 had any direct effect on the tumor, since 3.2.3 did not interact with either the surface of RKC cells, as demonstrated.

Table 2 Phenotype of NK and A-LAK cells from NK-depleted and control rats

<table>
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<th>Antibody</th>
<th>NK1.1-injected rats*</th>
<th>3.2.3-injected rats*</th>
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<tr>
<td>Spleen cells</td>
<td>3.2.3 dim*</td>
<td>8.30±4.21</td>
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<tr>
<td></td>
<td>3.2.3 bright*</td>
<td>11.14±7.23</td>
</tr>
<tr>
<td></td>
<td>OX-19*</td>
<td>76.09±0.00</td>
</tr>
<tr>
<td>A-LAK cells</td>
<td>3.2.3*</td>
<td>84.68±11.72</td>
</tr>
<tr>
<td></td>
<td>OX-19*</td>
<td>19.14±0.00</td>
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* Mean percentage of positive cells ± SD.

Type of cells stained.

Significantly different from NK1.1-injected controls (Student's t test, P < 0.05).

Significantly different from NK1.1-injected controls (Student's t test, P ≤ 0.05).
all rats were euthanized, and primary tumors were removed and weighed. Weights of tumors from individual rats are denoted as "o" for both NK1.1-injected and antibody) and then were implanted with RKC under the renal capsule. Rats were rats received injections of either 3.2.3 (to deplete NK activity) or NK1.1 (control agents because they maintain donor responsiveness and histol.

The xenograft models are excellent for studying chemotherapy (30-33), and human RCC xenografts in nude mice (34-37). The most common models are the mouse tumor. Renca interactions of the host immune system and the tumor.

DISCUSSION

Several murine models for RCC are currently used to study the interactions of the host immune system and the tumor (29). The most common models are the mouse tumor, Renca (30–33), and human RCC xenografts in nude mice (34–37). The xenograft models are excellent for studying chemotherapy agents because they maintain donor responsiveness and histology over several transplantation generations (37) but are limited in their usefulness in studying relevant host-tumor interactions because the tumors are placed in an immunodeficient foreign host environment. The Renca model shares many characteristics with RKC, but it is an extremely fast-growing tumor. The slower growth of RKC more closely mimics human RCC and provides a larger window of time for immunological manipulation and testing of potential therapies. The larger size of the rat simplifies surgical procedures and allows experiments requiring sequences of measurements over time, including in vivo imaging, e.g., with X-rays (38), to be performed without sacrificing the animals. RKC provides an excellent model for studying basic interactions between the host and RCC, as well as a vehicle for preclinical trials of immune-based therapies.

Previous reports have indicated that the depletion of NK cells inhibits the ability of host mice and rats to clear injected tumor cells from the circulation and allows enhanced growth of experimentally induced metastases (12–17). Our experiments differ in that we tested the effects of in vivo NK cell depletion on the establishment and growth of an implanted solid primary tumor. We observed a significant increase in the size of RKC in rats that were treated with the anti-NK cell MAb 3.2.3 in vivo. In an ongoing study, we are quantifying the spontaneous pulmonary metastases in NK-depleted and control rats and have found that NK depletion corresponds to an increase in the volume of metastatic tumor as well as the primary tumor.

The lytic ability of splenic and PB NK cells is often suppressed by human (39–41) and other animal (42–44) tumors. We found that splenic and PB NK activity did not significantly decrease in rats bearing RKC, even at late stages of tumor growth, although a trend toward lower NK activity from RKC bears’ spleens was repeatedly observed. Whether this decrease is biologically significant cannot be determined from these experiments, but rapid tumor progression between 6 and 8 weeks appeared to be correlated with a decrease in NK activity. However, the generation of A-LAK cells from late-stage tumor bear-

Peripheral blood NK activity was monitored over the course of RKC progression in rats that received weekly and biweekly injections of the specific anti-rat NK antibody 3.2.3 and the control, anti-mouse NK cell MAb NK1.1. As Table 2 and Fig. 9 indicate, near the end of the experiments, the NK activity of the 3.2.3-injected rats was no longer significantly lower than that of the NK1.1-injected control rats. It is likely that the rats developed anti-mouse antibodies and were able to clear the foreign antibodies more quickly. However, when we used the biweekly injection protocol (Fig. 9), NK activity was still significantly suppressed 6–8 weeks after implantation, during the rapid growth and early metastasis of the tumor. The marked effect of partial depletion of NK cells on the growth of RKC suggests that NK cells play an important role in the control of tumor growth at an early time point in the tumor progression. A potential therapy regime involving in vivo stimulation of NK tumor development. We also observed that NK activity of NK1.1-injected control rats decreased significantly 7 weeks after tu-

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We confirmed that the effector cells in the rats’ spleens and A-LAK cells were 3.2.3+ NK cells by injecting 3.2.3 into the rats in vivo. Fresh SCs from these rats exhibited both lower lytic activity against YAC-1 target cells and fewer 3.2.3 bright+ cells than from control rats. A-LAK cells generated from the spleens

of 3.2.3-injected rats also had less lytic activity and significantly fewer 3.2.3+ cells than A-LAK cells generated from control rats. In both SC and A-LAK cells, the level of lytic activity was positively correlated with the percentage of 3.2.3+ NK cells, demonstrating that the effectors, in both cases, were NK cells. Higher percentages of OX-19+ T-cells observed in SCs and A-LAK cells from 3.2.3-injected rats probably reflect the fact that T-cells constituted a larger fraction of the total cells present after the 3.2.3+ cells were removed.

The role of NK cells in the progression of RKC was explored by examining the direct effect of NK cells on RKC cells. We demonstrated that cultured RKC cells were lysed by control rat splenic NK cells and IL-2-stimulated A-LAK cells in vitro (Fig. 7B). This knowledge, together with our results showing that NK and A-LAK activity is not decreased until late-stage tumor growth in RKC-bearing rats, indicates that stimulation of NK cells may have a significant direct effect on the growth and metastasis of RCC in vivo. Both IL-2-stimulated LAK cells (4-7) and TILs (5, 6, 8) have been used with moderate success as a potential immunotherapy of RCC. We suggest that RKC offers advantages unmatched by any other model.

Our results suggest that an in vivo enhancement of NK activity will inhibit the growth of RKC and that therapy would be most effective before the progression is very advanced and there is much metastatic disease. Given the severity of RCC and its resistance to therapy, it is of critical importance to study relevant animal models both to define the mechanisms behind these phenomena and to create a vehicle for preclinical trials for potential immunotherapy of RCC. We suggest that RKC offers advantages unmatched by any other model.

REFERENCES


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