Induction of Transplantable Mouse Renal Cell Cancers by Streptozotocin: In Vivo Growth, Metastases, and Angiogenic Phenotype

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ABSTRACT

Interleukin-2-based regimens of biological therapy have shown some clinical promise for the treatment of kidney cancer in humans, although the mechanisms responsible for tumor regression occurring in these patients remain unclear. Preclinical insight into these mechanisms is limited by a paucity of orthotopic animal models of kidney cancer. We have used streptozotocin, an antibiotic and diabetogenic nitrosamine compound derived from Streptomyces achromogenes to induce new kidney tumors in BALB/c mice. Single or multiple doses of streptozotocin induced kidney tumors in up to 25% of mice by 50–90 weeks of age, with up to 18% characterized as renal cell carcinomas (RCCs). Several transplantable lines were obtained from the RCCs, and one of these lines was subsequently cloned. The initial tumor isolates and sublines were histologically reconfirmed to be RCCs, and all grew progressively but slowly (mean survival times, 57 to >100 days) in vivo after intrarenal implant. None of the primary isolates or sublines revealed mutations in either the VHL or Ras genes, although karyotype analysis and chromosome painting revealed the consistent presence of a submetacentric chromosome resulting from the fusion of chromosomes 16 and 19. Biological characterization of these tumors revealed several features analogous to the growth of human kidney cancers, including a propensity for the formation of lung metastases and high vascularity. This hypervascularity is evident by both gross and microscopic analysis and correlates with the expression of several proangiogenic genes. Overall, the features of orthotopic transplantability, slower in vivo growth (relative to the rapid growth rates of other transplantable mouse kidney tumors), propensity for lung metastases, and hypervascularity may make these tumors valuable models for the study of new therapeutic strategies based on antineovascular agents and antitumor cytokines.

INTRODUCTION

Approximately 30,000 new cases of RCC are diagnosed each year in the United States, and ~12,000 patients die of their disease annually (1, 2). Twenty to 30% of patients present with metastatic disease at the time of initial diagnosis (2, 3). The predominant cyt morphological form of kidney cancer is the clear cell type, accounting for about 70–80% of all kidney cancers (4). The molecular basis for most inherited and sporadic clear cell kidney cancers is inactivation of the VHL tumor suppressor gene on chromosome 3p25 (3, 5). The second most common form of kidney cancer is papillary RCC, which accounts for 10–15% of kidney neoplasms and is morphologically distinct from the more common clear cell type (4). Schmidt et al. (6) have recently related the hereditary form of papillary RCC to mutations in the c-met proto-oncogene. The prognosis for patients presenting with metastatic kidney cancer remains dismal, and biological approaches using interleukin 2 have proven to be as good or better than conventional chemotherapy (3, 7), suggesting that a better understanding of the interaction between the immune system and renal cancer cells may yield additional therapeutic benefits. However, there are relatively few preclinical tumor models of renal carcinoma (8). General attributes of an appropriate histopathologically characterized experimental model for kidney cancer would include spontaneous origin, slow growth rate, predictable spontaneous metastatic progression to regional lymph nodes and lungs, a well developed neovasculature, and a defined genetic etiology. The available models include the Renca adenocarcinoma of BALB/c mice (3, 9–12), a rat kidney carcinoma of spontaneous origin (13), the hereditary RCC model in Eker rats which derives from a defect in the tuberous sclerosis gene (14), and several human tumor xenograft models in athymic mice (15–18). These models all offer some advantages for the study of human kidney cancer but can also be limited to some degree by rapid growth rate in syngeneic rodents, which limits the available time period for the testing of biological therapy. There also is growing awareness that the organ microenvironment can exert a pronounced influence on the growth and metastasis of tumors by providing appropriate growth factors, tissue degradative enzymes that favor metastatic spread, enhanced expression of drug resistance mechanisms, and favorable disposition for neovascularization (reviewed in Refs. 17 and 18). In addition, there also is evidence that the effects of gene therapy approaches can be best assessed in orthotopic models (19). These findings have re-emphasized the need for orthotopic preclinical tumor models that may provide unique insight into critical biological events and important tissue-specific events during the use of biological therapy (20–24). For these reasons and given our focus on new approaches to biological therapy of RCC, we have induced and characterized several new, low-passage transplantable, orthotopic models of mouse RCC. These new tumor isolates have been induced by the nitrosamine compound, streptozotocin, an antibiotic and diabetogenic agent produced by Streptomyces achromogenes (25) that has been reported to induce a high frequency of RCCs in CBA/TfJ mice (26) after a single i.v. dose. Tumors induced in CBA mice by this method have been reported to share some ultrastructural features, including a degree of clear cell morphology, with human RCC (27).

BALB/c and C57BL/6 are two inbred mouse strains commonly used for preclinical tumor studies, and a wide variety of monoclonal antibody reagents are available for studying the mechanisms of immunologically mediated antitumor responses in these strains. Therefore, the studies reported in this paper were performed to determine whether streptozotocin could induce RCCs in BALB/c and C57BL/6 mice, adapt arising tumors to reproducible regrowth as orthotopic transplants, determine the heterogeneity of growth and progression within the new tumors, and to characterize them by histopathological and molecular analyses.

MATERIALS AND METHODS

Mice. BALB/cNcr and C57BL/6Ncr female mice at 6–8 weeks of age were obtained from the Animal Production Area of the National Cancer...
CHARACTERIZATION OF STREPTOZOTOCIN-INDUCED MOUSE RENAL CELL CARCINOMAS

Institute-Frederick Cancer Research and Development Center and maintained under specific pathogen-free conditions. Animal care was provided in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 86-23, 1985).

Induction of Primary Kidney Tumors by Streptozotocin. Two separate experiments using two different regimens were performed to determine the ability of streptozotocin to induce kidney tumors in BALB/c and C57BL/6 mice. In the first study, 180 mice of each strain were injected i.v. with 200 mg/kg streptozotocin (Calbiochem, La Jolla, CA). In the second study, 30 BALB/c mice initially received i.v. injections of 160 mg/kg streptozotocin; 75 days later, they were re-injected i.v. with 110 mg/kg streptozotocin, whereas another 30 BALB/c mice were injected once i.v. with 160 mg/kg. Other cohorts for this study consisted of C57BL/6 mice that received i.v. doses of 375 mg/kg on day 0 or 250 mg/kg i.r. on day 0 only. Mice were monitored for general appearance and were euthanized when moribund. All euthanized mice were grossly evaluated for tumor formation in the major visceral organs, and any tumors appearing in the kidney were resected and either: fixed in neutral buffered formalin, embedded in paraffin, sectioned at 4–6 μm, and stained with H&E; or for histopathological analyses, dissociated for orthotopic repassage

Isolation of Clonal Variants from Streptozotocin-induced Kidney Tumors. Tissue from early orthotopic tumor transplants was obtained, minced into small fragments, and dissociated by shaking in a mixture of hyaluronidase and collagenase. Tissue from early orthotopic tumor transplants was obtained, minced into small fragments, and dissociated by shaking in a mixture of hyaluronidase

Overall vascularity was performed by a recently described technique that allows direct visualization of tumor-associated blood vessels (29). Briefly, mice were euthanized, tails were snipped at the base to provide an open circulatory circuit below the diaphragm, and 3.0 ml of 50% latex suspension [1% heparin (1000 units/ml) and 40% PBS; Carolina Biological Supply, Burlington, NC] was infused into each mouse i.v. via the retroorbital plexus. After 10 min to allow the latex to begin to harden, the vascular supply leading to the tumor-bearing kidney was ligated, and the tumor-bearing kidney was resected en bloc and placed in 10% neutral buffered formalin until further use.

Ribonuclease Protection Assays. Total RNA from streptozotocin-induced tumor cells was isolated using Trizol reagent (Life Technologies, Inc.). Ribonuclease protection assays were performed using the RiboQuant Transcription and RPA kit (Pharmingen) according to the manufacturer’s instructions with the exception that the Multiprobe templates (Pharmingen), and M1 probes were purified using a TE micro select G25 spin column (5 prime–3 prime). The mouse M1 probe was prepared by PCR cloning of M1 fragment into TOPO vector (Invitrogen) using the sequence primer pairs: sense, 5’-GAAGTCCCGTGTCTTTTCC-3’; and antisense, 5’-GATTCAGGGTGCTTGGTGT-3’.

The protected bands were separated on a 5% sequencing gel (National Diagnostics) and analyzed using a Beckman Phosphorimager.

Chromosome Preparation and Chromosome Painting. Standard chromosome preparation and chromosome painting procedures were followed (30). Chromosome painting probes were labeled with biotin-dUTP or digoxigenin-dUTP by degenerate oligonucleotide primed-PCR. After hybridization with mouse chromosome paints, biotinylated DNA probes were detected with avidin coupled with FITC (Vector Laboratories) and digoxigenin-labeled probes with anti-digoxigenin coupled with rhodamine (Vector).

RESULTS

Induction of RCCs by Streptozotocin in BALB/c and C57BL/6 Mice. Several approaches were taken to determine whether streptozotocin could induce RCCs in BALB/c and/or C57BL/6 mice. In the first experiment, 180 mice of each strain received i.v. injections of a single dose of 200 mg/kg streptozotocin. During the first year of the experiment, a number of mice became moribund because of the diabetogenic effects of streptozotocin and/or tumor development. As shown in Table 1, 56 of 180 (31%) BALB/c mice developed single or multiple tumors and were euthanized for analysis between 51 and 92 weeks. A total of 44 mice (24%) exhibited tumors on the kidney [11 renal adenomas (6%) and 33 renal carcinomas (18%)], whereas 36 mice presented with tumors on the lung. Most of the lung tumors were histopathologically characterized as alveolar adenomas, alveolar carcinomas, or bronchiolar carcinomas. However, before transplanted isolates obtained in this study could be adapted to orthotopic transplant and frozen, they were lost to follow-up because of a mouse hepatitis virus infection in our animal facility that necessitated the depopulation of the entire animal facility. Only one RCC and one renal adenoma were detected in C57BL/6 mice, and the carcinoma did not grow upon transplantation and was lost to follow-up.

The data derived from the first experiment demonstrated the feasibility of using streptozotocin to induce primary RCCs in BALB/c mice. Therefore, a second experiment was initiated to reproduce these findings and obtain low-passage, transplantable isolates of mouse RCC for subsequent molecular and immunophysiological studies. This study used a slightly different design in an attempt to minimize the diabetogenic (31) effects of streptozotocin while retaining the ability to induce renal cancers. Thirty BALB/c mice received injections i.v. of streptozotocin (160 mg/kg on day 0 and 110 mg/kg on day 75), and 30 more received injections i.v. of 160 mg/kg streptozotocin on day 0. Because the incidence of tumors in C57BL/6 mice in the experiment reported above was very low and their resistance to streptozotocin-induced toxicity was high (data not shown), we chose to inject higher doses of streptozotocin (250 mg/kg i.r. on day 0 or 375 mg/kg i.v. on days 0 and 75) to mice of that strain. In these studies,
we found that histologically confirmed RCCs arose in 3 of 30 (10%) of BALB/c mice after two i.v. injections and in 1 of 30 (3%) of mice that received a single i.v. injection of streptozotocin. One of the RCCs obtained from the i.v. induction group was designated SIRCC-1, successfully adapted to serial orthotopic transplantation, and was frozen after the 2nd passage. The single primary renal tumor that arose in the group that received streptozotocin by the i.r. route (SIRCC-2) was also histopathologically characterized, successfully repassaged, and frozen for further analysis. Only one RCC was detected in the C57BL/6 mice (in the i.v. group), and it was not successfully adapted to serial transplantation. Overall, these results demonstrate that streptozotocin can reproducibly induce RCCs in BALB/c mice, but that these can be difficult to adapt to orthotopic transplantation. The ability of streptozotocin to induce renal tumors in C57BL/6 mice is very limited, suggesting that mice of this genotype are relatively resistant to this process.

**Heterogeneity of Primary Tumor Growth and Metastasis Formation by Cloned Sublines from SIRCC-1.** One of the limitations of currently available transplantable mouse RCCs is their very rapid growth rate in vivo, which results in a relatively brief time interval between the establishment of well developed primary tumors and the subsequent death of the mouse. These rapidly growing mouse tumors are thus of limited practical value for the study of some types of biological therapy, such as therapeutic vaccines, because the interval available for development and function of adaptive immune responses is inadequate; by the time an immune response can be developed, the mice are close to death and/or the level of tumor burden is insurmountable. Our decision to further characterize the SIRCC-1 isolate was partially based on its relatively slow progression in vivo, whereby a dose of \(1 \times 10^5\) cells injected orthotopically (i.r.) resulted in a mean survival time of \(\sim 3\) months (Table 2), but with metastasis to the lungs in 100% of the mice by days 17–19.

Because tumors are often heterogeneous because of inherent genetic instability and therefore can contain subpopulations of cells with somewhat different biological characteristics (17, 18) the SIRCC-1 tumor was cloned in vitro, and 11 resulting sublines were evaluated for RCC morphology by histopathology, in vivo growth rates, and propensity for metastasis.

The histopathological analyses for SIRCC-1 and SIRCC-2 and all 11 sublines derived from SIRCC-1 revealed a morphology consistent with RCC, but different isolates exhibited varying degrees of differentiation and predisposition for attempts at tubule formation. H&E-stained tissue sections showing the actual morphology of the SIRCC-1 and SIRCC-2 parental tumors and several SIRCC-1-derived sublines (1, 2, 3, 5, 11, 13, 15, 18, and 19) are shown in Figs. 1 and 2. The photomicrographs in Fig. 1 show the histopathological characteristics of the SIRCC-1 and SIRCC-2 parental tumor isolates. Specifically, in Fig. 1, A and B show \(\times 75\) and \(\times 300\) magnifications of the SIRCC-parental tumor, whereas Fig. 1C shows a \(\times 300\) magnification of a metastatic tumor nodule that arose in the lung from this primary tumor. In Fig. 1, D–F show the primary SIRCC-2 tumor in the kidney (Fig. 1D) and a metastasis in the lung at \(\times 75\) (Fig. 1E) and \(\times 300\) (Fig. 1F) magnifications. Both the SIRCC-1 and SIRCC-2 primary tumors (Fig. 1, B and D) showed evidence of rudimentary tubule formation. The photomicrographs shown in Fig. 2 (all at \(\times 300\)) illustrate the histopathological heterogeneity of sublines derived from the SIRCC-1 parental isolate. Clones 2 (Fig. 2A), 18 (Fig. 2C), and 19 (Fig. 2D) are moderately differentiated with evidence of tubule formation. Clone 8 (Fig. 2E) is also moderately differentiated, exhibits abundant eosinophilic cytoplasm, and contains large giant tumor cells. Clones 15 and 13 (Fig. 2, B and F), respectively, are less differentiated, and clone 13 (Fig. 2F) has a distinctive morphology characterized by areas of elongated tumor cells and pleomorphic tumor nuclei. In addition to the morphological heterogeneity of these sublines, the results shown in Table 2 demonstrate considerable heterogeneity in the growth of these SIRCC-1-derived sublines after orthotopic implant of \(1 \times 10^5\) cells into syngeneic BALB/c mice. Specifically, SIRCC-1-2, 1.5, 1.7, 1.10, 1.15, and 1.16 show mean survival times of \(\sim 70\) days or less, whereas SIRCC-1-8, 1.11, 1.13, 1.18, and 1.19 show mean survival times of about 80–100 days. In general, the SIRCC-1 sublines that lead to the shortest survival times are usually among the most efficient at forming well-established primary tumors (a primary tumor index of

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Table 1  Development of tumors in BALB/c and C57BL/6 mice after i.v. injection of streptozotocin

<table>
<thead>
<tr>
<th>Mouse strain injected</th>
<th>Overall tumor incidence (%)</th>
<th>Organ</th>
<th>Tumor type</th>
<th>Total obtained from 180 injected</th>
<th>% of total injected</th>
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<tbody>
<tr>
<td>BALB/c</td>
<td>56/180 (31)</td>
<td>Kidney</td>
<td>–</td>
<td>44</td>
<td>24</td>
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<tr>
<td></td>
<td></td>
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<td>11</td>
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<td></td>
<td>Carcinoma</td>
<td>33</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lung</td>
<td>–</td>
<td>36</td>
<td>20</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Adenoma</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carcinoma</td>
<td>27</td>
<td>15</td>
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<tr>
<td></td>
<td></td>
<td>Liver</td>
<td>–</td>
<td>3</td>
<td>2</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Adenoma</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Carcinoma</td>
<td>1</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lymph Node</td>
<td>–</td>
<td>3</td>
<td>&lt;2</td>
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<td></td>
<td></td>
<td></td>
<td>Lymphoma</td>
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<td>1</td>
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<tr>
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<td></td>
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<td>Metastatic carcinoma</td>
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<tr>
<td></td>
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<td>Other</td>
<td>Histiocytic sarcoma</td>
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<td>Osteocarcinoma</td>
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<td></td>
<td>Intraperitoneal carcinoma</td>
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<td>Pleomorphic tumor</td>
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<td>&lt;1</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Histiocytic sarcoma</td>
<td>1</td>
<td>&lt;1</td>
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<tr>
<td>C57BL/6</td>
<td>9/180 (5)</td>
<td>Kidney</td>
<td>–</td>
<td>2</td>
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<td></td>
<td></td>
<td>Adenoma</td>
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<td>Lung</td>
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<td></td>
<td></td>
<td>Histiocytic sarcoma</td>
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* A total of 180 BALB/c and 180 C57BL/6 mice received i.v. injections of streptozotocin on day 0 (200 mg/kg).
CHARACTERIZATION OF STREPTOZOTOCIN-INDUCED MOUSE RENAL CELL CARCINOMAS

Mutation Analysis of SIRC-1 Sublines. Some forms of human kidney cancer are associated with defined genetic mutations. The molecular basis for most inherited and sporadic clear cell RCC is inactivation of the VHL tumor suppressor gene (5). In addition, some nitrosamine-induced RCC in Wistar rats are also associated with VHL gene mutations (32), whereas some nitrosamine-induced RCC in F344 rats have been associated with overexpression of the K-Ras and N-Ras oncogenes (33). Therefore, we performed an initial analysis for VHL and Ras mutations in the SIRC-1 sublines. No sequence variants in the VHL gene were detected in exons 1, 2, or 3 for any of the subclones, whereas an analysis of Ras gene expression revealed no mutations in any of the clones at codons 12, 13, and 61, which are the sites of most frequent Ras mutations. These results demonstrate that the original SIRC-1 tumor did not arise as a result of VHL or Ras mutations and suggest that other genetic changes were responsible for the development of this tumor.

Karyotypic Analysis of SIRC-1 Parental Isolate and Selected Sublines. Because mutations in the VHL tumor suppressor gene or the Ras oncogene were not detected, a preliminary karyotype was performed on several of the SIRC-1 sublines (Table 3). The base karyotype for the parental and SIRC-1 sublines is polyploid. The mouse diploid number is 2n = 40 (19 autosomes and the sex chromosomes), whereas all cell lines exhibited high chromosome number >75. The very pleiotropic (Fig. 2F) 1.13 subline exhibited a metaphase chromosome number >110. The majority of chromosomes in each subline appear normal with G-banding, but all sublines contain at least some unclassified chromosomes. However, a submetacentric marker chromosome is consistently present, and chromosome painting

>3 by days 33–36), whereas those that are less aggressive also are somewhat slower (primary tumor index generally ~3 or less with the exception of the parental line) in forming large primary tumors (Table 3). An exception to this is the 1.8 subline, which is one of the more efficient sublines for primary tumor formation, yet affords the longest survival time. Furthermore, the propensity for the establishment of lung metastases also differs between isolates, with sublines 1.2, 1.5, and 1.7 being highly efficient in rapidly forming metastases (median number >100 by days 17–36), whereas sublines 1.10, 1.11, 1.18, and 1.19 were much less efficient (median numbers between 0 and 20 in the same time interval). Sublines 1.13 and 1.8 may be of some additional interest because they rapidly form metastases (by day 18) that then partially regress by day 36. In general, the faster growing sublines (1.2, 1.5, 1.7, 1.10, 1.15, and 1.16) also exhibited a propensity for an appreciable increase in the number of lung metastases between days 17–19 and 33–36, whereas most of the slower growing tumors (1.8, 1.11, 1.13, 1.18, 1.19, and the SIRC-1 parental line) exhibited either a general trend toward a reduction in metastases between days 17–19 and days 33–36 or a relatively poor ability to form lung metastases at either time point.

Overall, these data demonstrate that the parental SIRC-1 tumor line is composed of subpopulations of cells that differ in their aggressiveness (as defined by primary and metastatic growth) in vivo and may therefore also differ with regard to other biological properties, such as the expression of genes that may regulate their own growth, functions of various host cells, or some critical physiological interplay between tumor and host tissue that influences the biology of tumor progression.

Fig. 1. H&E photomicrographs of the SIRCC-1 (A–C) and SIRCC-2 (D–F) parental primary tumor isolates and spontaneously developed lung metastases. A, large tumor mass within the kidney (×75). B, high magnification of transplantable renal adenocarcinoma showing a few attempts at tubule formation (arrow). C, metastatic renal adenocarcinoma in the lung (×300). D, renal adenocarcinoma with areas of tubule formation (arrow; ×300). E, metastatic renal adenocarcinoma in the lung. Note uniform population of moderately differentiated tumor cells (×75). F, higher magnification of metastatic tumor in E showing solid pattern with no tubule formation (×300).

Fig. 2. H&E photomicrographs of sublines derived from the SIRCC-1 parental isolate. A: clone 2, moderately differentiated renal adenocarcinoma showing tubule formation (arrow; ×300). B: clone 15, less differentiated renal adenocarcinoma showing large tumor cells and no tubule formation (×300). C: clone 18, moderately differentiated renal adenocarcinoma (×300). D: clone 19, moderately differentiated renal adenocarcinoma showing tubule formation in center (arrow; ×300). E: clone 8, moderately differentiated renal adenocarcinoma with large giant tumor cells and abundant eosinophilic cytoplasm (×300). F: clone 13, poorly differentiated renal adenocarcinoma with areas of elongated tumor cells and pleomorphic tumor cell nuclei (×300).
shows that it derives from a fusion between mouse chromosomes 16 and 19 (Fig. 3). Thus, there is considerable karyotypic variability between the clones, as might be expected from the functional heterogeneity shown in Table 2. However, the consistent presence of the fusion between chromosomes 16 and 19 may support a common origin for these phenotypically and functionally disparate sublines. The consistent detection of the 16 of 19 submetacentric chromosomes may also ultimately provide some insight into the genomic events responsible for development of these renal cell cancers.

Neovascular Development in Tumors Derived from Various Isolates of SIRCC-1. Because the SIRCC-1 isolates exhibit appreciable differences in their overall aggressiveness, efficiency in forming primary tumors, and the propensity for formation of spontaneous lung metastases, studies were performed to determine whether there were detectable gross or molecular differences in the angiogenic phenotype of these sublines. The efficiency of these different RCC sublines in forming grossly visible tumor-associated neovascularatures was demonstrated using a latex infusion technique developed recently in our laboratory for this purpose (29). The results shown in Fig. 4 reveal efficient development of extensive tumor-associated neovascularatures in the parental SIRCC-1 tumor and the SIRCC-1 sublines. Specifically, in Fig. 4, A and B show the neovascularization of the SIRCC-1 parental isolate at day 30 after implantation of $1 \times 10^5$ tumor cells. Fig. 4A shows foci of tumor growth (↓) associated with an extensive and convoluted neovascularature (↓↓), whereas Fig. 4B shows a higher magnification of another SIRCC-1 parental tumor with a very highly branching vasculature (↓↓) feeding a large vessel (↓↓) that ultimately leads to a large area of intrarenal tumor growth (↓↓↓). In addition, Fig. 4B also shows several small discrete areas of tumor growth that are virtually covered with a finely fenestrated network of new tumor-associated capillaries (↓↓↓↓). Also visible in Fig. 4B is an area of kidney tissue showing the normal vasculature of the glomeruli (↓). In Fig. 4, C and D show low and higher power views of the new vasculature (↓) that has developed at day 22 to support the growth of the SIRCC-1.18 subline. The vasculature associated with the SIRCC-1.15 subline at day 36 (Fig. 4, E and F) shows a finely fenestrated, highly developed vasculature (↓) with numerous large vessels leading to areas of tumor growth (↓↓↓). Overall, these results illustrate the highly developed neovascularature that is associated with progressive growth of the SIRCC-1 tumor and its sublines and highlights this feature as a target for therapeutic intervention.

Molecular Phenotype of Angiogenesis-associated Genes in SIRCC-1 Sublines. The results presented in Fig. 4 illustrate the extensive neovascularature that develops in the parental SIRCC-1 tumor and its subline isolate to support the progressive growth of these tumors. The protracted survival times associated with all of these lines

<table>
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<tr>
<th>Subline</th>
<th>General growth characteristics</th>
<th>Mean no. of metastases on days 17–19 or 33–36</th>
<th>Chromosome</th>
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<tr>
<td>Parent</td>
<td>Slow</td>
<td>88/31</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Fast</td>
<td>137/169</td>
<td>84</td>
</tr>
<tr>
<td>5</td>
<td>Fast</td>
<td>3/334</td>
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</tr>
<tr>
<td>7</td>
<td>Fast</td>
<td>270/433</td>
<td>83</td>
</tr>
<tr>
<td>8</td>
<td>Slow</td>
<td>176/87</td>
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<td>114</td>
</tr>
<tr>
<td>18</td>
<td>Slow</td>
<td>0/15</td>
<td>77</td>
</tr>
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</table>

*The SIRCC-1 parental line and several sublines were either injected i.v. (1 × 10⁵ cells) in vivo for assessment of metastatic capacity at days 17–19 or days 33–36 or cultured in vitro for standard chromosome preparation and analysis, which was performed in either duplicate or triplicate for each cell line.
after transplantation and the very long survival times associated with some sublines suggest that these tumor models might be particularly useful for the detection of antineovascular therapeutics. The results shown in Fig. 5 present expression profiles for a number of angiogenesis-associated genes (34), as well as two antiangiogenic CXC chemokines (Mig and IP-10) that have been shown to be negative regulators of hematopoiesis. Tissue isolated from tumors growing in vivo (Fig. 5, Lane 1) shows clear expression of most proangiogenic genes (vascular endothelial growth factor, fibroblast growth factor 2, and transforming growth factor β1, and transforming growth factor β2) and several receptors for proangiogenic mediators (vascular endothelial growth factor receptors Flt1 and Flt4 and the angiopoietin R Tiel). In contrast, there was no or little basal in vivo expression of the antiangiogenic Mig and IP-10 chemokine genes. These results suggest that under normal conditions, the parental SIRCC-1 tumor and its clonal sublines exhibit an in vivo gene expression profile favorable for angiogenesis. Interestingly, most of the angiogenesis-associated genes were not detectable when the cells were cultured in vitro (Lane 2), and they were not upregulated by IFN-γ in the isolated SIRCC-1 sublines. The only exceptions to this were the ability of IFN-γ to up-regulate vascular endothelial growth factor, fibroblast growth factor 1, and Flt1 in SIRCC-1 parental cells. In many experimental tumor models, IFN-γ plays a significant role in the regulation of tumor growth by endogenous or externally induced immune responses (29, 35, 36), and at least some of the IFN-γ-induced antitumor effects can result from an up-regulation of various chemokine genes (29, 37–39). Culture of the SIRCC-1 parental cells and SIRCC 1.2 and 1.8 sublines in IFN-γ resulted in strong up-regulation of both Mig and IP-10 genes by 6 h (Fig. 5, Lane 3) and/or 24 h (Fig. 5, Lane 4). Some induction of both Mig and IP-10 genes by IFN-γ can also be detected by 24 h in sublines 1.15 and 1.18. Overall, these results show that the SIRCC-1 parental tumor and its subline isolates consistently express a gene profile that is skewed in favor of angiogenesis. However, the ability of IFN-γ to induce expression of the antiangiogenic Mig and/or IP-10 genes in all sublines suggests that IFN-γ-dependent biological therapies may be able to alter the angiogenic phenotype of these tumors and perhaps result in disease stability or tumor regression.

**DISCUSSION**

The development of new preclinical experimental approaches to the treatment of RCC has been limited by the paucity of available animal models with which to study the etiology and therapeutic response of this tumor type. In particular, the usefulness of the available mouse models has been limited by a variety of factors that may render them less than optimal for these purposes. For example, most widely used tumors arose many years ago and have been serially transplanted for hundreds of passages in heterotopic sites. Such tumors can be far removed from the histocompatibility background of their initial host of origin and are often highly aggressive and rapidly growing, which is in contrast to the more indolent nature of some human cancers. Rapidly growing tumors can limit the preclinical experimental study of some newer forms of biological therapy, including vaccine or dendritic cell-based approaches that require considerable time to engage an adaptive immune response. Furthermore, repeated heterotopic passage may alter the biology of tumors by removing them from the tissue-specific milieu of their organ of origin and alter critical tissue-specific events that may contribute to the success or failure of biological therapies. In this regard, the perceived value of various models has been re-evaluated by the growing awareness that the organ microenvironment can exert a pronounced influence on the growth and metastasis of tumors by providing appropriate growth factors, tissue degradative enzymes that favor metastatic spread, enhanced expression of drug resistance mechanisms, and favorable disposition for neovascularization (reviewed in Refs. 17, 18, and 40). Such orthotopic models have also been reported to favor the development of hetero-

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Fig. 3. Fluorescence in situ hybridization analysis of chromosomes from SIRCC-1 parental tumors. Fluorescence in situ hybridization to metaphases of the parental cell line with mouse chromosome paints reveals that the submetacentric marker chromosome (arrows) is derived from a fusion of mouse chromosome 16 (red) and 19 (green). The hybridization signals from these two mouse chromosomes are separated by a large, nonhybridized centromere.
geneous subpopulations of tumor cells, as are often observed in human cancers (41), and to be most suitable for the assessment of new biological (40) and gene therapy (19) approaches. These findings have re-emphasized the need for orthotopic preclinical tumor models that can provide appropriate insight into critical biological events and important tissue-specific events during the use of biological therapy (20–24, 40).

The results presented in our studies extend the previous observa-

Fig. 4. Gross visualization of tumor-associated neovasculatures during growth of SIRCC-1 primary tumors. All mice received injections i.r. on day 0 with $1 \times 10^7$ tumor cells from either SIRCC-1 parental tumors or selected subline isolates. Mice were euthanized at various times as detailed below and infused with liquid latex as described in “Materials and Methods”; tumor-bearing kidneys were excised and placed in formalin; and photographs were taken at a later date. A and B show that tumor-associated vasculatures developed by day 30 in the parental SIRCC-1 tumor. A, small foci of tumor growth (↓) associated with extensive neovasculature (↓↓↓). B, a higher magnification of another SIRCC-1 tumor showing a highly branched vasculature (↓) feeding a large neovessel (↓↓↓) leading to a large area of tumor growth (↓↓↓). Also shown are several foci of tumor growth covered with a complex microvasculature (↓↓↓↓↓↓) and an area of normal kidney tissue showing the normal vasculature of the glomerululi (↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓↓→
tions of Hard (26), who reported the ability of streptozotocin to efficiently induce renal carcinomas in CBA/T6 mice, by demonstrating induction of renal carcinomas in BALB/c mice. Our results also demonstrate an inherent resistance of C57BL/6 mice to streptozotocin-induced renal carcinomas. The molecular mechanism for the induction of RCC in BALB/c mice remains undefined, although our studies suggest that it is not dependent on either VHL mutations associated with human RCC (4, 5) or N-nitroso-dimethylamine-induced RCC in rats (32), or Ras mutations associated with methyl (methoxymethyl) nitrosamine in F344 rats (33). Our results do show that even heterogeneous sublines derived from the original SIRCC-1 tumor consistently present with submetacentric fusions of chromosomes 16 and 19. Further studies are in progress to investigate the possibility of mutations in other genes or other genetic events that may contribute directly to the etiology of these tumors.

The SIRCC-1 parental line and its sublines offer several advantages as a preclinical model for RCC. Specifically, the inherent heterogeneity in the cellular composition of the parental line, as evidenced by the widely differing growth and metastatic patterns of the SIRCC-1-derived sublines, provides a setting in which experimental therapies can be rigorously tested against a backdrop of widely varying tumor cell phenotypes. This would seem to be at least somewhat analogous to the documented biological heterogeneity of many human tumors. Furthermore, the availability of the various phenotypically distinct SIRCC-1-derived sublines may provide an opportunity to identify factors that could contribute to susceptibility or resistance to different forms of biological therapy. One possible opportunity for such studies could be in the evaluation of antiangiogenic therapies. Our results have shown evidence that the constitutive in vivo proangiogenic phenotype of these tumor sublines can be altered by exposure to IFN-γ to include expression of known antiangiogenic genes (e.g., Mig and IP-10). Furthermore, the clear propensity of some sublines to grow and metastasize progressively, whereas others show either a poor ability to initially form spontaneous metastases or show a stabilization/regression of the number of metastases, suggests that these lines may be useful for investigating the regulation of metastasis formation and progression. Alternatively, the disparate growth rates and metastatic frequencies of these various sublines also may suggest a differential ability of the host immune response to regulate their progression. Such effects could be a result of varying immunogenetic differences between the various sublines, suggesting possible qualitative or quantitative differences in expression of tumor-associated antigens.

Overall, these SIRCC-1 tumor isolates provide newly derived, slow-growing tumors that are completely histocompatible with the syngeneic mice of origin. These features provide new opportunities for the study of antiangiogenic and adaptive immune-based approaches to cancer therapy in a setting where the expected physiological relationship between the tumor and its appropriate tissue of origin is preserved.

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