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Establishment and Characterization of Five New Human Renal Tumor Xenografts

A. J. M. C. Beniers,* W. P. Peelen,*
H. E. Schaafsma,† J. L. M. Beck,†
F. C. S. Ramaekers,† F. M. J. Debruyne,*
and J. A. Schalken*

From the Departments of Urology and Pathology,† University Hospital Nijmegen, Nijmegen, The Netherlands*

Ten different human renal cell carcinoma (RCC) primary tumors were xenografted into BALB/c nu/nu mice. Five of the tumors (NU-10, NU-12, NU-20, NU-22, and NU-28) gave rise to serially transplantable tumors that were further characterized. Histology, DNA index, immunohistochemical characteristics, growth rate, and clonogenic potential were followed from primary tumor to the 5th to 15th transplant passage. Only one of the tumors (NU-20) showed remarkable instability for all tested parameters in the first five transplant passages. Histology of the other tumors was essentially the same to the histology of the primary tumors, although differences between human and host-derived vessels were apparent. DNA index values in general showed a trend toward an aneuploid character of the xenografts. Immunohistochemical analyses showed a loss of intensity of staining but a concomitant rise in the fraction of positively staining cells with antibodies against cytokeratins, vimentin, tumor-associated antigens, and human leukocyte antigen (HLA) class I antigens. Human leukocyte antigen class II antigen expression showed a loss of intensity as well as a decrease in the fraction of positive cells. Tumor doubling time was lowest in transplant passage number 0, and stable growth was noticed in transplant passages 1 through 4. Clonogenic potential of four of the lines was higher for the xenografts than for the primary tumors. The authors conclude that, on xenografting, histologic characteristics of the primary tumor are essentially conserved. Progression in the first transplant passages, however, results in tumors with a more aggressive character. (Am J Pathol 1992, 140:483–495)

Human renal cell carcinoma (RCC) rarely responds to conventional therapy with cytostatic drugs or hormones and clinically this tumor seems to have a unique immunobiology as evidenced by the occasional spontaneous regressions of its metastases,[1] noticed in about 0.8% of the cases. This suggests that host factors may be capable of modifying the course of RCC, which is further evidenced by the 20% to 30% response rates found after therapy with different human cytokines. Therefore studying antineoplastic effects against human RCC in a model system will never be able to mimic the human situation in all its aspects, because in *in vitro* systems, only direct effects can be measured, and in *in vivo* systems host factors, to some extent, will always impair histologic or immunologic aspects of the tumor. Studying syngeneic tumors in rodent systems circumvents the problem of the immunologic aspects, but these tumors can differ from human tumors in their biologic characteristics and so will not be highly representative of the human situation.

Since the first report on cultivation of RCC cell lines by Richter and Akin,[2] many reports described the establishment and characterization of new RCC cell lines.[3] *In vitro* cultivation, however, usually is associated with progression to a more aggressive status because of the intrinsic genetic instability of the tumor. Transplantation of human tumors *in vivo* became possible after initial observations that athymic nude mice could support progressive growth of human tumors,[4] and nude mice have since also been used to establish human RCC xenografts.[5,6] Not all tumors thereby readily form transplantable tumors, and immunologic reactions by the nude mouse against the human tumors[7] may be responsible for at least part of the xenotransplantation failures, which is evidenced by the fact that immunosuppression of nude hosts enhances the xenotransplantability of human tumors.[8,9] Tumor characteristics also may play a very important role in the transplantability of the tumors and therefore may determine the success rate of xenotransplantation.

To evaluate changes in tumor characteristics during

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Address reprint requests to Dr. J. A. Schalken, Urological Research Laboratory, Department of Urology, University Hospital Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands.

xenotransplantation leading to the selective establishment of RCC xenografts, we subcutaneously transplanted tumor pieces into the right flank of nude mice, directly after excision of the primary tumor. We compared the tumor lines with their primary tumors and evaluated their establishment, not only by means of histology or DNA index, but also by means of various other parameters such as immunohistochemical characteristics, tumor doubling times, and clonogenic potential.

Materials and Methods

Tumors

Ten different primary human renal tumors were tested for their xenotransplantability in the nude mouse. Five tumors (NU-10, NU-12, NU-20, NU-22, and NU-28) gave rise to serially transplantable tumors (>5 passages), one tumor did not give rise to a growing tumor at all, and the other four tumors stopped growing in the first to third transplant passages. A description of the tumor history is given only for the serially transplantable tumors.

NU-10

This tumor originates from a 78-year-old man with long-term complaints of pain in the right flank and hematuria. Examination disclosed a T3 tumor with diameter of 9.5 cm in the right kidney and multiple lung metastases. Lung metastases showed spontaneous regression after nephrectomy, but 1 year later the patient was found to have multiple brain metastases. Material for xenotransplantation was taken from the primary tumor.

NU-12

This tumor originates from a 53-year-old man with short-term complaints of pain in the left flank. Examination disclosed a yellow-colored T3 tumor with a maximum diameter of 7 cm and multiple white to brown-colored satellite tumors with maximum diameters of 1 cm in the left kidney. Tumor growth was also localized in a hilar lymph node, and two metastases were apparent in the left lung. Four months after nephrectomy, the lung metastases in the left lung had disappeared completely without any therapy, but a 7-mm diameter metastases had formed in the right lung. Four months later also this metastasis had disappeared completely. One year after nephrectomy, however, two local recurrences of 4 and 2 cm and two new lung metastases had formed. Material for xenotransplantation was taken from the 7-cm diameter primary tumor.

NU-20

This tumor originates from a 41-year-old man with short-term pain complaints in his right flank. Examination disclosed a T2 tumor with a maximum diameter of 8.5 cm in the right kidney and two lung metastases in the right lung. Three months after nephrectomy, a slight progression of these metastases could be measured. One year after nephrectomy, the lung metastases were clearly progressed. No new lung metastases had formed, but multiple liver metastases were present at that time. Material for xenotransplantation was taken from the primary tumor.

NU-22

This tumor originates from a 46-year-old man with short-term complaints of coughing and cachexia. Examination disclosed a cystous T3 tumor with a maximum diameter of 9 cm in the right kidney. Metastases already existed in the retroperitoneal lymph nodes, adrenal, and both lungs. Possible small metastases also existed in the liver. Three months after nephrectomy, this patient also was found to have brain metastases. Material for xenotransplantation was taken from the primary tumor.

NU-28

Material for xenotransplantation was taken from a lung metastasis of 6 cm in diameter grown after interferon–alpha/gamma therapy in a patient with a long history of a tumor in the right kidney. This 16-year-old boy entered the clinic after complaints about erythrocyturia, and examination disclosed a T2 tumor with a maximum diameter of 5 cm in the right kidney, with retroperitoneal lymph node metastases as well as metastases in both lungs. Lung metastases grew progressively after radical nephrectomy and removal of retroperitoneal lymph nodes. Positive intrathoracic lymph nodes (mediastinum) were removed from the right lung 5 months after nephrectomy, and 4 months later positive mediastinal lymph nodes were removed. Six months later, no macroscopically visible tumor mass was found anymore. Ten months after the last removal of positive nodes, however, small metastases were visible again in abdomen and mediastinum. The patient then received a combination of alpha- and gamma-interferon, and 8 weeks after start of the therapy, dimensions of the 'intrapulmonale' metastases had stabilized, whereas the retroperitoneal tumor mass had clearly regressed. Nine months after therapy, the retroperitoneal tumor mass had completely disappeared but the intrapulmonale metastases showed a minor progression. Three months after the therapy had stopped, there was a clear progression of this lesion in the left lung

again, and this lesion with a maximum diameter of 6 cm was removed surgically. Material for xenotransplantation was taken from this metastasis. Ten months after this, the patient was still free of any macroscopically visible metastases.

Mice

Six-week-old male BALB/c nu/nu mice (Bomholtgård, Ry, Denmark) were used for the xenotransplantations. Husbandry and feeding were the same as described previously.[10]

Xenotransplantation

Tumor material was taken as soon as possible after nephrectomy. Tumor pieces were transported in unsupplemented RPMI-1640 medium (Gibco, Paisly, United Kingdom) and after removal under sterile conditions of necrotic and hemorrhagic fragments and connective tissue, vital tumor pieces of about 1 × 1 × 1 mm were cut from the tumor mass. One tumor piece was transplanted subcutaneously into the right flank of each ether-anesthesized mouse after making a 5-mm dorsal incision into the skin. After transplantation, the incision was closed with a metal clip (agrave Michel, Instruvet, Amerongen, The Netherlands). Five mice were used for each transplantation. Transplant generations were performed under the same conditions as for the primary tumors after the transplanted tumor pieces reached dimensions of about 1 cm. Corresponding fragments of primary tumors and all serial passages were either fixed in formalin or frozen directly in liquid nitrogen for further histologic analyses. For analyses of DNA index and testing of the clonogenic potential, fresh tumor material was used.

Evaluation of Tumor Growth

Each tumor was measured twice a week with a sliding caliper in three dimensions: maximum diameter (L), diameter at right angles to the length (W), and thickness (H). The volume of the tumors, expressed as the tumor size index (TSI) was calculated by the following equation:

$$TSI = L \times W \times H/2$$

Histopathologic Examination

For histopathologic evaluation of the tumors, surgical specimens and fragments of the different passages in the nude mice were fixed in 4% formaldehyde and embedded in paraffin. Sections (5 μ) were stained with hematoxylin and eosin (H&E) by standard techniques and evaluated by light microscopy.

Preparation of Single-cell Suspension

When tumors reached sizes of about 1 cm^3, the mice were killed by cervical dislocation, and tumor material was suspended in unsupplemented RPMI-1640 medium. After careful removal of central necrosis, blood clots, and connective tissue, the tumors were cut into pieces of about 3 to 4 mm^3. The tumor pieces then were minced with scissors into a 300-μ metal sieve and continuously washed with RPMI-1640 medium in a petri dish. The minced tumor tissue then was passed twice through a 40–70 nylon filter (Ortho Diagnostics, Beerse, Belgium) to obtain a single-cell suspension. The cells were centrifuged at room temperature at 400*g* for 5 minutes, after which the supernatant was discarded. After resuspension of the cell pellet, cell density and viability were determined using the trypan blue exclusion test and simultaneously counting colored and uncolored cells using a Bürker Türk hemocytometer.

Flow Cytometric (FCM) Analysis of Cellular DNA Content

Suspensions of mechanically disaggregated tumor tissues were centrifuged at 400*g* for 5 minutes. The supernatant was discarded and, under constant shaking, 70% ethanol (−20°C) was added rapidly to the cell pellet. The final concentration was about 3.10^6 cells/ml. Approximately 1.10^6 cells in 70% ethanol were centrifuged at 400*g* for 5 minutes, and the cell pellet was resuspended in 1 ml of a propidium iodide (PI) solution (20 mg/liter A-grade Calbiochem-Boehringer, La Jolla, CA) in 150 mmol/l (millimolar) sodium phosphate buffer, pH 7.4). One milliliter of a 1% RNase solution (RNase type A, Sigma, St. Louis, MO; in 150 mmol/l sodium phosphate buffer pH 7.4) was added to 1 ml of this cell suspension and subsequently incubated for 10 minutes at 37°C. Finally, the cell suspension was filtered through a 49-μ filter, and the cells were kept in the dark at room temperature before FCM analysis.

A cytofluorograph 50H (Ortho, Westwood, MA) equipped with an argon ion laser (Spectra Physics, Mountain View, CA) was used for the DNA measurements. The fluorochrome PI was excited at 488 nm, and fluorescence was measured using a 630-nm longpass filter. Data were recorded as 2048 channel histograms and stored in a PDP 11/34 computer (Digital, Marlboro,

MA) for subsequent data analyses. For ploidy measurements, chicken red blood cells (CRBC) were added to the samples as internal standard, and a mixture of human lymphocytes and CRBC served as external standard. The DNA content was expressed as a DNA index (DI) with human lymphocytes equivalent to 1.00. The coefficient of variation for the G0/G1 peak of malignant and nonmalignant cells ranged from 3% to 6%.

Human Tumor Colony-forming Assay

For the detection of the growth potential of tumor cells in soft agar, a modified two-layer soft agar culture method as originally described by Hamburger and Salmon was used.[11] Briefly, tumor cell suspensions were plated at a concentration of 5.10^5 tumor cells per dish for primary tumor cell suspensions or 1.10^5 for xenograft cell suspensions in the upper layer of the two-layer agar culture method, in which the percentages of agar in the bottom and top layers were 0.5% and 0.3%, respectively. The cells were cultured immediately after preparation of the single-cell suspension, and growth potential was quantified using an Omnicon Fas II automated colony counter (Milton Roy Inc., Rochester, NY). A day 0 count directly after plating was made to correct for occasional cell clusters. For dynamic colony growth development, we used the 'temporal growth pattern' method, giving an estimation of growth over a certain period of time. Briefly, 24 dishes were plated from each sample to allow frequent colony counting without having to return the counted dishes to the incubator. The dynamic growth of colonies was detected and followed by counting two or three dishes twice a week over a period of 4 to 6 weeks. Using this method, the growth potential in culture is described by growth curves, indicating the number of colonies counted as a function of time.

Immunohistochemistry

Two-micron (immunofluorescence) or 4-μ (immunoperoxidase) thick frozen sections of tumor tissue that was stored in liquid nitrogen were dried in the air during the night and next fixed in acetone (10 minutes, room temperature). The indirect immunofluorescence technique was performed as described by Ramaekers et al.[12] Briefly, after being washed in phosphate-buffered saline (PBS) for 10 minutes, the tissue sections were incubated with 10% normal goat serum (NGS) in PBS at room temperature (30 minutes) and subsequently with the primary antiserum for 1 hour. After repeated washings in PBS (3 times, 10 minutes), the fluorescein isothiocyanate conjugated goat anti-mouse IgG and goat anti-rabbit IgG (1:25 in 10% NGS, Nordic, Tilburg, The Netherlands) were applied to the monoclonal and polyclonal antibodies conjugated sections, respectively. After incubation for 30 minutes and extensive washing in PBS (3 times, 10 minutes), the tissue was mounted in Gelvatol (Monsanto, St. Louis, MO) containing 100 mg/ml 1.4-diazobicyclo-(2.2.2)-octane (DABCO, Janssen Pharmaceutica, Beerse, Belgium). In control experiments, 10% NGS was used instead of the primary antiserum.

The direct immunoperoxidase technique was performed as follows: the acetone-fixed slides were incubated for 1 hour at room temperature with direct peroxidase (HRPO)-labeled monoclonal antibody (MAb). After repeated washings in PBS (3 times, 10 minutes), 3-3'-diaminobenzidine (DAB; 6 mg/10 ml, 0.65% imidazole in PBS) and hydrogen peroxide to a final concentration of 0.01% was applied. After incubation for 5 minutes and extensive washing with tap water, the slides were counterstained with hematoxylin, and mounted with Permount (Fisher Scientific, New York, NY).

The following antibodies directed against human intermediate filament proteins, human leukocyte antigen (HLA) class I and II antigens, and tumor-specific antigens were used in this study:

1) pKER. An affinity-purified rabbit antiserum to human skin keratins that reacts with virtually all epithelial tissues, but not with nonepithelial tissues.[12] This broadly cross-reacting polyclonal antibody was used for the detection of the epithelial nature of the tumors. It was also used for comparison of the staining with the monoclonal antibodies against cytokeratins, to ascertain the distinction between human tumor and mouse stromal cells.

2) pVim. An affinity-purified rabbit antiserum to bovine lens vimentin.[12] This broadly cross-reacting polyclonal antibody was used for the detection of the intermediate filament protein present in mesenchymal cells. It also was used for comparison of the staining with the RV 202 monoclonal antibody, to ascertain the distinction between the human tumor and mouse stromal cells.

3) RCK 102 (IgG1). A cytokeratin antibody that recognizes cytokeratins 5 and 8 and as a result stains virtually all epithelial tissues.[13]

4) RCK 105 (IgG1). A MAb reacting with cytokeratin 7, staining a subgroup of glandular epithelia and their tumors, next to transitional bladder epithelium and bladder carcinomas. This antibody does not react with renal cell carcinoma (Grawitz tumors).[14]

5) RCK 106 (IgG1). A MAb reacting with cytokeratin 18. In general, this antibody recognizes columnar epithelial cells from digestive, respiratory, and urogenital tracts, endocrine and exocrine tissues, and mesothelial cells, as well as their tumors. Generally no reaction is found in squamous epithelia.[13]

6) RV 202 (IgG1). A MAb reacting with vimentin, specific for mesenchymal cells.[13]

7) W6.32 (IgG2a). A MAb directed against the human histocompatibility leukocyte antigens (HLA) of class 1 (A, B, C).[15]

8) B8.11.2 (IgG2b). A MAb directed against the human histocompatibility leukocyte class II antigen (HLA-DR).[16]

9) RC-38 (IgG2a). A MAb directed against a heat-labile antigen expressed on cell membranes of primary and metastatic renal cell carcinoma cells.[17,18]

10) G 250 (IgG2a). A tumor-associated antigen that does not react with normal proximal tubular epithelium but recognizes an antigen preferentially expressed on cell membranes of primary and metastatic renal cell carcinoma cells.[17,18]

The direct labeled antibodies W6.32 and B8.11.2 were provided by Dr. G. N. P. van Muyen, Department of Pathology, University Hospital Nijmegen, The Netherlands, and the RC-38 and G250 MAbs were provided by Dr. S. O. Warnaar, University of Leiden, The Netherlands.

Mycoplasm Test

Possible contamination of the xenografts by mycoplasm was routinely checked with Hoechst stain 33258 3 days after attachment of single-cell suspensions of the xenografts to glass slides in RPMI-1640 medium supplemented with 10% fetal calf serum. Attached cells were fixed in Carnoy's fixative (methanol/acetic acid 3:1) and next stained with Hoechst stain solution in phosphate citric acid buffer. Cells were analyzed by means of immunofluorescence microscopy.

Results

Histology of the Primary Tumors and Their Xenografts

We reviewed the histology of the primary tumors and their xenografts up to the 5th to 15th transplant generation using histopathologic criteria as described by Thoenes et al.[19,20]

NU-10

The primary tumor (Figure 1) showed a solid, both trabecular and acinar pattern of predominantly clear cells, admixed with some granular cells. Areas of both G1 nuclei (small, round nuclei with inconspicuous nucleoli) and G2 nuclei (enlarged, moderately polymorphic nuclei with prominent nucleoli) were present. Mitotic figures were rare (<1/10 high-power fields [HPF] [40×]). Only focal necrosis and fibrosis were present. The capillaries were inconspicious.

The transplant passages in the nude mouse, up to passage 15, showed consistently the features of the primary tumor of a solid pattern with both clear and granular cells with G2 nuclei and a similar low mitotic index. Only the small thin-walled blood vessels were frequently severely dilated as compared with the primary tumor.

NU-12

The primary tumor (Figure 1) consisted of large solid trabecular fields of large clear cells with rather small, polymorphic nuclei (G2) that were frequently multinucleated. Nucleoli were small, and mitotic figures were rare (<1/10 HPF). The thin-walled vessels in the septa were moderately dilated. Extensive necrosis was found.

The transplant passages up to no. 10 showed consistent features similar to the primary tumor, except for the multinucleated cells, which were scarce in the xenografts.

NU-20

The primary tumor (Figure 1) was solid with a trabecular pattern. The cells were predominantly granular, and the nucleoli were moderately pleomorphic with inconspicious nucleoli and dispersed multinucleated forms (G2). Mitoses were rare (<1/10 HPF). The thin-walled vessels were dilated, and some fibrosis and necrosis was present.

The transplant passages up to passage 10 showed increasing cell polymorphism up to G3 with multinucleated cells, increasing numbers of mitoses (up to 10 to 12/10 HPF) and necrosis with leukocytic infiltrates.

NU-22

The primary tumor (Figure 1) showed a solid, both acinar and trabecular pattern of predominantly granular cells admixed with clear cells. The nuclei were enlarged and polymorphic with prominent nucleoli (G2). Mitoses were observed, as many as five per HPF. The small vessels were inconspicuous. Necrosis and fibrosis were not extensive.

The transplant passages up to passage 10 consistently resembled the primary tumor very well. Mitoses were found in as many as three per 10 HPFs.

NU-28

The primary tumor (Figure 1) and the retroperitoneal lymph node metastasis showed similar features of a solid

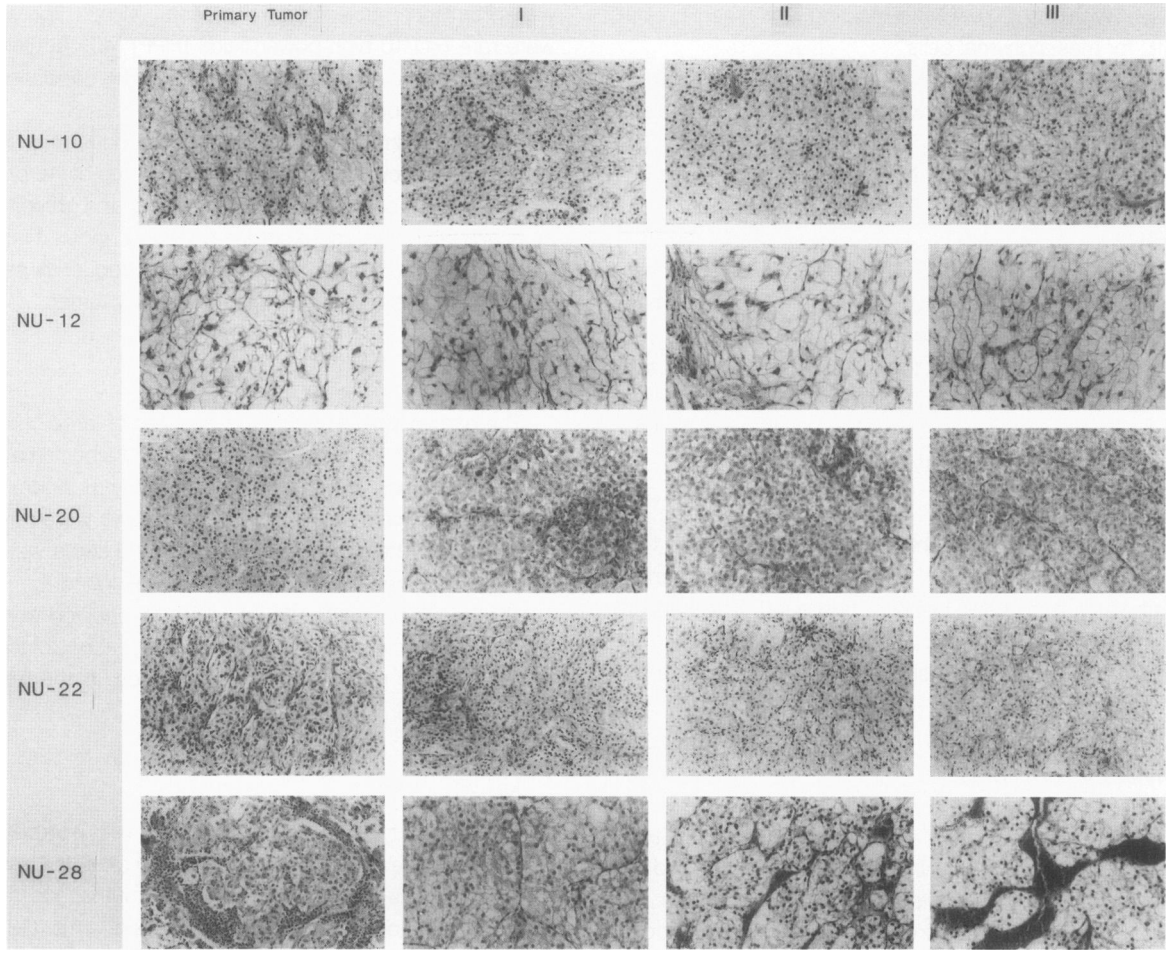

Figure 1. *Histology of primary tumors and their xenograft transplant passages no. 0 and 5–15. The slides (5 micron) were stained with hematoxylin-eosin by standard techniques and observed at ×40 magnification under a Zeiss Axioskop microscope. I = transplant passage 0; II = transplant passage 5 of NU-10, NU-12, NU-20, NU-22, transplant passage 3 of NU-28; III = transplant passage 15 of NU-10 and NU-12, transplant passage 10 of NU-20 and NU-22, transplant passage 5 of NU-28.*

tubular pattern with extensive necrosis and cellular leukocytic infiltrates. The tumor cells were predominantly granular cells admixed with some clear cells. The nuclei were large, polymorphic, irregular, and frequently multinucleated (G3), and the nucleoli were prominent. Mitoses were rare (<1/10 HPF). The lung metastasis excised 2 years later gave rise to the xenograft that was more solid with less extensive necrosis. More clear cells were encountered. Nuclear atypia was less severe (G2), and in the septa many leukocytes were present. Mitoses were also frequent.

The xenografts up to passage 5 showed less nuclear polymorphism, and multinucleated cells were not present (G2). Also no leukocytic infiltrates were encountered. Mitotic index was also low, as found in the lung metastasis. The thin-walled vessels were dilated and the first three passages showed an increase in vessel diameter.

This slow-growing tumor gives rise to many intravascular localizations of small nests of solid tumor with large granular cells with G2 nuclei and and only rare mitoses in the lungs of the mice 3 to 4 months after subcutaneous transplantation of small tumor fragments.

Flow Cytometric Analyses of Cellular DNA Content

Data about DI values of the primary tumors and their xenografts are summarized in Table 1.

NU-10

The primary tumor as well as the xenografts were shown to be aneuploid. In both the primary tumor and in the different xenografts passages, two cell populations are present: one with a diploid and one with an aneuploid DNA content. This pattern is seen in all other primary tumors and their xenografts except for the NU-22 tumor.

Table 1. *Flowcytometric Analyses of the Primary Tumors and Their Xenografts*

Tumor	Transplant generation	Aneuploidy	D.I. G1 diploid	D.I. G1 tumor
NU-10	Primary tumor	Y	1.08	1.54
	Passage no. 0	Y	0.96	1.50
	Passage no. 15	Y	0.97	1.53
NU-12	Primary tumor	S	0.97	0.97
	Passage no. 0	Y	0.89	1.68
	Passage no. 15	Y	0.93	1.57
NU-20	Primary tumor	N	0.92	1.00
	Passage no. 0	Y	0.88	1.50
	Passage no. 10	Y	0.90	2.06
NU-22	Primary tumor	N	0.96	1.00
	Passage no. 0	—	—	—
	Passage no. 10	N	0.93	1.05
NU-28	Primary lung metastasis	Y	0.95	1.60
	Passage no. 0	Y	0.89	1.63
	Passage no. 5	Y	0.89	1.60

DI = DNA index; Y = yes; N = no; S = suspect.

NU-12

The primary tumor has a predominant diploid character. However, a small triploid tumor peak can be identified in the histogram (data not shown). The xenografts from passage 0 up to passage 15 have an aneuploid character, with DI values of about 1.6.

NU-20

The primary tumor has a diploid DNA index. In transplant passage 0, an aneuploid peak is seen (DI value of 1.5). Transplant generation 10 has a DI value of 2.06.

NU-22

Like the NU-20 tumor, the primary tumor is diploid, with a DI value of 1.00. This tumor remains its diploid nature in all transplant generations tested so far. Because of a shortage of tumor material, flow cytometric analysis of transplant generation 0 was not possible.

NU-28

The lung metastasis removed 2 years after nefrectomy, which gave rise to the xenograft, is clearly aneuploid with a DI value of about 1.6. The subsequent xenografts from passages 0 to 5 were shown to maintain this near triploid DNA content.

Colony-forming Capacity in Soft Agar

Data about the colony-forming capacity of the primary tumors and their xenografts are summarized in Table 2. The cells from all xenografts have gained anchorage-independent growth potential in this culturing assay. The primary tumors of the NU-20 and NU-22 did not give rise to colony formation, but, because their xenografts did, sensitivity of the system will probably have been too low to detect the colony-forming capacity of the cells isolated from the primary tumors. Plating efficiency (PE) of the xenografts NU-10, -12, -20, and -22 was at least 20-fold

Table 2. *Clonogenic Potential of Cells of the Primary Tumors and Their Xenografts in a Double-layer Soft-agar Culturing System*

Tumor	Material	No. of cells per dish × 1000	No. of colonies after 4 weeks	Plating efficiency (%)
NU-10	Primary tumor	500	134	0.1
	Xenograft p.5	100	695	2.0
	Xenograft p.15	100	740	2.0
NU-12	Primary tumor	500	160	0.1
	Xenograft p.5	100	275	1.0
	Xenograft p.1	100	240	1.0
NU-20	Primary tumor	500	0	0.0
	Xenograft p.5	100	150	1.0
	Xenograft p.10	100	130	1.0
NU-22	Primary tumor	500	0	0.0
	Xenograft p.5	100	268	1.0
	Xenograft p.10	100	280	1.0
NU-28	Lung metastasis	500	154	0.1
	Xenograft p.5	100	31	0.1

Colonies were counted by an Omnicon Fas-II automated colony counter and plating efficiency was calculated as percentage of viable cells (i.e., not staining cells with trypan blue) giving rise to a colony (colony diameter ≥ 60 micron) after 4 weeks of incubation at 37°C and 6% CO_2.

higher than their primary tumors. Plating efficiency of the NU-28 xenograft, however, was the same as the PE of the corresponding tumor (lung metastasis), the primary tumor, and the retroperitoneal lymph node metastasis, which all were shown to have a PE of 0.1%.

Tumor Doubling Times

Tumor doubling times were constant after the first (NU-22), third (NU-20), or fourth transplant generation (NU-10, -12, -28). Table 3 summarizes the doubling times for the first five transplant generations of the different tumors. This table shows that doubling time is longest for the primary tumor pieces (transplant generation 0).

Intervals between two passages are reflected by the tumor doubling times, although there are remarkable differences in tumor growth when tumor dimensions pass about 1.0 cm. NU-10 tumor grows to about 1.3 cm, but stops growing thereafter. The tumor can stay in the mice for about 1 month beyond this point before forming elaborate necrosis. The NU-12 tumor will grow with a tumor doubling time of 4 days, until its dimensions reach about 1.5 cm. Thereafter, because of the formation of a cyst, tumor dimensions change much faster, until maximum dimensions of about 2 cm are reached. Extensive necrosis then forms most of the tumor. The NU-20 tumor is not limited in its growth as the NU-10 and NU-12 tumors are. This tumor grows until the mouse dies of cachexia. Tumor dimensions will be about 2.5 cm at this point, and extensive necrotic fields form most of the tumor. The NU-22 tumor stops growing at about 1 cm. Like the NU-10 tumor, this tumor can remain in a dormant state for about 1 month before becoming extensively necrotic. The NU-28 tumor, which is the slowest growing tumor, is, like the NU-20 tumor, not restricted in its growth by the dimensions of the tumor (>2.5 cm). The mice do not show any signs of cachexia despite the intravascular colonies and micrometastases that are found in the lungs at that time of the development.

Metastatic Capacity

Lungs, liver, and lymph nodes were checked for metastases by means of histologic analyses of paraffin-embedded material. Four of the tumors (NU-10, -12, -20, -22) do not form any metastases. The NU-28 tumor gives rise to multiple tumor colony formation in the lungs of the mice. Sporadic outgrowth of these colonies into the surrounding lung tissues giving rise to micrometastases of about 300 to 700 μ is also noticed in all transplant generations.

Table 3. *Tumor Doubling Times of the Xenografts in BalbC* nu/nu *Mice*

Tumor	Transplant generation	Tumor doubling time (days)
NU-10	0	9
	1	8
	2	6
	3	6
	4	4
	5	4
NU-12	0	11
	1	9
	2	6
	3	5
	4	4
	5	4
NU-20	0	6
	1	5
	2	5
	3	3
	4	3
	5	3
NU-22	0	7
	1	6
	2	6
	3	6
	4	6
	5	6
NU-28	0	11
	1	10
	2	10
	3	9
	4	8
	5	8

Tumor pieces of 2-mm cubes were implanted subcutaneous into the right flank of the mice and tumor dimensions were measured with calipers twice a week.

Immunohistochemical Analyses

Data about the immunohistochemical analyses of the primary tumors and their xenografts are summarized in Table 4. This table shows that all tumors stain with RCK 102. The expression on the NU-10 tumor remains the same from primary tumor to xenograft passage no 15. In the NU-12 tumor, however, there is a selection for the positive cells between passages 1 and 5. In the NU-20 tumor, the intensity of the staining decreases from primary tumor to xenograft passage 0 (3 and 1, respectively). Also between the first and fifth passage there is a selection for positive cells. Loss of intensity but selection for positive cells is seen also in the NU-22 and NU-28 tumors. Intensity of staining appears to be higher on the lung metastases than on the primary kidney tumor of the NU-28 xenograft.

Except for sporadically staining cells, none of the tumors show staining with RCK 105. Loss of intensity of staining in the first five passages of the tumors is not as evident for RCK 106, except for the NU-28 tumor. The NU-20 tumor even shows enhancement of RCK 106 expression between the first and fifth passages. All tumors, however, show a positive selection for RCK 106 expression cells in the first five passages.

A loss of intensity of staining with RV 202 is seen in the NU-10, NU-22, and NU-28 tumors. The NU-20 tumor shows an increased intensity of staining with RV 202, concomitantly with a rise in percentage of cells staining with this antibody. Also, the NU-12 shows a positive selection for RV 202–positive cells.

Staining with the polyclonal antibodies pKer and pVim shows the same patterns as seen with the monoclonal antibodies.

Human leukocyte antigen class I expression remains present on all cells of the different passages. Intensity, however, is diminished in all tumors from passages 0 to 1, except for the NU-28 tumor, on which the intensity of the class I staining remains the same in the succeeding passages.

Staining with the HLA class II antibody is rapidly lost in most tumor cells except for the NU-12 tumor. The low intensity of staining is already partly lost in passage 0 of the NU-10 tumor, and in passage 1 almost all cells have lost any detectable antigen expression. Staining in the NU-12 tumor remains constant in the different passages, but staining is completely lost in passage 1 of the NU-20 tumor. Like the NU-10 tumor, HLA class II expression is also almost completely lost between passages 1 and 5 of the NU-22 tumor. The NU-28 tumor shows only a loss in intensity of the staining and not in percentage of positive cells.

Table 4. *Immunohistochemical Analyses of the Primary Tumors and Their Xenografts*

Tumor	Transplant generation	RCK 102		RCK 105		RCK 106		RV 202		pKer		pVim		W6.32		B8.11.2		G 250		RC-38	
		Int.	%	Int.	%	Int.	%	Int.	%	Int.	%	Int.	%	Int.	%	Int.	%	Int.	%	Int.	%
NU-10	Primary tumor	1	0	0	0	1	0	3	4	2	1	2	2	3	4	1	3	2	3	2	4
	Passage 0	1	0	0	0	1	0	3	4	2	1	2	2	3	4	1	2	2	4	2	4
	Passage 1	1	0	0	0	1	1	2	4	2	1	1	2	2	4	1	0	2	4	2	4
	Passage 5	1	0	0	0	1	1	2	4	2	1	1	2	2	4	1	0	2	4	2	4
	Passage 15	1	0	0	0	1	1	2	4	2	1	1	2	2	4	1	0	2	4	2	4
NU-12	Primary tumor	1	3	0	0	1	3	1	3	1	3	1	2	3	4	1	4	1	2	1	2
	Passage 0	1	3	0	0	1	3	1	3	1	3	1	2	3	4	1	4	1	3	1	4
	Passage 1	1	3	0	0	1	4	1	4	1	3	1	2	2	4	1	4	1	3	1	4
	Passage 5	1	4	0	0	1	4	1	4	1	3	1	3	2	4	1	4	1	3	1	4
	Passage 15	1	4	0	0	1	4	1	4	1	3	1	3	2	4	1	4	1	3	1	4
NU-20	Primary tumor	3	2	0	0	2	1	3	3	3	1	3	2	2	4	1	2	2	2	1	2
	Passage 0	1	2	0	0	2	1	1	4	1	1	1	3	2	4	1	1	3	4	2	4
	Passage 1	1	2	0	0	2	2	2	4	1	2	1	4	1	4	0	0	3	4	2	4
	Passage 5	1	3	0	0	3	4	3	4	2	4	2	4	1	4	0	0	3	4	2	4
	Passage 10	1	3	0	0	3	4	3	4	2	4	2	4	1	4	0	0	3	4	2	4
NU-22	Primary tumor	3	3	0	0	2	1	3	4	3	2	1	3	3	4	1	3	2	3	2	2
	Passage 0	1	4	0	0	2	3	2	4	1	3	1	3	3	4	1	2	1	3	1	4
	Passage 1	1	4	0	0	2	3	2	4	1	3	1	3	2	4	1	1	1	3	1	4
	Passage 5	1	4	0	0	2	4	2	4	1	4	1	3	2	4	1	0	1	3	1	4
	Passage 10	1	4	0	0	2	4	2	4	1	4	1	3	2	4	1	0	1	3	1	4
NU-28	Lung metastasis	3	1	0	0	3	1	3	3	2	0	3	2	2	4	2	3	2	3	2	2
	Passage 0	1	3	0	0	1	3	2	3	1	2	1	2	2	4	1	3	2	4	2	4
	Passage 1	1	3	0	0	1	3	2	3	1	2	1	2	2	4	1	3	2	4	2	4
	Passage 5	1	3	0	0	1	3	2	3	1	2	1	2	2	4	1	3	2	4	2	4

Staining with the antikeratin monoclonal antibodies RCK 102, RCK 105, RCK 106, the antivimentin monoclonal antibody RV 202 and the polyclonal antibodies against cytokeratin and vimentin (K40 and K36 resp.) was analysed by means of indirect fluorescence microscopy. Staining with the W6.32 (anti HLA class-I), B8.11.2 (anti HLA class-II), G250 and RC-38 monoclonal antibodies (both tumor associated) was followed by means of direct immunoperoxidase staining (DAB staining). Intensity of staining was graded according to the following scale: 0 = no visible staining present, 1 = just visible staining, 2 = clear visible staining and 3 = strong staining (Int, left figures). The percentage of tumor cells expressing the particular antigen (%, right figures) was estimated within the following ranges: 0 = 0%, 1 = 1–25%, 2 = 26–50%, 3 = 51–75% and 4 = 76–100% positive tumor cells.

Except for the G250 staining of the NU-22 tumor, a selection for G 250– and RC-38–positive cells is shown by all tumors in their early passages in the nude mouse. A loss of intensity for both G250 and RC-38 is shown by the NU-22 tumor only, and as for the staining with the anticytokeratin and antivimentin antibodies, intensity of staining with the G250 and RC-38 antibodies is also enhanced in the transplant passages of the NU-20 tumor as compared with the primary tumor.

Tumor Characteristics of the Nonserially Transplantable Tumors

Tumor characteristics of the primary tumors as described for the serially transplantable tumors were performed also for the nonserially transplantable tumors, to see whether a relation could be found between histologic and molecular characteristics of a tumor and its capacity to form a serially transplantable tumor. A lower HLA class I expression on the primary tumors of the nonserially transplantable tumors was the only obvious difference between the two groups. One of the tumors (NU-24) showed that not only intensity of staining with the HLA class I antibody was low, but also the percentage of staining cells diminished from 100% in the primary tumor to about 50% in transplant passage 1, which is opposed to the findings from the serially transplantable tumors in which the HLA class I antigen was found in about 100% of the primary tumors and all succeeding transplant passages.

Mycoplasm Screening

None of the cultures showed any signs of contamination with mycoplasm.

Discussion

A disadvantage of studying human cancer in models is that none of the models will be representative in all aspects. *In vitro* models do not enable us to study the histologic and many of the physiologic aspects of the tumor, and in the *in vivo* models, the syngeneic rodent tumors, for instance, have the disadvantage of the different physiologic and metabolic aspects between human and rodent tumors. A model enabling *in vivo* studies on human tumors, the nude mouse model system, has a disadvantage that the intrinsic T cell deficiency may impair with many aspects of the transplanted tumors. However, because of the first description of the nude mouse in 1966 by Flanagan[21] and the demonstration of the congenital defect of the thymus by Pantelouris,[22] many reports described the use of this model for the transplantation of human tumors.

Histology and Tumor Doubling Time

Tumor histology remained essentially the same in the different transplant passages; however, some histologic and cytologic changes occurred during transplantation. The NU-20 tumor showed most apparent changes in cell morphology. In this tumor, there was an increasing nuclear polymorphism during succeeding transplant passages and an increase of the numbers of mitoses and multinucleated cells that was opposite to the findings in the NU-12 and NU-28 tumors, which showed less multinucleated cells and also less nuclear polymorphism (NU-28) in the succeeding transplant passages. Differences in vascular structure were most pronounced between the primary tumors and their xenografts. Blood vessels appeared to be very thin and dilated in the xenografts (NU-10, NU-28). A remarkable increase in vessel diameter in the first three transplant passages was shown by the NU-28 tumor. Changes in vessel structure were noticed by other authors as well[23] and may be explained by the fact that blood flow and vessel structure are host related because the vascular system and stroma in xenograft tumors are known to be host derived. The differences in vessel structure also may explain part of the differences in growth characteristics between the primary tumor transplants (passage 0) and the succeeding xenografts. Table 3 shows that tumor doubling time is highest in transplant passage 0 and decreases thereafter until constant growth is reached. The tumor transplants taken directly from the primary tumor contain human endothelial cells and stromal parts, which will be replaced by host cells. So, before newly formed host-derived capillaries will be functional in the tumor, blood supply and oxygenation will be insufficient for normal tumor growth. As seen in the NU-28 transplant passages, diameter of the newly formed vessels may increase further in the first transplant passages. Such tumor-induced vessels are known to have a different structure and hence possess different hemodynamic properties.[24] Furthermore, the vascular density in the primary tumor in humans and in xenografts is likely to be strongly influenced by the dissociation curve for oxygen from hemoglobin. The partial pressure of oxygen ($_pO_2$) 50 values for mouse and human hemoglobin are 41 and 25 mmHg, respectively.[25] This suggests that anatomically identical vascular networks would result in a less effective tissue oxygenation in mice.[23] Because a change in vessel diameter was not noticed as clearly in the other tumors, intrinsic factors also play a role in tumor vascularization, which is in accordance with the conclusions drawn by Solesvik et al[26] in a study of the

vascular anatomy of five different human melanoma xenografts. Also the results of flow cytometry, soft agar culture, and immunohistochemistry indicate that some clonal selection of the primary tumor occurs in the nude mice. This clonal selection also may play an important role in the noticed differences in tumor-doubling time between succeeding passages.

Molecular Characteristics

Flow cytometric analyses show that DI values can show considerable change in the subsequent transplant passages, which is not in accordance with findings by other authors,[27] who found preservation of DNA characteristics after passage in nude mice.

Interpretation of FCM data regarding primary renal cell carcinoma (RCC), however, need careful consideration. Recently it became evident that a wide spread in DI can exist within an individual tumor.[28] The consensus at the moment is that for a valid DI determination of a primary renal tumor, at least six samples need to be evaluated. At the start of this studies (3 years ago), the DI was established from single specimens. Hence it is conceivable that the xenografts arose from subpopulations not abundantly present in the primary tumor. It is interesting to see that in the samples analyzed for FCM one of the tumors remains diploid (NU-22), whereas others progress to aneuploidy or tetraploidy. Recent studies suggest that aneuploidy may arise from several mechanisms, either by acquisition of DNA abnormalities resulting in peridiploid tumors or by a gain of chromosome numbers and subsequent chromosomal loss, resulting in near triploid tumors. In this aspect, the NU-10 and NU-28 tumors arose from near-triploid tumors and retain the same DNA content during the subsequent transplant passages. The NU-22 tumor resulted from a diploid primary tumor piece, and this tumor was shown to retain its diploid character during xenotransplantation. The NU-12 and NU-20 tumors are interesting because they developed from apparently diploid primary tumors, whereas during xenotransplantation they gave rise to aneuploid tumors. The NU-12 was shown to be hypertriploid in passage 0 and appears to remain its triploid character in passage 15. The NU-20 tumor, however, has triploid DNA content in passage 0, but appears to be tetraploid in passage 10. Most probably, the triploid and tetraploid tumors evolved from minor different tumor fractions not apparent in the preceding transplant passages, although a tetraploid peak, for instance, could not be detected in the histogram of transplant passage 0.

Staining with the cytokeratin antibodies RCK 102, RCK 105, and RCK 106 fulfills the characteristics of RCC tumors.[13,14] Also, most of the tumor cells stain with the vimentin antibody RV 202, and this too is a characteristic of RCC cells.[29] Because we did not perform double-labeling studies, we cannot exclude the possibility that some cells of the NU-10 tumor stain only with anticytokeratin (RCK 106) or only with antivimentin. The concomitant keratin and vimentin expression of most of the cells of the other tumors is obvious from the staining patterns with both types of antibodies. Staining with the polyclonal antibodies against keratin and vimentin are comparable with the staining with the monoclonal antibodies. Because of the lower intensity of staining by the polyclonal antibodies, however, some low-keratin or vimentin-expressing cells may have been overlooked. The cytokeratin and vimentin staining patterns show that, except for the NU-20 tumor, some particular changes occur during the first passages of the tumors. In most tumors there is a loss of intensity of staining in passage 0, but concomitantly there is a selection of the keratin- or vimentin-expressing cells that may even continue in the following passages (eg, NU-22). Again this pattern is seen also with the polyclonal antibodies. The NU-20 tumor, however, shows an increase in staining intensity with both cytokeratin (RCK 106) and vimentin antibodies, together with a positive selection of staining cells, which shows that this tumor is very unstable regarding the cytoskeletal filaments.

Human leukocyte antigen class I and class II expression is down-modulated in all transplant passages, except for the NU-12 tumor, which only loses some intensity of class I expression. Class I remains present on almost all cells in the different passages, but class II is lost on most of the cells of the NU-10 and NU-22 tumor and is even completely lost on cells of the NU-20 tumor. Down-regulation or loss of HLA class II expression on xenografting is not uncommon and is found for melanoma cells as well.[30]

Staining with the antitumor antibodies G250 and RC-38 shows a pattern that is very much like the staining with the monoclonal and polyclonal antibodies against cytokeratins and vimentin. An increase in positive cells is seen in almost all cases for both G250 and RC-38 on xenotransplantation. A loss of intensity, however, is seen only in the NU-22 tumor. A gain of intensity of staining, however, is again shown by the NU-20 tumor, which means that except for the HLA class I and class II antigens this tumor shows an increase in intensity of staining of all other tested antigens on xenotransplantation, whereas for the other tumors intensity of staining of these antigens is constant or diminished on xenotransplantation.

No essential differences between the histologic and molecular characteristics between the serially and non-serially transplantable tumors could be measured, except for a lower HLA class I expression in the nonserially transplantable tumors. Nude mice possess high levels of

natural killer (NK) cells, and reduction of the NK activity in nude mice has been shown to enhance the growth of transplanted tumor growth in nude mice.[31] Conversely in autologous systems, NK cells are known to be activated by cells expressing low levels of HLA class I antigens.[32] The fact that the low HLA class-I–expressing cells do not form serially transplantable RCC tumors in the nude mouse is therefore an interesting finding because to our present knowledge it is not known whether there is a functional relation between human HLA class I expression and murine NK cell activity.

Colony-forming Capacity

The colony-forming capacity in soft agar of the xenograft passages is indicative for the increase in tumorigenic characteristics of the xenografts. The enrichment for viable tumor cells, as well as the progression to a more aggressive character of the xenografts, may explain the differences (up to 20 times) in PE between the primary tumors and their xenografts.

We conclude that xenografting human renal tumor tissue into nude mice gives rise to tumors that in general give a good representation of the primary tumor, however, presenting the more aggressive character of the primary tumors. Histology of the primary tumors was essentially preserved during xenotransplantation in which host-derived blood vessels replaced the human vasculature. Molecular characteristics of the tumor cells, however, may change to a greater or lesser extent during the first transplant passages in which there is a trend for a selection for cytokeratin-, vimentin-, and tumor-associated antigen-expressing cells. The DNA index shows a trend toward aneuploidy, which means that during transplantation a selection for the most tumorigenic cells is made.

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