Multiparameter Analysis of Four Human Renal Cell Carcinoma Xenografts in Nude Mice


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Abstract. Four human renal cell carcinoma xenografts (RC2, RC14, RC43, NC63), maintained in nude mice for several years, were investigated in a multidisciplinary study, using (immuno)histochemical, biochemical and ultrastructural techniques. Histological, cellular, nuclear and biological characteristics were investigated. All tumors showed histologically recognizable features of human renal cell carcinomas, although marked differences between the four tumors were seen, both at the histological and ultrastructural level. Flowcytometric analysis of tumor cell suspensions allowed DNA quantification as well as the detection of subpopulations. Immunohistochemical staining procedures using tissue specific antibodies against intermediate filament proteins revealed two populations of tumor cells. Most tumor cells in three of the xenografts coexpressed cytokeratins and vimentin, while in RC43 most of the tumor cells expressed only vimentin. Northern blot analysis showed a higher expression of vimentin mRNA in all tumors as compared to normal kidney tissue. RC43 showed a three-fold higher level of vimentin mRNA than the other xenografts. Growth potential in the human tumor cloning system was evaluated by temporal growth pattern analysis. These experiments showed that the xenografts resemble human primary renal cell tumors in different ways, and reflect different characteristics that can be present in human renal cell carcinoma.

Recent developments in cell biology have led to the identification of new tumor cell parameters that allow a more accurate tumor characterization. In renal cell carcinoma, histological classification alone can so far not provide the clinician with sufficient information to predict the course of the disease (1). Additional information has become available from biochemical and cell biological techniques such as the analysis of cellular DNA by flow cytometry (2, 3, 4). Moreover, the immunohistochemical identification of different cellular constituents has given further insight into renal cell tumor biology (5, 6, 7, 8). Differences in gene expression in renal cell carcinomas as compared to normal kidney have also been investigated (9).

Since they are used as a model for the human situation, we have investigated some of these parameters in four different human renal cell tumor xenografts which were continuously transplanted in nude mice, in order to establish the biological properties of these tumor lines.

Although they do not completely resemble the human situation, these human xenografts are more closely related to it than the transplantable experimental animal tumors (10, 11). Human renal cell carcinoma xenografts in nude mice retain their histological and electron microscopical features (12, 13), and show a chromosomal stability (14). The grade of nuclear atypia, the predominant cell type and abnormalities in cholesterol metabolism also remained the same (15), while HLA expression, too, remained consistent (16). The mitotic index, however, was reported to be higher than that of the original tumor (17).

For our study, four xenografts (RC2, RC14, RC43 and NC63), originating from patients with metastatic disease at the time of diagnosis, were established in nude mice. These tumors have been described as having comparable histology to the original tumors (18). We further evaluated the four tumors by immunohistochemical methods, flow cytometry, growth potential, and by the Southern (19) and Northern blot technique, in order to evaluate the vimentin gene expression in relation to immunohistochemical findings.

Materials and Methods

Renal cell carcinoma xenografts in nu/nu athymic mice were established at the Department of Urology of the Erasmus University, Rotterdam (18).
Table I. Results of the multidisciplinary analysis of four established xenografts of renal cell carcinomas in nude mice.

<table>
<thead>
<tr>
<th>In vivo doubling time</th>
<th>Histology</th>
<th>EM</th>
<th>pVim</th>
<th>pKer</th>
<th>Vimentin mRNA</th>
<th>Aneuploidy fraction</th>
<th>DNA index</th>
<th>Gross HTCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>( RC_2 )</td>
<td>11.2±0.7 d</td>
<td>G+C</td>
<td>c=m</td>
<td>+</td>
<td>+</td>
<td>Elevated</td>
<td>9.0%</td>
<td>1.47</td>
</tr>
<tr>
<td>( RC_{14} )</td>
<td>3.8±1.2 d</td>
<td>C</td>
<td>c=m</td>
<td>+</td>
<td>+</td>
<td>Elevated</td>
<td>7.5%</td>
<td>1.935</td>
</tr>
<tr>
<td>( RC_{43} )</td>
<td>4.0±1.4 d</td>
<td>G+C</td>
<td>c=m</td>
<td>++</td>
<td>-</td>
<td>Elevated</td>
<td>47.7%</td>
<td>2.263</td>
</tr>
<tr>
<td>( NC_{65} )</td>
<td>3.6±1 d</td>
<td>G+C</td>
<td>c=m</td>
<td>+</td>
<td>+</td>
<td>Elevated</td>
<td>23.0%</td>
<td>2.041</td>
</tr>
</tbody>
</table>

Meaning of symbols: G = granular cell type; C = clear cell type; c = epithelial characteristics; m = mesenchymal characteristics; _ = negative; + = positive; ++ = strongly positive; d = days; = is equal to.

Four of these tumors \( RC_2, RC_{14}, RC_{43} \) and \( NC_{65} \) were evaluated in a multidisciplinary study in order to investigate several biological characteristics and potentials of these tumors.

**Histology.** Tissue material of a representative part of the tumor was prepared for routine histological examination using hematoxylin and cosin (H.E.) staining and a periodic acid Schiff (P.A.S.) staining.

**Electron microscopy.** Tumor material was cut in small pieces and fixed in a cacodylate buffered mixture of glutaraldehyde and paraformaldehyde. Following postfixation in osmic acid and dehydration in graded ethanol, they were embedded in Epon. Double contrast ultrathin sections were examined in a Philips EM 300.

**Immunohistochemistry.** 5 - 7 Micron thick frozen sections of the renal cell carcinoma xenografts snap frozen in liquid nitrogen were fixed in methanol at -20°C for 5 min and thereafter dipped in acetone at room temperature. The indirect immunofluorescence technique was performed as described previously (7).

The following antisera were used in this study: 1) An affinity purified rabbit antiserum to human skin keratin (pKer) for the detection of the epithelial nature of tumor. 2) Affinity purified rabbit antiserum to bovine lens vimentin (pVim), the intermediate filament protein present in mesenchymal cells. 3) A monoclonal antibody to cytokeratin 18 (RGE 53) specific for glandular epithelial cells (19).

**Flow cytometric analysis.** Flow cytometric analysis was performed on single cell suspensions of the xenografts in combination with the two parameter labeling technique for the differentiation of subpopulations in the tumors as described previously (8). Ethanol fixed cell suspensions were split into three fractions. One sample was stained with PI and used for cell kinetic studies. A second sample was used for estimation of the DNA - index, with chicken red blood cells as internal standard. The third sample was labeled using tissue specific markers (cytokeratins and Vimentin) for the detection of tumor cell subpopulations.

**Northern blot analysis.** Vimentin mRNA level with actin as a standard was detected using pVim and pAct1 as DNA probes (20). Preparation of the DNA probe and its nick translation (21) was carried out as described previously (22). Primer extension reactions were according to Messing and co-workers (23). The specific activity of the probes used in the hybridization studies was 2 - 5 x 10^6 cpm/ug. Agarose gel electrophoresis and hybridization analysis was performed as described previously (22).

Total cellular RNA was isolated according to the procedure described by Auffray and Rougeon (24). Upon poly(A) selection by oligo(dT) - cellulose chromatography, the mRNA was dissolved in sodium phosphate buffer \( 10 \text{ mM, pH 7.0} \) containing \( 50\% \) DMSO and \( 1 \text{ M} \) glyoxal, and heated to 50°C for 1 h. RNA was size fractionated to Hybond N (Amersham) for hybridization analysis as described previously (25).

**Human tumor clonogenic cell culture system (HTC,8).** For the detection of growth potential in the soft agar double layer cell culture system, single cell suspensions of the tumors were prepared. Tissue selected for soft agar culture was processsed according to the detailed description published by Salmon (26). A modified two layer soft agar culture method as described previously (27, 28) was used. The cells were cultured immediately after preparation of the single cell suspension and cell growth estimation using the Omnicron FAS II automated colony counter (Milton Roy Inc., Rochester, New York, USA) as described previously (29). For dynamic colony growth development evaluation, we used the "Temporal Growth Pattern" (TGP), giving an estimation of the growth over a certain period of time (28).

**Results**

All four xenografts investigated are known to be derived from primary metastatic human renal cell carcinomas and were encoded \( RC_2, RC_{14}, RC_{43} \) and \( NC_{65} \) (Table I).

**Histology.** \( RC_2 \) was composed of tumor cells of varying size and organized in a somewhat trabecular arrangement. The cytoplasm of the tumor cells showed large vacuoles, optically empty. Irregularly shaped nuclei contained macronucleoli with a marked variation in shape. Mitoses, mostly abnormal, were often observed. The tumor was classified as a mixed cell type Grawitz tumor of moderate differentiation (Figure 1C).

\( RC_{14} \) consisted almost entirely of cytoplasmic rich cells, arranged in a tubular and alveolar way. In between the tumor cell nests many optically empty spaces of areas filled with erythrocytes were present. Mitoses, many of which were atypical, were often observed. The tumor cells contained mostly large vacuoles. It was classified as a well differentiated pure clear cell type renal cell carcinoma (Figure 1A).

\( RC_{43} \) showed tumor cells with a spindle - like appearance. Some of the tumor cells showed cytoplasmic vacuoles, while most of them revealed a fine granular cytoplasm. Nuclei were polygonal, sometimes of a bizarre shape, containing mostly
Figure 1. Representative examples of the histology of the human renal cell carcinoma xenografts in nude mice of (A) RC13, (B) NC38, (C) RC2, and (D) RC25. (*) 10.)
Figure 2. (A) RC, electron microscopy of three tumor cells with indented nuclei. The electron-lit cytoplasm contain numerous organelles and glycogen particles. The cell membranes show interdigitations of microvilli with desmosomal contacts (arrows). (x 7000).

(B) RC, The tumor cells often show highly irregular nuclei. The scanty cytoplasm contains polymorphic mitochondria and few lipid droplets. They are partly in close apposition without junctional complexes, or are provided with loosely interdigitating microvilli of varying shape. (x 8000)
irregularly shaped macronucleoli. Occasionally polynuclear cells were observed. The tumor was classified as a poorly differentiated mixed type Grawitz tumor (Figure 1D).

NC$_{65}$ consisted of tumor cells arranged in tubular solid structures interspersed with extensive areas of stromal tissue and necrosis. The tumor showed cytoplasm rich cells with polygonal nuclei containing macronucleoli of irregular shape. The cytoplasm contained mostly vacuoles but eosinophilic granular structures were also observed. The tumor was classified as a moderately differentiated mixed type Grawitz tumor (Figure 1B).

**Electron microscopy.** All tumors contained cells with well developed nuclei and organelles. Junctional complexes as well as glycogen were demonstrated in varying amounts (Figure 2A). The epithelial characteristics were distinct in all the cases except for RC$_{30}$. The latter differed in various aspects, e.g. more polymorphic nuclei, vary few junctions and lipid droplets (Figure 2B).

**Immunohistochemistry.** Expression of both the epithelial and mesenchymal type of intermediate filament proteins in human renal cell carcinoma is well established by rabbit antisera to cytokeratin and vimentin, respectively (5, 6, 8). The xenografts RC$_{2}$, RC$_{14}$ and NC$_{65}$ (Figure 3) were also stained by both these antibodies in the clear cell as well as the granular cell component. Using the RGE 53 monoclonal antibody to cytokeratin 18, coexpression of this adenocarcinoma marker and vimentin could be observed in tumor cell areas, thus rendering these three xenografts completely indistinguishable from original human renal cell carcinomas. In RC$_{30}$, however, a reaction with the vimentin antibody was almost exclusively found and there was no staining with the cytokeratin antibodies.

**Flow cytometric DNA analysis.** The flow cytometric DNA analysis revealed in all xenografts the presence of two cell populations, one with a diploid and one with an aneuploid DNA content. The diploid cells probably represent stromal and inflammatory cells. A two parameter flow cytometry technique, using Propidium Iodide for DNA quantitation and FITC for the detection of cytokeratins or vimentin using the indirect immunofluorescence technique, allowed quantification of the aneuploid fractions and calculation of the DNA index for each tumor separately from the non-tumor cells (Table 1).

The flow cytometric data obtained with NC$_{65}$ have been illustrated elsewhere (8).

Flow cytometric data obtained for RC$_{30}$ are shown in Figure 4. In these FCM studies this tumor was also found to react almost exclusively with the vimentin antisera and not with the cytokeratin antisera. For further details see legends to Figure 4.

**Northern and Southern blot analysis.** The expression of vimentin was also studied by Northern blot analysis (Figure 5), which is indicative of the amount and length of vimentin mRNA. Using pVim1 as a molecular probe, and increased level of vimentin mRNA was found in all tumors with respect to normal kidney tissue. RC$_{30}$ showed a vimentin mRNA level that was three - fold that of the other tumors, which is in good agreement with the results of the immunohistochemical analysis using vimentin antibodies. By restriction enzyme analysis of the gene encoding vimentin, it was found that no abnormalities in the vimentin gene in the tumor tissue could be found (data not shown).

**Human tumor cloning system.** In double layer soft agar HTCS all tumors showed a temporal growth pattern (TGP). The peak of growth colonies was obtained after about 17 days, and colonies showed an increase in both size and number (Figure 6).
Discussion

At the moment no consistently effective treatment for renal cell carcinoma exists, be it hormonal, chemotherapy or immunotherapy (30). Investigations attempting to identify and test effective agents with in vitro assays have so far been unsuccessful, in part because of technical problems and the refractory nature or renal cell carcinoma in response to currently available chemotherapeutic agents (31). Additional difficulties in clinical evaluation of new treatment protocols are caused by the relatively rare occurrence of the tumor and the pluriuniform spectrum of its clinical presentation with marked differences in prognosis (32). The search for new approaches for the treatment of renal cell carcinoma necessitates the use of a reproducible biologic testing system in combination with a renal tumor model system that has predictable behaviour in vitro and in vivo (33). In addition, it is necessary that the characteristics of the model show as close resemblance as possible to the human tumor if the treatment results in the model are to be transferred to the in vivo situation in man.

The human tumor model systems in nude mice are considered to be of a higher value than spontaneously evolved murine tumors such as those described by de Vere White and Olsson (34, 35).

As mentioned before, the xenografts investigated so far
have been shown to retain histological (18), ultrastructural (17) and cytogenetical (14) features of the original renal cell tumors. For the evaluation of the four xenografts described here, RC2, RC14, RC15 and NC65, their original human renal tumors were not available for comparison. Histologically, however, they were recognized as typical renal cell carcinomas with different characteristics, varying from moderately differentiated (RC2, NC65) to poorly differentiated (RC15) tumors consisting of both granular and clear cells. RC14 consisted of a well differentiated pure clear cell type tumor.

At an ultrastructural level, all these tumors revealed epithelial properties as described by Kurth et al (17). RC15 differs from the other tumors in the fusiformic aspect of most cells with their polymorphic nuclei and mitochondria, while it is also noteworthy that the amount of desmosomal contacts is very low when compared to the other tumors.

Recent studies (5, 6, 8) have described the intermediate filament protein pattern of human renal cell carcinoma. These led to the conclusion that renal cell carcinomas consist of cell populations expressing different intermediate filament patterns, i.e. cells containing only cytokeratins or only vimentin, but also cells co-expressing cytokeratins and vimentin. It was anticipated that these different biological characteristics may give rise to difference in chemosensitivity.

Immunohistochemical studies and DNA flow cytometric analyses of the xenografts confirmed these findings, as in each case an abnormal DNA stemline expressing cytokeratin and vimentin expression (albeit in different amounts) could be detected. Evaluation of vimentin expression by RNA analysis showed increase of transcription in all tumors. RC15 showed a vimentin expression three-fold higher than that of the other three tumors. In the HTCK cells all tumors could be cultured, possibly due to the fact that they had already been selected for their in vitro growth potential by their successful growth as xenografts in nude mice.

From this study, it can be concluded that the four xenografts contain a variety of biological properties that also occur specifically in human renal cell carcinoma. It is clear that the individual xenografts are different and that none of these tumors unites all characteristics. Therefore one should realize that successful results in chemosensitivity tests with one of these tumor models can not immediately be transferred to the human situation of renal cell carcinoma in general. Characterization of each of these xenografts was also performed to see if these tumor models represent the different subpopulations of renal cell carcinoma tumor cells. Future studies comparing different biological parameters of the tumors, such as oncogene expression and in vitro chemosensitivity, will have to show whether these tumors represent subtypes of renal cell carcinoma. If a differentiated response of treatment protocols is observed, these xenografts may be useful tumor models for the study of new experimental chemotherapeutics.
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References