Multidisciplinary Evaluation of Rat Renal Cell Carcinoma

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Abstract. The rat renal cell carcinoma system as described by deVere White and Olsson in 1980 is used widely as a model for its human counterpart. The tumor arose spontaneously in a male Wistar Lewis rat and its behaviour has been shown to be stable during multiple passages. We have compared this tumor with the human renal cell carcinoma using a multidisciplinary approach. Light microscopy and electron microscopy showed a great resemblance of this rat tumor to a human renal cell carcinoma of the clear cell type with the ultra-structural presence of desmosomes. With the use of tissue specific antibodies against intermediate filament proteins, it could be shown that their expression is comparable to human renal cell carcinoma, i.e. coexpression of vimentin and different cytokeratins in the tumor cells. The cells could also be shown to contain cytokeratin 18. An aneuploid cell population in the tumor, expressing both vimentin and keratin, could be characterized by DNA flow cytometry in double labeling experiments. Comparison of normal and malignant rat kidney tissue by Northern blot analysis revealed increased levels of vimentin mRNA. In conclusion, this tumor model seems to have several histological and biological properties in common with the human renal tumor.

Metastatic renal cell carcinoma in man has a mortality rate of about 70%, in the first year and virtually no five year survivals. For this reason, one is reluctant to treat these patients with experimental cytotoxic chemotherapy. This treatment may, in a number of cases, not only fail to induce an increased survival time but may even decrease the quality of life.

For this reason, it is preferable to test experimental treatment protocols in animal models. For the extrapolation of results that are obtained in an animal model to the human situation, the animal model system should closely resemble the human renal cell carcinoma.

deVere White and Olsson have described a rat renal adenocarcinoma model system that has been used in several studies, amongst which chemosensitivity tests (1, 2, 3). This rat renal cell tumor arose spontaneously in the kidney of a male Wistar Lewis rat. It has been maintained by transplantation in the flank of syngeneic rats and has shown more or less predictable growth characteristics over several years.

In order to be able to use the rat renal cell tumor as a model system for human renal cell carcinoma, we analysed the animal tumor in a multidisciplinary approach and compared its cell biological properties with those of human renal cell carcinomas.

Materials and Methods

The rat renal tumor was maintained as follows (2): donor animals with large tumor nodules (3 - 6 cm in diameter) were anaesthetized with ether. Under sterile conditions, the tumor was dissected ex vivo. The normal tissue was excised from the tumor nodules and the tumor tissue cut into pieces of about 0.06 gr.

In recipient animals an 0.5 cm incision was made in the flank and the tumor fragment was implanted subcutaneously. The average tumor take with this method was more than 90%, and the tumor did not metastasize from this site. Wistar Lewis rats from OLAC (England) were used for further maintenance. We therefore evaluated the original tumor as well as the tumor in the English Wistar Lewis rats. In these latter animals the tumor could easily be kept, and it preserved the same macroscopic characteristics as in the original Wistar Lewis rats.

Histology. Tissue material of a representative part of the tumor was prepared for routine histological examination using hematoxylin and eosin (H. and E.) and periodic acid-schiff (P.A.S.) staining. Histological classification was in accordance with the human renal cell tumor classification (4).

Electron microscopy. The tumors were cut into 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 contrasted ultrathin sections were examined in a Philips EM 300.
Immunohistochemistry. 5 - 7 Micron thick frozen sections of the rat renal cell carcinoma, 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 (5).

The following antisera were used in this study: 1) An affinity purified rabbit antiserum to human skin keratin for the detection of the epithelial nature of the tumor. 2) An affinity purified rabbit antiserum to bovine lens vimentin, the intermediate filament protein of mesenchymal cells. 3) A monoclonal antibody to cytokeratin 18 (RGE 53) specific for glandular epithelial cells (6).

FCM analysis. Cell analyses were performed using a cytofluorograph 50 OHI (Ortho Instruments, Westwood, MA) as described previously (7). All data were stored in a correlated (list) mode on a PDP 11/34 computer (Digital, Marlboro, MA) for subsequent data analysis.

The normal human DNA index in our laboratory is 2.65 to 2.75 times the fluorescence intensity of chicken red blood cells (CRBC) (8). Cell cycle analysis was performed as described by Baisch et al. (9). As a control, cells incubated only with an FITC-conjugated second antibody were analyzed.

Northern and Southern blot analysis. Expression of vimentin at the mRNA level, using actin mRNA as a standard, was evaluated using pVim1 and pAct1 as probes (10). Preparation of the DNA probe and nick translation (11) was performed as described previously (12). Primer extension reactions were according to Messing and co-workers (13). The specific activity of the probes used in the hybridization studies was (2 - 5) x 10^6 cpm/µg. Agarose gel electrophoresis, Southern blot (14) and hybridization analysis was performed as described previously (12).

Total cellular RNA was isolated according to the procedure described by Aulfay and Rougeon (15). Upon poly (A) selection by oligo (dT) - cellulose chromatography, RNA was dissolved in sodium phosphate buffer (10 mM, pH 7.0), which containing 50% DMSO and 1 M glyoxal, and heated to 50°C for 1 h. RNA was size fractionated by agarose gel electrophoresis (1.0% agarose gel) and transferred to Hybond N (Amer sham) for hybridization analysis as described previously (16).

Results

Transplantation in vivo. The rat renal tumor model system described in this paper showed a 90% acceptance rate after subcutaneous transplantation of tumor pieces during ten passages. No differences were found between the original Wistar Lewis rat and the rats finally used in this study.

The tumors showed a reproducible growth curve as shown in Figure 1, which illustrates an example of a growth curve in vivo of the fourth passage in three different rats.

The mean growth in these three tumors is 9.1 cm^3 (longest diameter * height * width x 0.52 x 10^3) (17) at day 28.

Light microscopy. The rat renal cell carcinoma was composed of solid tumor areas containing cells with a large cytoplasm of varying size. The cytoplasm of the cells was strongly eosinophilic and contained multiple vacuoles. Nuclei, mostly eccentrically localized and round to ovaly shaped, often showed invaginations and contained one or more macro - nucleioli of an irregular or round shape. Extensions, part of which showed an atypical appearance, could be observed. Between the tumor cells solid areas of spindle - formed cells with oval nuclei and an eosinophilic granular cytoplasm were seen. The histological appearance fitted well with that of a clear cell type Grawitz tumor (renal cell carcinoma).

Electron microscopy. The tumor mass was composed of clustered electron-dense cells. Electron-light cells were also found interspersed in between the dark cells (Figure 2c). Generally the nuclei contained several nucleoli. Incomplete basal laminae surrounded islets of mainly dark tumor cells. Glandular-like formations with lumina were also observed. Mostly, smooth cell membranes were in close contact showing small desmosomes at several places and few fascia adherens - like junctions (Figure 2d). The organelles were well developed and few bundles of intermediate-sized filaments were present.

Apart from some lysosomes, small-sized next to large lipid droplets, as well as electron - lucent vacuoles, were always observed. Most striking was the presence of glycogen particles in varying amounts. Compared to the dark tumor cells, the light cells were irregularly shaped and showed long projections. Junctional complexes were present between both types of cells (Figure 2e,f). Generally the rough endoplasmatic reticulum appeared to be more abundant in these cells and was arranged in whorl - like patterns. Distinct Golgi areas
and intermediate-sized filaments were observed. The interstitium with fibroblasts consisted of collagenous fibrils and amorphous electron-dense plaques randomly distributed. Macrophages were frequently found enclosing the peripheral localized tumor cells.

Ultrastructurally the same tumor cell characteristics were found in both the American as well as in the English strain of rats.

**Immunohistochemistry.** It has recently been shown by several investigators (7, 18, 19, 20, 21) that human renal cell carcinomas express both the epithelial and the mesenchymal type of intermediate filament proteins. Rabbit antisera to cytokeratin and vimentin, respectively, have been shown to react with such tumors. These antibodies also stained both types of intermediate filament proteins in the rat renal cell tumor (Figure 2a, b), in both the clear cell and the granular cell part.
Using a monoclonal antibody to cytokeratin 18, (RGE 53) coexpression of this adenocarcinoma marker and vimentin in tumor cell areas was clearly demonstrated. Thus, these results show that, as far as intermediate filament expression is concerned, this rat renal cell tumor model is completely comparable to the human renal cell tumor.

Flow cytometric DNA analysis. The flow cytometric DNA analysis showed the presence of a mixture of two cell populations, one with a diploid and one with an aneuploid DNA content (figure 3a). In order to characterize these two cell populations, the two parameter flow cytometric technique with propidium iodide (P.I.), for the quantitation of DNA and labeling for vimentin of cytokeratin was used. Vimentin proved to be present in both the aneuploid tumor cells and the diploid cells (Figure 3b). Cytokeratin was expressed only...
in the aneuploid tumor cells (Figure 3c). The specific labeling of the tumor cells with the cytokeratin antibody allowed determination of the proliferative fractions of the neoplasm without any disturbance of the stromal component. The cytokinetic results for the aneuploid cells indicate an S fraction of about 20% and a G2+M fraction of about 18% (n=4) (Figure 3d). The distribution of normal rat kidney cells was G1=82%, S=5.6% and G2+M= 12.4%.

Northern and Southern blot analysis. Analysis of vimentin expression in rat renal tumors and normal rat kidney tissue at the mRNA level was performed using pVim1 as a molecular probe. Actin expression was used as a standard using pAct1 as a probe. It was shown that there was a threefold increase of vimentin expression in the tumor compared to normal kidney tissue of the same rat (Figure 4). Results of southern blot analysis using pVim1 did not reveal any abnormality in the structure of the vimentin gene (data not shown).

Discussion
The purpose of this study was a multidisciplinary analysis and
characterization of a rat renal cell tumor model system with special emphasis on the question of whether or not this tumor model is comparable to the human renal cell tumor. Such comparability would imply that this tumor could eventually be used as a model system in cancer drug studies. Such a model for renal cell tumor should have the following properties: a) it must have originated (spontaneously) from rat renal cell tissue; b) it must be maintainable in a stable form during long term passages; c) the tumor should have histological, biological and biochemical characteristics identical or very similar to the human renal cell tumor.

The murine tumor evaluated in this paper originated in a male Wistar Lewis rat and was maintained over several years by subcutaneous transplantation as described earlier (2). According to these authors, the tumor arose spontaneously, was hormone independent and could be readily transplanted. In our hands, in 90% of all tumor transplantsations a reproducible tumor growth curve could be monitored in the recipient animals.

As far as histological and biochemical characteristics are concerned, we can state that this rat renal cell tumor model shows properties that closely resemble the human renal cell tumor. The histology as deduced from paraffin sections is that of a typical clear cell type Grawitz tumor. Electron microscopic examinations of the rat tumor revealed the presence of two different cell types. Ultrastructural studies also showed that this mixed type of renal cell tumor is composed of electron-dense and electron-light tumor cells. The electron-dense cells showed a more epithelial organization with occasional formation of glandlike structures.

Incomplete basal laminae mostly surrounded islets of electron-dense cells. The electron-light cells showed a more interspersed distribution. All cells show the presence of high amounts of glycogen and lipids, a feature which is typical for the human clear cell component of the renal cell tumor. Whether or not the two different cell types seen in the EM in the rat renal cell tumor have their human counterparts in the clear cell and granular cell types remains to be answered (4).

From the intermediate filament studies it appeared that most rat renal cell tumor cells co-express cytokeratins and vimentin. The presence of cytokeratins, as detected by a broadly cross-reacting rabbit antiserum, shows the epithelial nature of the rat tumor. The occurrence of cytokeratin 18 within the tumor, as demonstrated by a monoclonal antibody (RGE 53), suggests an adenocarcinomatous character. It furthermore supports the assumption that this tumor has its origin in (part of) the ductular structures of the kidney.

As well as cytokeratin type of IFP, the tumor cells also contain vimentin. Double label experiments have shown that renal cell tumor cells can express both cytokeratin and vimentin while others express only cytokeratins, thus confirming our earlier studies on human renal tumors in which the same properties of intermediate filament expression were seen (18).

In general, the vimentin type of IFP preferentially occurs in mesenchymal cell types, but it has also been demonstrated to be present in some epithelial neoplasms, among them the Grawitz type of kidney tumor. This phenomenon thus adds further evidence of the renal cell carcinoma nature of the tumor studied here. It should be kept in mind, however, that the fact that this tumor is constantly transplanted may also be partly responsible for this vimentin expression. At the RNA level, a three-fold increase of the vimentin mRNA in the rat tumor as compared to normal rat kidney tissue could also be shown. The increase of the vimentin mRNA level is probably due to the greater number of cells expressing the vimentin gene, however, the possibility cannot be excluded that overexpression is responsible for this phenomenon. No structural disorders in the genomic organization were seen. In human renal cell tumors too, a drastic increase of the vimentin mRNA level has been detected.

By means of two dimensional DNA flow cytometric analysis, the DNA content of the vimentin and cytokeratin positive cells could be compared and the proliferative fraction of the tumor cells could be analyzed separately from (cytokeratin negative) stromal and inflammatory cells (7). The coexpression of vimentin and cytokeratin can be seen in the aneuploid fraction which was found in this renal cell tumor, while the diploid fraction proved to be cytokeratin negative. These data are comparable to findings in human renal cell tumor (7, 18) and they give support to the biological comparability of the rat and human renal cell tumor.
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