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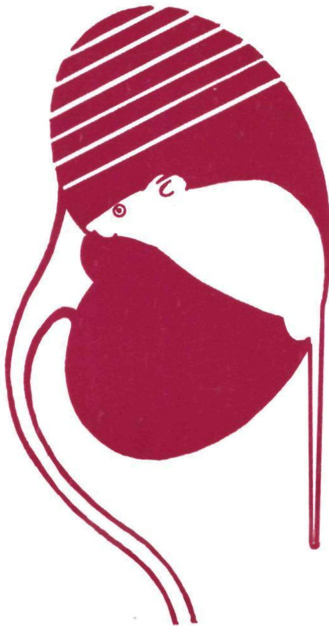
A COMPARATIVE STUDY
OF RENAL CELL TUMOR MODEL SYSTEMS



H.F.M. KARTHAUS

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CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Karthaus, Herbert Ferdinand Marie

A comparitive study of renal cell tumor model systems / Herbert
Ferdinand Marie Karthaus. - (S.l.: s.n.)(Helmond: Wibro).-Ill.
Proefschrift Nijmegen. - Met lit. opg. - Met samenvatting in het Nederlands.
ISBN 90-9001512-4
SISO 605.91 UDC 616.61-006.6(043.3)
Trefw.: nierkanker.

A COMPARATIVE STUDY OF RENAL CELL TUMOR MODEL SYSTEMS

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE KATHOLIEKE UNIVERSITEIT TE NIJMEGEN, OP GEZAG VAN DE RECTOR MAGNIFICUS PROF. DR. B. M. F. VAN IERSEL VOLGENS BESLUIT VAN HET COLLEGE VAN DECANEN IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 27 FEBRUARI 1987 DES NAMIDDAGS TE 1.30 UUR PRECIES

DOOR

HERBERT FERDINAND MARIE KARTHAUS

GEBOREN TE UTRECHT

Druk Dissertatiedrukkerij Wibro, Helmond

Promotores : Prof. dr. F.M.J. Debruyne
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The investigations presented in this thesis were performed in the Department of Urology and Pathology of the St. Radboud Hospital and in the Molecular Oncology Section of the Department of Biochemistry, University of Nijmegen, The Netherlands.

These studies were financially supported by the Kidney Foundation of The Netherlands, projectno's C 85.531, 563, and Rhône-Poulenc Netherlands.

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aan Noor
Frederike en Wouter

Aan mijn ouders en
schoonouders

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CHAPTER I

INTRODUCTION

INTRODUCTION

Renal cell carcinoma or renal adenocarcinoma represents an intriguing problem in tumor behaviour and tumor cell biology. This type of carcinoma is a disease of older adults, the median age at diagnosis being about 55 years. It may remain asymptomatic until relatively late in its natural history. Also one third of the patients is known not to present with urological symptoms which can result in a delayed diagnosis. About 30% of patients have overt metastases at the time of diagnosis (1, 2, 3). In addition, the clinical course and prognosis of metastatic renal cell carcinoma is extremely variable and unpredictable, although generally poor with a median survival time of 8-10 months after the time of diagnosis (1). Spontaneous regression of metastases has been reported, mostly concerning lung metastases disappearing after nephrectomy (4). The incidence of spontaneous regression, however, is very rare and at the moment it is impossible to identify such patients in an early stage of the disease. A special entity in metastatic renal cell carcinoma is a primary tumor with a solitary metastasis, which has an identical prognosis as non-metastasized renal cell carcinoma when treated aggressively (5).

One of the phenomena suspected to be responsable for the capricious behaviour of the tumor is a high degree of immunological host-tumor interaction (6). However our actual knowledge about renal cell tumor biology is still fragmentated, making a strategic approach in therapy very difficult.

At the moment it appears that the limits of therapeutical modalities for localized disease have been reached in radical nephrectomy (7). It is known that surgical treatment, performed in an early stage of disease, is the only approach that can lead to a cure in renal cell carcinoma. Unfortunately one third of these patients prove to have clinical imperceptible metastases at the time of surgery rendering the prognosis for these patients negligible with a 5 year survival of 0-8% (2, 7).

Logically, the identical surgical approach in clinically advanced cases does not guarantee a definite cure. Nor does pre- or

postoperative combination with irradiation, chemotherapy, hormonal therapy or immunotherapy improve the prognosis so far (8, 9, 10, 11, 12).

In light of this knowledge one can conclude that progression in the treatment of renal cell carcinoma is to be made by

1) development of new techniques for earlier diagnosis,
2) advances in systemic therapy, and
3) a better understanding of cell biological properties and clinical behaviour of the tumor.

This thesis has been written in order to shed some light on aspects of the biological, biochemical and histochemical properties of renal cell carcinoma. For this purpose a spontaneous rat renal cell tumor (13) and several renal cell carcinoma xenografts in nude mice (14) were studied. Routine techniques such as light microscopy and electron microscopy as well as flow cytometric DNA measurements, determination of intermediate filament expression, activated oncogene analysis and growth studies in the double layer soft agar culture system were performed.

In order to be able to compare the results of these studies with the results obtained in human tumors, also flow cytometry and oncogene studies were performed on primary human renal cell carcinomas.

A brief inventory on what is known about renal cell carcinoma, its clinical behaviour, its prognostic factors, and its histological and biological properties is made below.

RENAL CELL CARCINOMA

Epidemiology

Malignant renal cell carcinomas account for 3% of all cancers in human and renal adenocarcinomas constitute 85% of all renal tumors (15). The median age at diagnosis of renal cell carcinoma is 55 years (16) and is two or three times more common in men than in women (17). The

incidence of renal cell carcinomas is highest in the Nordic countries and lowest in the Orientals (18).

Clinical presentation

About 70% of the patients with a renal cell carcinoma present with urological symptoms such as hematuria, flank pain or a palpable mass. A combination of these three symptoms, called "the classical triad", occurs only in 9% of all cases (7, 19).

Ten percent of all patients with renal cell carcinoma present with primary symptoms as a result of metastases in lungs, bones or brain (7). In turn 6% of patients renal cell carcinoma is accidentally encountered in the course of a diagnostic procedure for other reasons (20). Renal cell carcinoma is frequently accompanied by aspecific signs of malignancy including weightloss, fatigue and gastrointestinal symptoms (25%), fever, secondary to tumor necrosis or tumor pyrogens (20%), anemia (30%) and a raised E.S.R. (erythrocyte sedimentation rate) (39%) (21).

Also paraneoplastic syndroms such as erythrocytosis (10%) and hypercalcemia (5%), as a result of bone metastases or ectopic or primary hyperparathyroidism are seen (21).

In 15% of the patients the so called Stauffer syndrome is found. This is represented by a hepatic dysfunction (detected as elevated serum levels of indirect bilirubin, $alpha_2$ globulin and alkaline phosphatase combined with hypoprothrombinemia) in absence of metastases, which resolves spontaneously after nephrectomy (22).

In addition renal cell carcinoma can present with a variety of symptomatology such as vaginal bleeding secondary to vaginal metastasis, priaprism as a result of penile metastases, breast masses secondary to breast metastases, varicocele following obstruction of the spermatic or ovarian vein by tumor mass or several syndroms of ectopic hormone production (21, 31).

Metastatic behaviour

The metastatic route of renal cell carcinoma is threefold (21), lymphogenous to regional and juxtaregional nodes,
2) lymphohematogenous, and
3) hematogenous.

The lymphogenous metastases affect mainly the renal hilar lymphactics and the regional, paracaval, paraaortal and parailiacal lymph nodes. The lymphohematogenous pathway follows the thoracic duct into the supraclavicular venous angle.

The hematogenous metastases spread mostly directly via the renal vein and via pararenal and paravertebral anastomoses. Also retrograde spread via the spermatic or ovarian veins is possible (27).
This phenomenon may account for the odd clinical presentations mentioned above.

When metastasized at the time of diagnosis, renal cell carcinoma shows in 70% metastases limited to one organ, usually the lung (45%) or the bone (22%) (7, 23). Autopsy, performed on patients with renal cell carcinoma, shows in 81-95% of cases multiple metastases. The incidence of involvement of different sites is noted as follows: lung (67-76%), lymphnodes (40-66%), bones (mostly lytic of character) (42%), liver (41%), contralateral kidney (23%), ipsilateral adrenal (17%) and contralateral adrenal or brain (11%) (24, 25, 26).

To identify the prognosis of the subsets of renal cell carcinomas with or without metastases, many efforts were made to relate clinical staging and cellular, histological and biochemical characteristics to prognosis.

Classification of anatomical extent

To express and determine the relative value of the anatomic extent of renal cell carcinoma two methods are presently in use. The first is the Robson classification scheme (2), a major step forward at the time of its presentation in 1969, made by establishing the basic principles

for a delineation of the anatomic extent of the disease. The second scheme is the TNM staging system as proposed by the U.I.C.C. (28) in 1978, which more explicitely defines the anatomic extent of the disease including venous involvement.

Several studies (7, 21, 29, 30) confirmed the stage related prognosis which means that an advanced tumor stage, local tumor invasion and invasion of vena renalis or cava and even regional lymph node invasion are unfavourable signs.

Histopathological classification

Renal cell carcinoma has been proposed to originate from the proximal tubular epithelium of the nephron (21).

Macroscopically, renal cell carcinomas are solid tumors of a yellow to grey color depending on their lipid content. In large tumors often areas of tumor cell necrosis are found. There is no preference for localization in certain areas of the kidney (26).

Microscopically, tumor structures vary from tubular, papillary, cystic to a solid and anaplastic character. The histological cell type patterns are to be classified in four subsets based on cytoplasmic properties (26):

1) clear cell tumors (25%) consisting of cells containing large amounts of lipids and glycogen (34).
2) granular cell tumors (15%) containing cells with granular cytoplasm as a result of abundant, mostly abnormal, mitochondria and cellular organelles (34).
3) mixed type tumors (46%) containing both clear cells and granular cells.
4) sarcomatoid or spindle cell tumors (14%).

Statistically clear cell tumors appear to have a somewhat favourable prognosis as compared to granular and mixed type tumors, but this is not a consistant finding in literature (3, 32). An overall 5 year survival rate is reported to be 50%. Spindle cell tumors, however, have a significantly worse prognosis showing a 5 year survival rate of

23% (3).

The histological grading varies from highly differentiated (G1, 28%), via intermediate (G2, 32%) to undifferentiated (G3, 40%) (15). The significance of the histological degree of differentation for the prognosis of renal cell carcinoma is generally recognized (29, 30). This means that roughly grade I tumors of the primarily non-metastasized renal cell carcinoma clearly show the best prognosis (30, 32).

In the German literature a combination of staging and grading is proposed by Hermanek et al. (33), identifying groups with survival curves that are clearly distinguishable from each other (30).

Ultrastructural grading is found to add little to light microscopic grading; neither could adenomas be distinguished from adenocarcinomas. Ultrastructural analysis is most helpful, however, in tumor classification. Also ultrastructural similarities between tumor cells and cells of the proximal convoluted tubule are encountered (34). Moreover several features of renal cell carcinoma such as lipid and glycogen content, which disappear upon routine histological processing, are easily identifiable (34).

Nuclear grading is based on the impression that distinctly larger nuclei in renal cell carcinoma are an expression of an aneuploid state and therefore represent a morphologic sign of biologic anaplasia. For this purpose the relative sizes of the nuclei of the cancer cells are categorized. If more than 5% of the entire nuclear content shows a variation factor in the nuclear area surface greater than two, the survival rates of patients in the same stage of disease (according to Robson) (2) differ significantly and it is suggested that foci of cells with larger nuclei in primary renal cell carcinoma can be interpreted as the morphologic manifestation of tumor agressiveness. As such, nuclear grading has been reported to be of dominant prognostic value in supplement to the Robson staging system (35, 36).

Marker studies

Recently monoclonal antibodies detecting cell surface antigens, expressed in distinct domains of the normal adult human nephron, have been identified (37). They are supposed to be valuable reagents for the definition of cellular origins and subsets of renal cell carcinoma in relation to prognosis and therapy. In analogy to bladder carcinoma, renal cell carcinomas have been examined for the presence of A.B.O. (H) blood group antigens, T-antigen and C.E.A. (carcinoembryonic antigen), but no valuable results were established (38).

The latest developments in renal cell tumor characterization concern flow cytometric DNA content analysis, the study of cytoskeletal structures and activated oncogene identification.

Flow cytometric analysis allows us to study factors such as the DNA content and heterogeneity of a certain tumor by using a two parameter analysis (39). Also the ploidy of a certain specific tumor fraction can be determined. The DNA content may be a very important parameter in diagnostic pathology, as a change in DNA content may be one of the main characteristics of malignant transformation or progression and may be of prognostic value in renal cell carcinoma (40).

Studies on the composition of the cytoskeletal structures in tumors may provide information about their cell composition and histogenesis. The cytoskeleton consists of a fibrous matrix of proteins spanning the cytoplasm between nucleus and plasma-membrane and may have important functions in many vital processes of the cell. One group of proteins has been shown to be of particular importance for the characterization of different tumors and tissue types. These are the so-called intermediate sized filaments (I.F.; 7-11 nm diameter) represented by five different protein types with a tissue specific distribution (41). In tissues most cells express only one characteristical I.F. protein. Specific I.F. protein expression is generally preserved upon neoplastic transformation (42).

I.F. expression in renal cell carcinoma shows interesting aspects, in that a coexpression is seen of two I.F. proteins, i.e. cytokeratin, a

marker for epithelial cells, and vimentin, which is predominantly found in mesenchymal cells (43, 44, 45). In contrast, this coexpression is normally not found in the mature human kidney. In the proximal tubular cells of the nephron only cytokeratin is encountered. In the fetal stage however, the embryonal mesenchyme expresses vimentin suggesting that some tumor cells reexpress their embryonal I.F. pattern (44).
The correlation of these findings with the biological behaviour of renal cell carcinoma may lead to a better understanding of the characteristics of renal cell tumors and their subfractions.

Recently another challenging field in the progressing knowledge of tumorigenesis has emerged in the form of the discovery, that common molecular mechanisms may be responsible for the origin of cancer. In the normal cell these mechanisms are regulated by genes called proto-oncogenes. They fullfill regulatory functions in cell growth and differentiation. By means of somatic mutations (either spontaneous or induced by environmental factors) they can be abnormally activated and thus turn into oncogenes, capable of deregulating the cell, for instance in favour of cell proliferation.

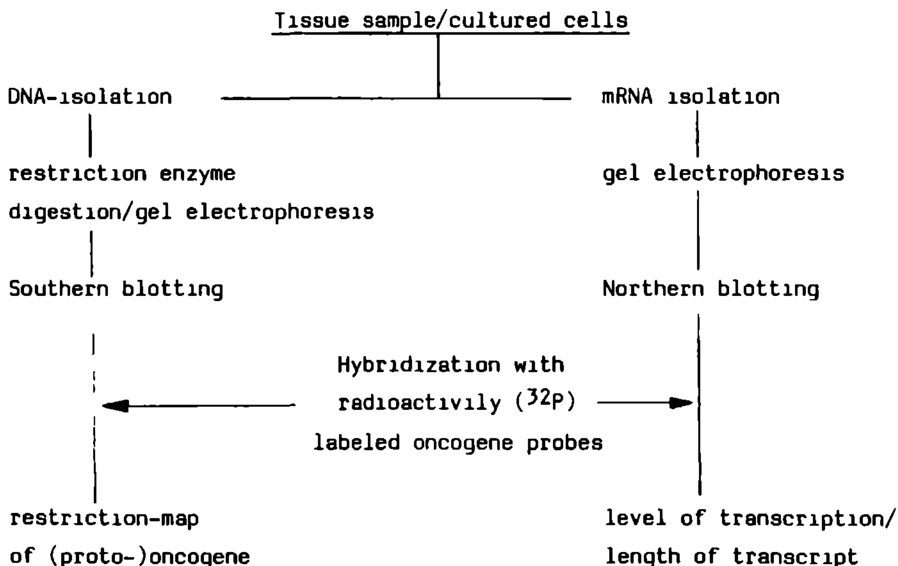
Up to this moment more than fourty oncogenes have been characterized. Mainly based upon localization of gene products within the cell the proto-oncogenes are divided in categories (46). One category consists of so called nuclear oncogenes and one of cytoplasmic oncogenes (46). The nuclear group is the smallest one in which among others the proto-oncogenes c-*myc*, c-*myb* and p53 are classified. These genes tend to have immortalization abilities and are generally weak in inducing anchorage independence of fibroblasts in culture. Their encoded proteins are usually bound to nuclear structures. In their action a deregulation of transcription seems to be involved (47). The encoded proteins of c-*myc* and c-*myb* are structurally homologous (48). p53 is a phosphoprotein that is overexpressed in transformed cells (49). The expression of p53 is apparently necessary for maintaining cells in a transformed state in certain cases (50).

The larger cytoplasmic group is characterized by localization of their gene products in the cytoplasm. These oncogenes are thought to be

involved in the event of transformation and anchorage independence of fibroblasts in culture. Their mode of functioning seems to be rather divergent (46). Members of this group are for instance the family of ras genes, the fes oncogene and the abl oncogene. Their activation can be the result of a mutation of the structure of their encoded protein. (For instance, the p21 ras protein can be activated by a point mutation, which affects the GTP-ase binding properties.)
The p21 protein of c-ras is localized at the inner surface of the cell membrane and has been shown to reveal homology to the G-proteins, which are known to function as signal transducers of cell surface receptors (51). The c-fes and the c-abl oncogene belong to a group that is characterized by a tyrosine specific protein kinase activity. In this group the functional analogy is more obscure, however, some might encode receptor-like proteins.

The analysis of activated oncogene expression in renal cell carcinoma is therefore important as a fundamental approach that may lead to a better understanding of tumorigenesis. Moreover, this analysis could possibly be of diagnostic value.

Schematically the analysis of the structure and expression of proto-oncogenes is performed as follows:



The study of (proto-)oncogenes in relation to renal cell carcinomas reported in this thesis is confined to the experiments outlined sofar. One should realize that this is only the onset of a research program for a team of molecular oncologists. Once a typical expression pattern of one or more genes for a certain type of cancer is established, one should discriminate between expression at an abnormal level, and synthesis of an altered gene product. In both cases one wants to know the causal relationship, if any, between the observation and the development of the cancer. In the former case the research will be aimed at the elucidation of the genetic sequences responsible for the abnormal expression and of the factors that in their turn act as effectors in the regulation of these genes. In the latter case the nature of the modification should be studied in an attempt to understand the malignant properties of the oncogene. The history of medicine, although loaded with empirical therapies, is witness to the fact that other new insights eventually lead to substantial improvement of the treatment of patients. This view has inspired our research and is the justification of molecular oncology.

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CHAPTER II

MULTIDISCIPLINARY EVALUATION OF RAT RENAL CELL CARCINOMA

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Submitted.

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 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 ultrastructural 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 the double layer soft agar human tumor cloning system rat renal cell tumors showed a positive growth capacity. In conclusion this tumor model seems to have several histological and biological properties in common with the human renal tumor.

INTRODUCTION

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 therapy may, in a number of cases, not only fail to induce an increased survival time, but may even decrease the quality of life.

Therefore it should be preferred 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 (deVere White et al., 1980, 1982 and 1983). This rat renal cell tumor arose spontaneously in the kidney of a male Wistar Lewis rat. It has been maintained by tranplantation in the flank of syngeneic rats and has shown more or less predictable growth characteristics over several years.

In order to be able and use the rat renal cell tumor as a model system for human renal cell carcinoma we analized 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 (deVere White et al., 1980): Donor animals with large tumor nodules (3-6 cm in diameter) were anesthetized with ether. Under sterile conditions, the tumor was dissected en bloc. The normal tissue was excised from the tumor nodule 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 in this way, 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 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 to the human renal cell tumor classification (Bennington and Beckwith, 1975).

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 aceton at room temperature. The indirect immunofluorescence technique was performed as described before (Ramaekers et al., 1983a).
The following antisera were used in this study : 1) An affinity purified rabbit antiserum to human skin keratins 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 (Ramaekers et al., 1983).

FCM analysis: cell analyses were performed using a cytofluorograph 50 OH (Ortho Instruments, Westwood, MA) as described before (Feitz et al., 1986a). All data were stored in 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 fluorescense intensity of chicken red blood cells (CRBC) (Hiddeman et al., 1984) Cell cycle analysis was done as described by Baisch et al. (1975). As a control, cells incubated only with an FITC-conjugated second antibody were analyzed.

Human tumor clonogenic culture system (H.T.C.S.): For the detection of the growth potential of tumor cells in the soft agar double layer cell culture, the culture system as described by Hamburger (1977) was used. Tissue selected for this test was processed according to Salmon et al. (1978). The cells were cultured immediately after preparation of the single cell suspension and growth potential was quantified using the Omnicon FAS II automated colony counter (Milton Roy Inc., Rochester, New York, USA) (Herman et al., 1983). For dynamic colony growth evaluation a "Temporal Growth Pattern" (T.G.P.) was used which gives a description of the growth over a certain period of time. The briteria that had to be met for these evaluations are described by

Feitz et al. (1986b) and Verheijen et al. (1985). Also a plating efficiency can be calculated by the formula:

$$\left(\frac{\text{number of colonies}}{\text{amount of cells/disk}} \times 100\right).$$

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 (Dodemont et al., 1982). Preparation of the DNA probe and nick translation (Rigby et al., 1977) was performed as described before (Schalken et al., 1985). Primer extension reactions were according to Messing and co-workers (Messing et al., 1982). The specific activity of the probes used in the hybridization studies was (2-5) x 10^8 cpm/µg. Agarose gel electrophoresis, Southern blot (Southern, 1975) and hybridization analysis was performed as described before (Schalken et al., 1985).

Total cellular RNA was isolated according to the procedure described by Auffray and Rougeon (1980). 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 (Amersham) for hybridization analysis as described before (Steenbergh et al., 1984).

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 reproducable growth curve as shown in Fig. I, which illustrates an example of a growth curve in vivo of the fourthpassage in three different rats.
The mean growth in these three tumors is 9,1 cm^3 (longest diameter

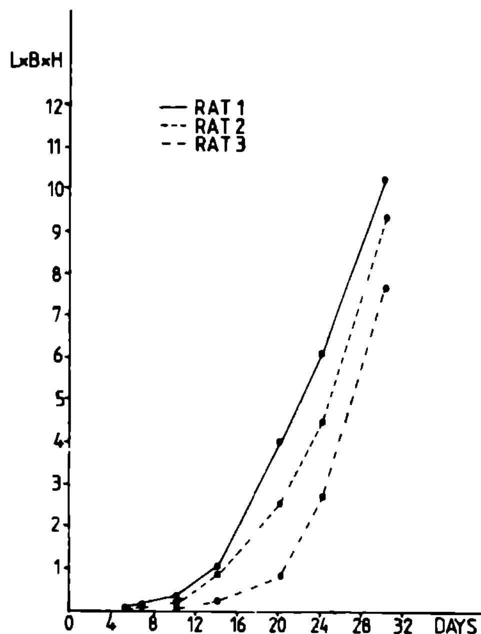

Fig I: Example of in vivo growth curve of tumor nodules in three different rats during the fourth passage. Tumor volumes are calculated according to the formula described by Janik et al. (1978): length x width x height x ($0{,}52 \times 10^{-3}$) (symbol:LxBxH).

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Fig II: Immunohistochemistry and electron microscopy of the rat renal cell tumor. (a) Positive reaction for cytokeratin and (b) vimentin polyclonal rabbit antiserum in the tumor cells (x 230). (c) Low magnification of glandularlike formation of epithelioidly arranged electron dense tumor cells with few light cells interspersed. Note mitotic figure in light cell (arrow). (x 2000). Asterisks indicate glycogen areas. (d) Part of two electron-dense tumor cells with adherence-like junctions and intermediate-sized filaments. Asterisks indicate glycogen. Arrow points to incomplete basal lamina. (x 18500). (e) Detail of an electron-dense and electron-light tumor cell with a small desmosome. At open arrows intermediate-sized filaments are shown. (x 24000). (f) Two electron-light tumor cells with desmosomes and intermediate-sized filaments (open arrows); black arrows indicate glycogen particles.(x 29500).

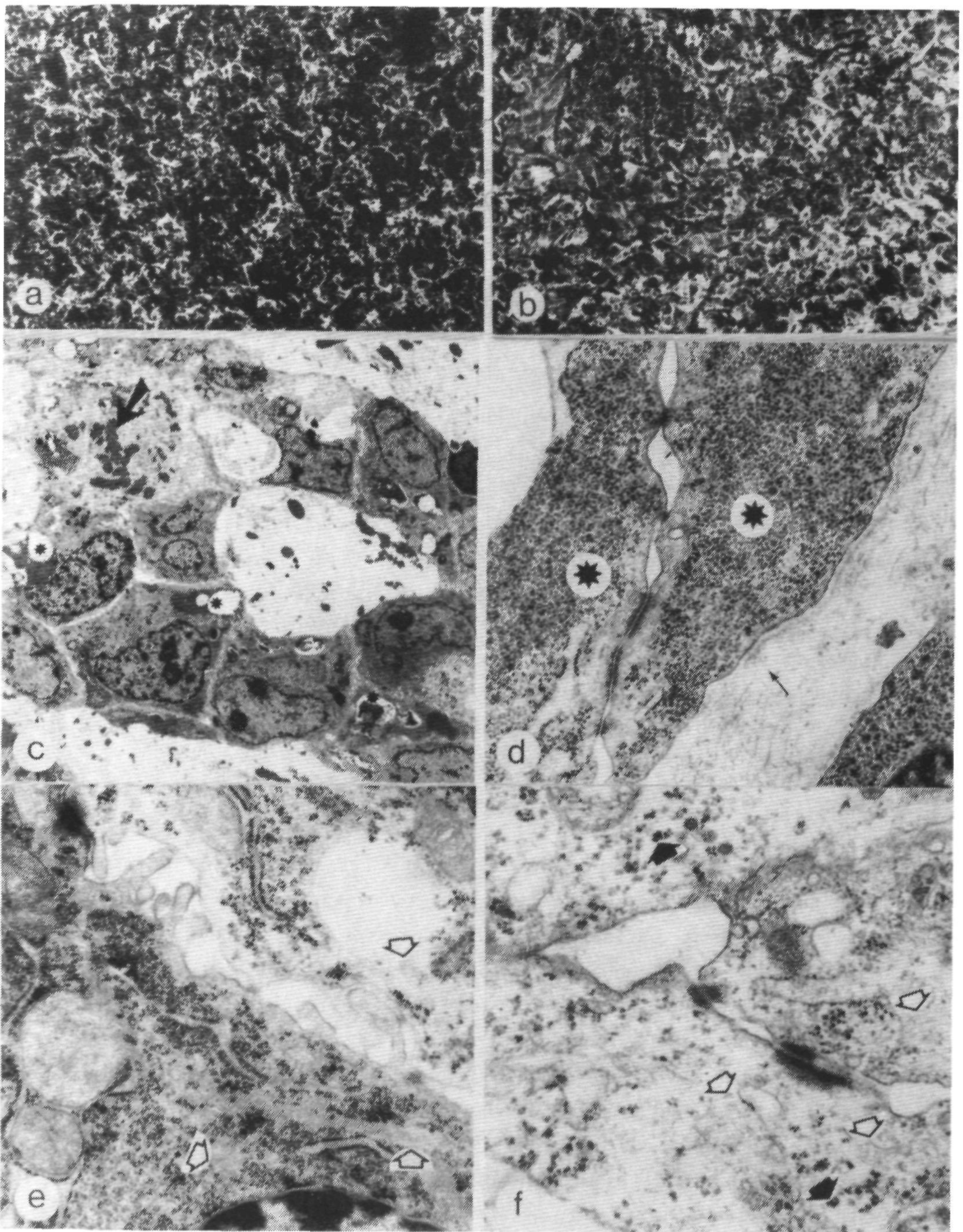

Fig. II

versus height and width x 0,52x10-3) (Janik et al., 1978) 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 excentrically localized and round to ovally shaped, showed often invaginations and contained one or more macronucleoli of an irregular or round shape. Extensions, part of which showed an atypical appearence, 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 (Fig. IIc). Generally the nuclei contained several nucleoli. Incomplete basal laminae surrounded islets of mainly dark tumor cells. Glandular-like formations with lumina were observed, too. Mostly, smooth cell membranes were in close contact showing small desmosomes at several places and few fascia adherens-like junctions (Fig. IId). The organelles were well developed and few bundles of intermediate-sized filaments were present.

Apart from some lysosomes, small-sized next large lipid droplets as well as electron-lucent vacuoles were always observed. Most strikingly was the presence of glycogen particles in varying amounts. Compared to the dark tumor cells the light cells were irregularly shaped, showing long projections. Junctional complexes were present between both types of cells (Fig. IIe, 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 described by several investigators (Herman et al., 1983, Holthofer et al., 1983, Feitz et al., 1986a, b) 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 did also stain both types of intermediate filament proteins in the rat renal cell tumor (Fig. II a, 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. Therefore, 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 (Fig. IIIA). In order to characterize these two cell populations, the two parameter flowcytometric technique with propidium jodide (P.I.), for the quantitation of DNA and labeling for vimentin or cytokeratin was used.Vimentin proved to be present both in the aneuploid tumor cells as well as in diploid cells (Fig. IIIB). Cytokeratin was expressed only in the aneuploid tumor cells (Fig. IIIC). 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) (Fig. IIID). The

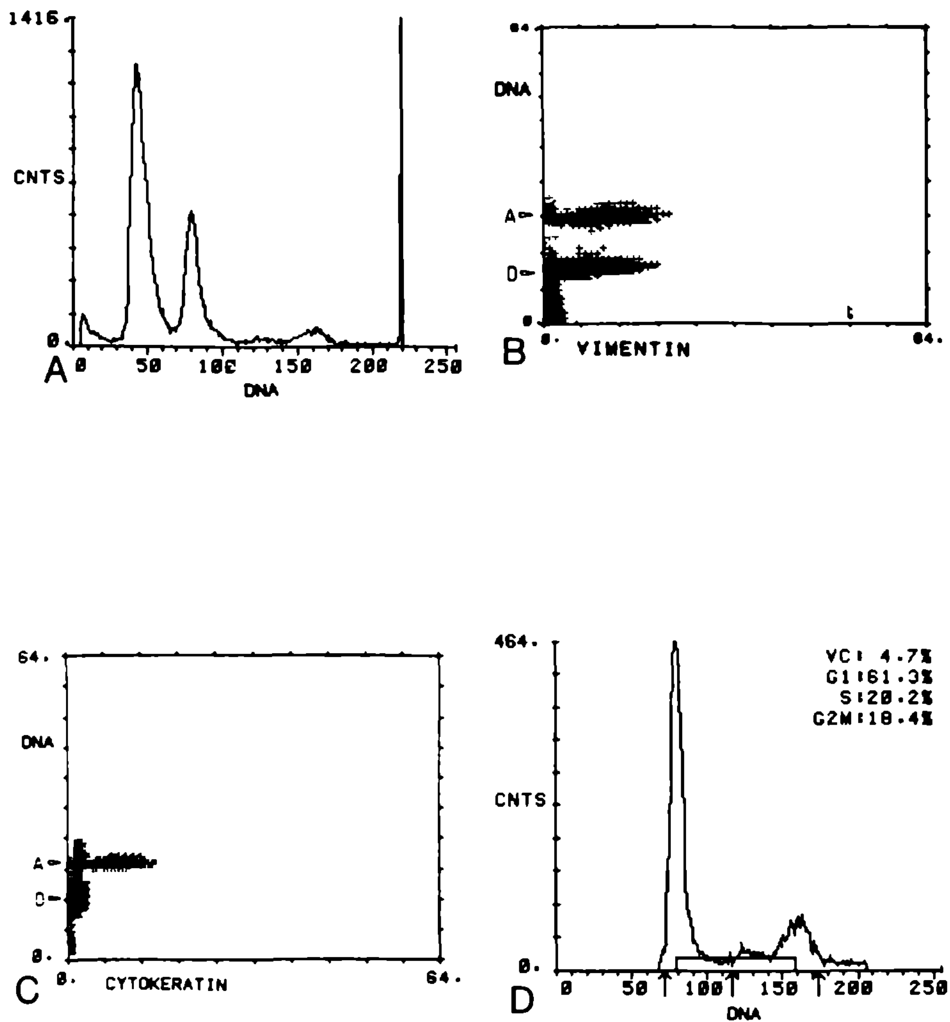

Fig 3 : Example of a two-parameter flow cytometric (FCM) analysis of a rat renal cell tumor using the cytokeratin antibody pKer and the vimentin antibody pVim in addition to DNA-staining with PI. (A) One-dimensional FCM DNA pattern of the cell suspension stained only with PI. (B) A two-dimensional projection of the DNA content and the vimentin content of the rat tumor. (C) A two-dimensional projection of the DNA content (as PI fluorescence) and the cytokeratin content (as FITC fluorescence) from the same cell suspension as shown in A. Note that the tumor fraction is cytokeratin- and vimentin-positive. (D) DNA histogram of the cytokeratin-positive cells, present in the cell suspension. The cell cycle parameters of the tumor cell suspension are indicated in the upper right corner.

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 (Fig. IV). Data of southern blot analysis using pVim1 could not reveal any abnormality in the structure of the vimentin gene (data not shown).

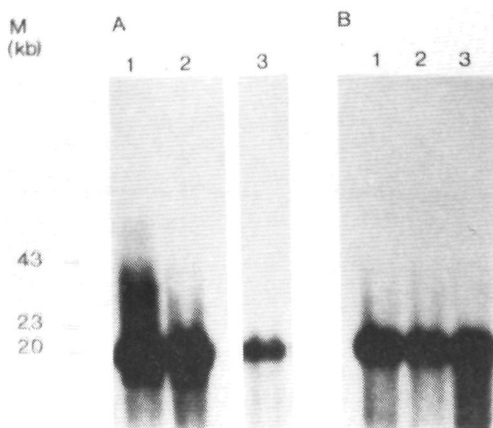

Fig IV: Northern blot analysis of vimentin (blot A) and actin (blot B) mRNA levels in the original rat renal cell tumor (lanes A1 and B1), in a tumor specimen of the tenth passage (lanes A2 and B2) and in normal rat kidney tissue (lanes A3 and B3). Poly(A) selected RNA from the tumor was size-fractioned by agarose gel electrophoresis and analyzed by the Northern blot technique. Molecular probes used were pVim1 for vimentin and pAct1 for actin. Molecular weight markers included were single strand HindIII digested labda DNA molecules.

H.T.C.S. Results

In the Human Tumor Cloning System using the double layer soft agar method culture the rat renal tumor cells gave a reproducable Temporal Growth Pattern (T.G.P.) during the different passages. The peak growth of colonies was obtained after about 16 days and colonies showed an increase not only in number, but also in the size (Fig. V). A plating efficiency of 0.015% was noticed when 500,000 cells/disk were plated.

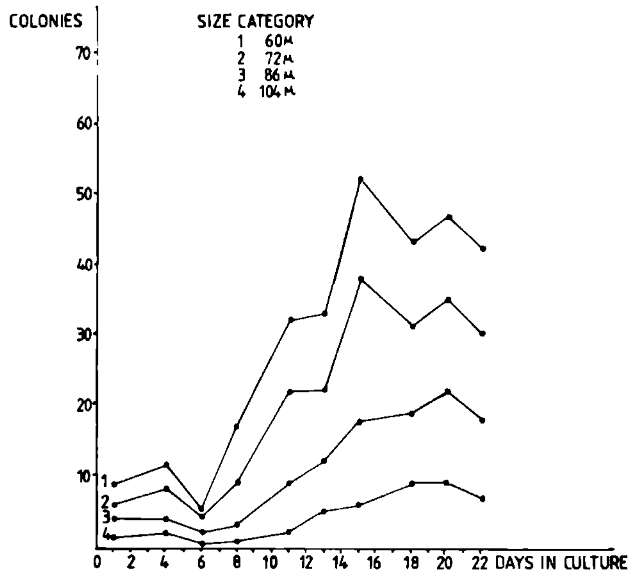

Fig V: Temporal growth patterns in the double layer soft agar human tumor cloning system of four size-categories of tumor colonies of a cell suspension of the rat renal cell tumor after three passages.

DISCUSSION

The purpose of this study was a multidisciplinary analysis and characterization of a rat renal cell tumor model system with special emphazis to the question whether or not this tumor model is comparable to the human renal cell tumor. This 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:

1) it must have originated (spontaneously) from rat renal cell tissue;
2) it must be maintainable in a stable form during long term passages;
3) 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 before (deVere White and Olsson, 1980). According to these authors the tumor arose spontaneously, was hormone independent and could be readily transplanted. In our hands, in 90% of all tumor transplantations a reproducable 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 epitheloid organization with occasional formation of glandlike structures.
Incomplete basal lamina 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 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 (Bennington and Beckwith, 1975).

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.
Next to 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 (Herman et al., 1983).

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

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 (Feitz et al., 1986a). 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 the findings in human renal cell tumor (Herman et al., 1983; Feitz et al., 1986a) and they give support to the biological comparability of the rat and human

renal cell tumor.

Finally, the tumor was also tested in the Human Tumor Cloning System (Hamburger and Salmon, 1977) in order to evaluate its growth capacity and behaviour in this in vitro culture system, using a dynamic culture evaluation method. Cell suspensions of tumors of different passages showed a reproducable Temporal Growth Pattern (Verheijen et al., 1985). In summary we can conclude that the rat renal cell tumor model described here shows a similar histological and biochemical profile as compared to human renal cell tumor and is therefore a suitable model for studying effects of new or experimental drugs on renal cell tumor, provided that the precautions necessary for comparing each animal model with the human counterpart are kept.

ACKNOWLEDGEMENTS

We want to thank Mr. H. Croes, Mr. P. Peelen and Mr. O. Moesker for excellent technical assistance. We also want to express our gratitude to Dr. Richard K. Babayan, M.D., Boston, Massachusetts for providing the tumor.

This study was supported by the Kidneyfoundation of The Netherlands. Projectno.: C 85.563.

Title: Chemosensitivity modification and cellpopulation characterisation of primair human and model systems of the Grawitztumor.

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MULTIPARAMETER ANALYSIS OF FOUR HUMAN RENAL CELL CARCINOMA XENOGRAFTS IN NUDE MICE

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Submitted.

ABSTRACT

Four human renal cell carcinoma xenografts (RC_2, RC_{14}, RC_{43}, N_{65}), maintained in nude mice during several years, were studied in a multidisciplinary set up, 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 vimentins, while in RC_{43} 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. RC_{43} showed a three-fold higher level of vimentin mRNA then the other xenografts. Growth potential in the human tumor cloning system was evaluated by temporal growth pattern analysis.

These experiments showed that in the xenografts resemble human primary renal cell tumors in different ways, and reflect different characteristics that can be present in human renal cell carcinomas.

INTRODUCTION

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 sofar 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). Also the immunohistochemical identification of different cellular constituents has given further insight in renal cell tumor biology (5, 6, 7, 8).

Also differences in gene expression in renal cell carcinomas as compared to normal kidney have been investigated (9).
Since they are used as a model system 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). Also the grade of nuclear atypia, the predominant cell type and abnormalities in cholesterol metabolism remained the same (15). While also HLA expression remained consistent (16). The mitotic index, however, was reported to be higher than that of the original tumor (17).

For our study, four xenografts (RC_2, RC_{14}, RC_{43} and NC_{65}), originating from patients with metastatic disease at the time of diagnosis, were established in nude mice. These tumors have been described to have a 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, 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).

Four of these tumors (RC_2, $RC14$, $RC43$ and NC_{65}) were evaluated in a multidisciplinary set-up 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 hematoxilin and eosin (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 ethanols 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 renal cell carcinoma xenografts snap frozen in liquid nitrogen were fixed in methanol at -20°C for 5 min. and thereafter dipped in aceton at room temperature. The indirect immunofluorescence technique was performed as described before (7).

The following antisera were used in this study : 1) An affinity purified rabbit antiserum to human skin keratins (pKer) for the detection of the epithelial nature of a certain tumor. 2) An 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 done 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 before (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 pVim1 and pAct1 as DNA probes (20). Preparation of the DNA probe and its nick translation (21) was carried out as

described before (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 10^8 cpm/µg. Agarose gel electrophoresis and hybridization analysis was performed as described before (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 mM, pH 7.0), 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 (Amersham) for hybridization analysis as described before (25).

Human tumor clonogenic cell culture system (HTC_3S): 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 processed according to the detailed description published by Salmon (26). A modified two layer soft agar culture method as described before (27, 28) was used. The cells were cultured immediately after preparation of the single cell suspension and cell growth estimation using the Omnicon FAS II automated colony counter (Milton Roy Inc., Rochester, New York, USA) as described before (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 and made an optical empty impression. Irregularly shaped nuclei contained macronucleoli with a marked variation in shape. Mitoses, mostly abnormal, were often

Table I: Results of the multidisciplinary analysis of the four established xenografts of renal cell carcinomas in nude mice.

	in vivo doubling time	histology	EM	pVim	pKer	RGE53	vimentin mRNA	aneupl. fraction	DNA index	growth HTCS
RC_2	11.2 ± 7 d	G+C	e = m	+	+	+	elevated	9.0%	1.47	++
RC_{14}	3.8 ± 1.2 d	C	e = m	+	+	+	elevated	7.5%	1.935	++
RC_{43}	4.0 ± 1.4 d	G+C	e < m	++	-	-	elevated	47.7%	2.263	+
NC_{65}	3.6 ± 1 d	G+C	e = m	+	+	+	elevated	23.0%	2.041	++

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

observed. The tumor was classified as mixed cell type Grawitz tumor of moderate differentation (Fig. IC).

RC_{14} consisted almost completely of cytoplasm rich cells, arranged in a tubular and alveolar way. Inbetween the tumor cell nests many optical empty spaces or areas filled with erythrocytes were present. Mitoses, from many of which 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 (Fig. IA).

RC_{43} showed tumor cells with a spindle-like appearance. A part of the tumor cells showed cytoplasmic vacuoles, while most of them revealed a fine granular cytoplasm. Nuclei were polygonal, sometimes of a bizar shape, containing mostly irregularly shaped macronucleoli. Occasionally polynuclear cells were observed. The tumor was classified as a poorly differentiated mixed type Grawitz tumor (Fig. ID).

NC_{65} consisted of tumor cells arranged in tubular or 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 also eosinophilic granular structures were observed. The tumor was classified as a moderately differentiated mixed type Grawitz tumor (Fig. IB).

Electron microscopy. All tumors contained cells with well developed nuclei and organelles. Junctional complexes as well as glycogen were demonstrated in varying amounts (Fig. IIA). The epithelial characteristics were distinct in all the cases except for RC_{43}. The latter differed in various aspects e.g. more polymorphic nuclei, very few junctions and lipid droplets (Fig. IIB).

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} (Fig. III) were also stained by both these antibodies in the clear cell as well as the granular cell component. Using the RGE53

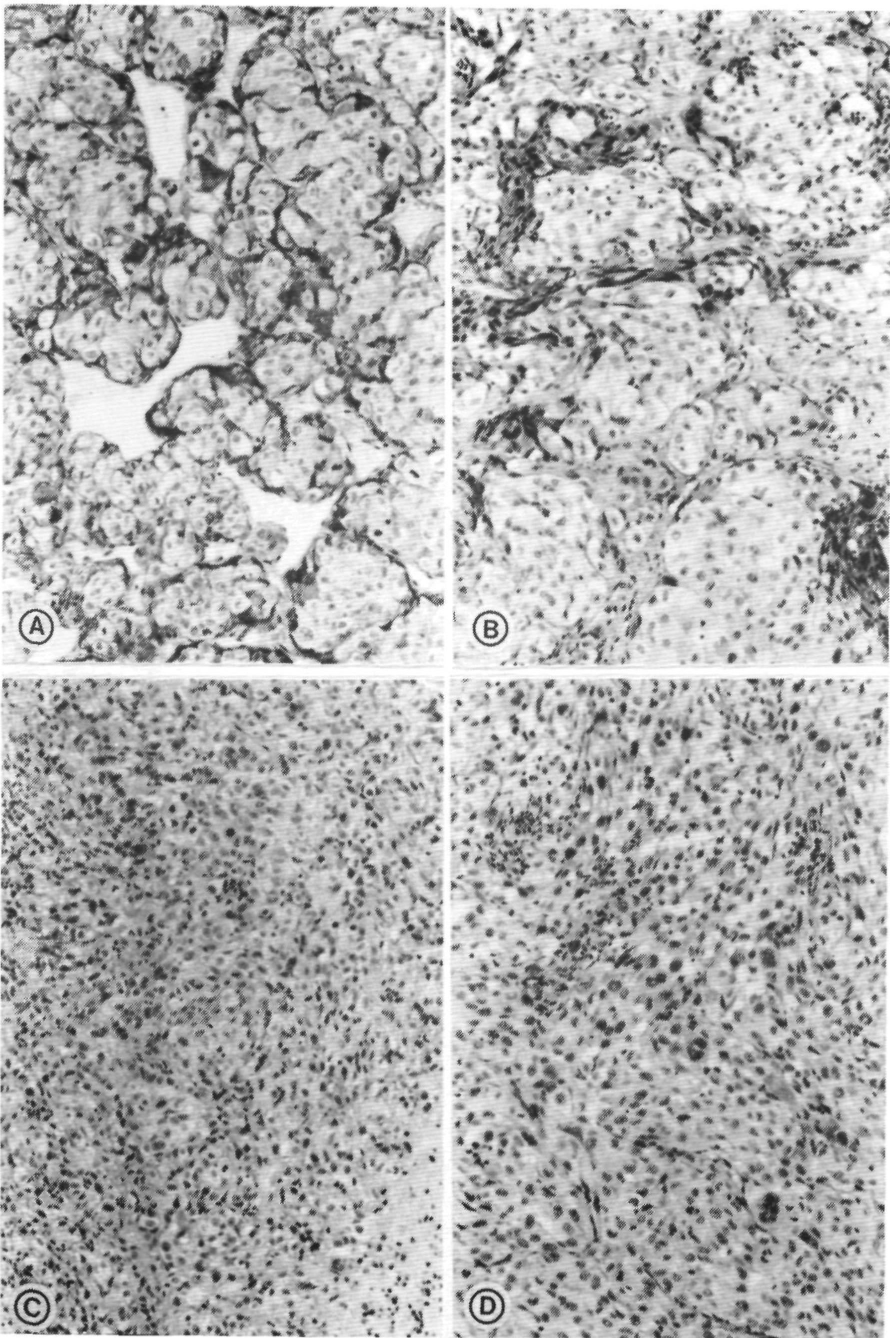


Fig. I: Representative examples of the histology of the human renal cell carcinoma xenografts in nude mice of (a) RC14, (b) NC65, (c) RC2 and (d) RC_{43}. (x 10)

moncolonal antibody to cytokeratin 18, coexpression of this adenocarcinoma marker and vimentin could be óbserved in tumor cell areas, thus rendering these three xenografts completely indistinguishable from original human renal cell carcinomas. In RC_{43}, however, almost exclusively a reaction with the vimentin antibody was found and 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 most likely represent stromal and inflammatory cells. A two parameter flow cytometry technique, using Propidium Jodide 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 I).

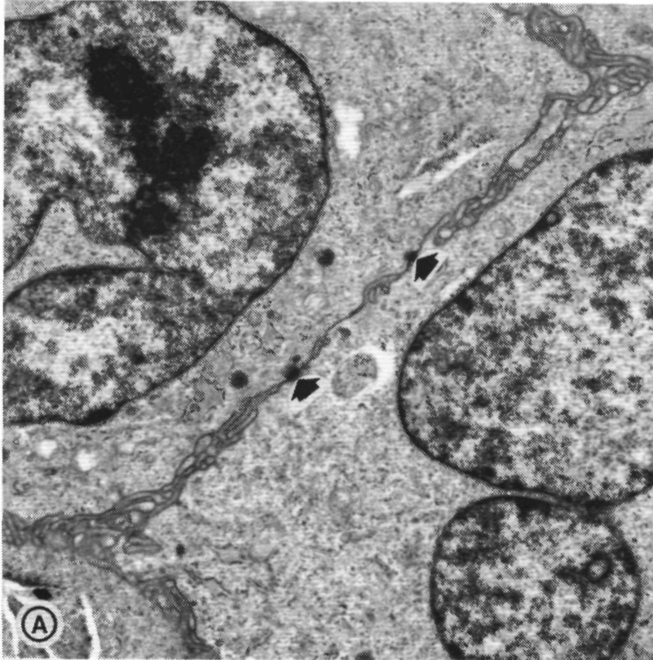
The flow cytometric data obtained with NC_{65} have been illustrated elsewhere (8).

Flow cytometric data obtained for RC_{43} are shown in Fig. IV. This latter tumor was also in these FCM studies found to react almost exclusively with the vimentin antiserum and not with the cytokeratin antiserum. For further details see legends to Fig. IV.

Northern and Southern blot analysis. The expression of vimentin was also studied by Northern blot analysis (Fig. V), which is indicative for the amount and length of vimentin mRNA. Using pVim1 as a molecular probe an increased level of vimentin mRNA was found in all tumors with respect to normal kidney tissue. RC_{43} 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 shown 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 HTC_3S 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 (Fig. VI).



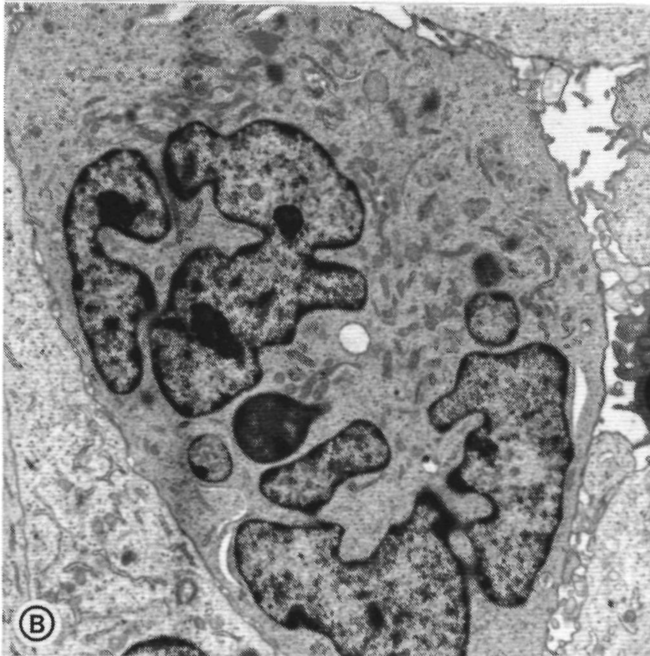
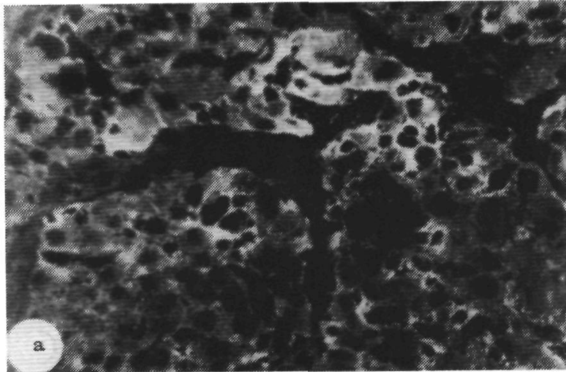
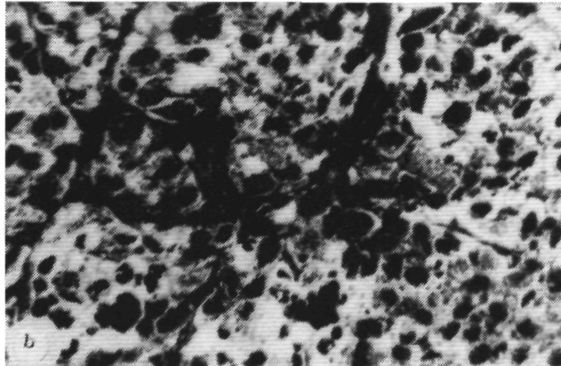
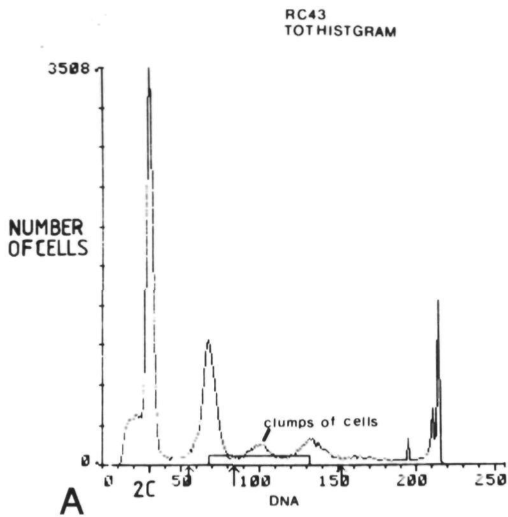


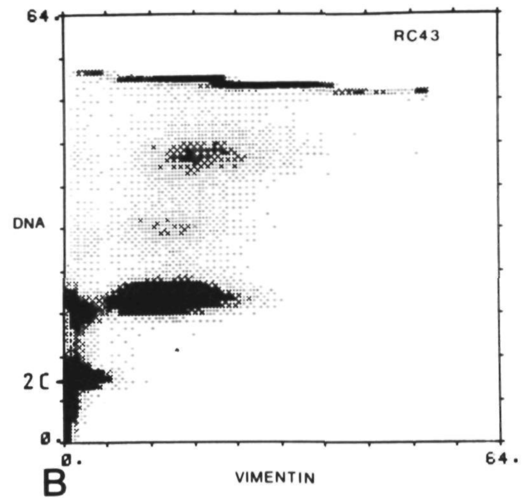
Fig. II: (a) RC_{14}. Electron microscopy of three tumor cells with indented nuclei. The electron-light cytoplasm contain numerous organelles and few glycogen particles. The cell membranes show interdigitations of microvilli with desmosomal contacts (arrows). (x 7700)
(b) RC_{43}. The tumor cells show often highly irregular nuclei. The scanty cytoplasm contains polymorfic mitochondria and few lipid droplets. The cells are partly in close apposition without junctional complexes or are provided with loosely interdigitating microvilli of varying shape. (x 5500)

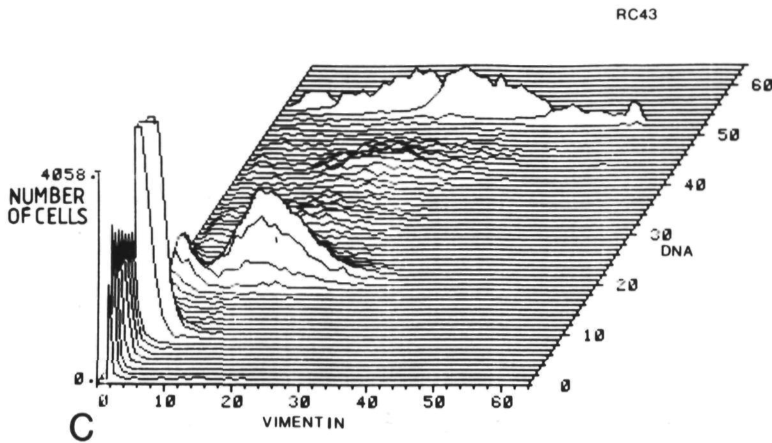
Fig. III: Immunofluorescence micrograph of the NC_{65} human renal cell tumor xenograft in a nude mouse stained with the cytokeratin antiserum pKer(a) and with the vimentin antiserum pVim(b) note the co-expression of both types of intermediate filament proteins in tumor cells. (x 200)











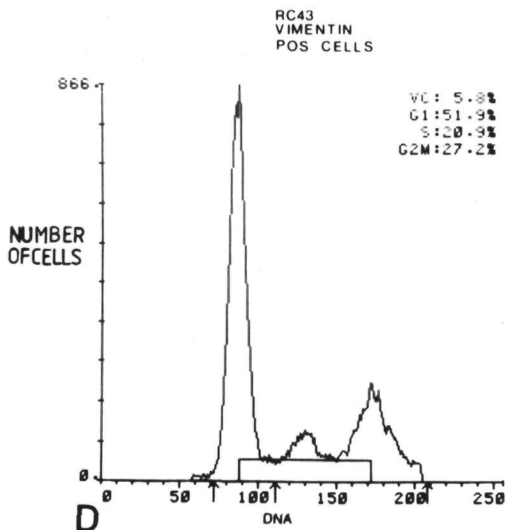


Fig. IV: Examples of FCM analyses of a human renal cell tumor xenograft in a nude mouse (RC_{43}), using the vimentin antibody pVim in addition to DNA staining with PI.

(A) One-dimensional FCM DNA pattern of the total cell suspension stained with PI.

(B) A two-dimensional projection of the DNA content and the vimentin content of cells present in the tumor cellsuspension. Note that the tumor fraction is strongly vimentin positive.

(C) A three-dimensional projection of the results of the same experiments as in (c).

(D) DNA histogram of the vimentin positive cells present in the cell suspension. The cell cycle parameters are indicated in the right upper corner.

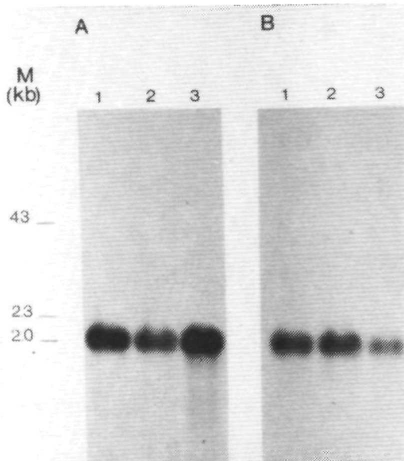


Fig. V: Northern blot analysis of vimentin (blot A) and actin (blot B) expression in NC_{65} (lane A_1 and B_1), in RC_2 (lane A_2 and B_2) and in RC_{43} (lane A_3 and B_3). Poly(A) selected RNA from the tumor was size-fractioned by agarose gel electrophoresis and analyzed for the presence of Vimentin mRNA and Actin mRNA. Molecular probes used were pVim1 for vimentin and pAct1 for actin. The molecular weight markers included were single strand Hind III digested lambda DNA molecules.

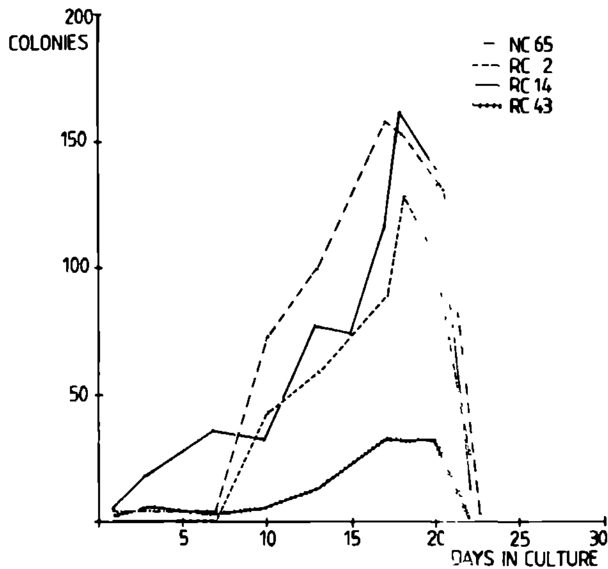


Fig. VI: Temporal Growth Patterns in the double layer soft agar human tumor cloning system of all four xenografts. Of each tumor cell suspension 5 x 10^5 cells/dish were plated. Obtained growth patterns were corrected for the tumor fraction percentage of each specimen as established by flowcytometry.

DISCUSSION

At the moment no consistingly effective therapy, be it hormonal, chemotherapy or immunotherapy for renal cell carcinoma exists (30).

Investigations attempting to identify and test effective agents with in vitro assays have sofar been unsuccessful, in part because of technical problems and the refractory nature of renal cell carcinoma to currently available chemotherapeutic agents (31). Additional difficulties in clinical evaluation of new treatment protocols are caused by the relatively rare occurence of the tumor and the pluriform 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 reproducable 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 a resemblance as close as possible to the human tumor if the treatment results in the model have to be transferred to the in vivo situation in man.

The human tumor model systems in nude mice are estimated of a higher value than the spontaneously evolved murine tumors as for instance

described by deVere White and Olsson (34, 35).

As mentioned before, the xenografts investigated sofar 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 human xenografts, RC_2, RC_{14}, RC_{43} and NC_{65} described here, 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 (RC_2, NC_{65}) to poorly differentiated (RC_{43}) tumors consisting of both granular and clear cells. RC_{14} 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). RC_{43} differs from the other tumors in the fusiformic aspect of most cells with their polymorfic 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 differences in metastatic behaviour of the subpopulations or a difference in chemosensitivity.

Immunohistochemical studies and DNA flow cytometric analyses of the xenografts confirmed these findings as in all four cases an abnormal DNA stemline expressing cytokeratin and vimentin expression (albeit in different amounts) could be detected. Evaluation of vimentin expression by RNA analysis showed an increase of transcription in all tumors. RC_{43} showed an even three-fold higher vimentin expression than the other three tumors. In the HTC_3S all tumors could be cultured, possibly due to the fact that they already were selected for their in

vitro growth potential by their succesful growth as xenografts in nude mice (36).

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 succesful 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 to treatment protocols is observed, these xenografts may be useful tumor models for the study of new experimental chemotherapeutics.

ACKNOWLEDGEMENTS

We want to acknowledge Mr. P. Peelen, Mr. O. Moesker, Mr. H. Croese for excellent technical assistance and Dr. C.J. Romijn, Dept. of Urology, Dijkzigt Hospital, Rotterdam, for supplying of the tumor material.

The study was supported by The Netherlands Kidney Foundation.

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EXPRESSION OF PROTO-ONCOGENES IN XENOGRAFTS OF HUMAN RENAL CELL CARCINOMAS

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Submitted.

ABSTRACT

In a recent paper, we described the expression pattern of proto-oncogenes in primary human renal cell carcinomas (12). To test the possibility of using xenografts as a useful alternative for such studies, we have analyzed xenografts of a number of human renal cell carcinomas in nu/nu mice. Xenografts included RC2, RC14, RC21, RC43 and NC65. Northern blot analysis indicated that c-ras was expressed in all these xenografts. The identity of the ras transcripts in the individual xenografts was further specified as c-Ha-ras, c-Ki-ras or N-ras. Expression of c-myc and the p53 gene was also found in a number of these tumors. Only RC21 failed to express the c-myc or the p53 gene. In all xenografts, a 3.0 kb c-fes/fps mRNA was present. In RC2, RC14, RC21 and RC43, low levels of the 4.8 kb abl transcript were detectable. Transcripts of myb and sis could not be detected in any of the xenografts. The results indicated that the expression pattern of a variety of proto-oncogenes in xenografts of human renal cell carcinomas was similar to that in the primary tumors.

INTRODUCTION

Molecular oncology has led to the discovery of genes whose malignant potential becomes apparent upon retroviral transduction of these genes. Because of this characteristic, the genes are also thought to be implicated in the onset and development of naturally ocurring tumors. Collectively, they are called proto-oncogenes. Up to this moment, more than fourty proto-oncogenes are characterized (3, 4). Recently, classification of proto-oncogenes in a nuclear and cytoplasmic group was proposed on the basis of the site of action of their gene products (27). The nuclear group is the smallest one and include c-myc, c-myb and the p53 gene. An important characteristic of these genes is their immortalizing capacity. In general, the nuclear group of proto-oncogenes is weak in inducing anchorage independent growth of fibroblasts in tissue culture (27). There are indications that these genes are involved in regulation of gene expression and that disturbances in their own expression patterns (in place and/or in time) is a major factor in tumorigenesis (2, 15). The gene products of

c-myc and c-myb are structurally homologous (6, 20). p53 is a phosphoprotein that is overexpressed in some transformed cells (11, 29). The cytoplasmic group of proto-oncogenes is larger and its members are thought to be a major factor in processes involved in malignant transformation. Proto-oncogenes of this group include for instance the ras-gene family, c-fes and c-abl. It has been suggested that in general their malignant activation is the result of mutations (15). The gene products of c-abl and c-fes are tyrosine-specific protein kinases (10, 22, 24) and the ras gene product resembles the G-protein and probably acts as a signal transducer (4, 27). A proto-oncogene that does not belong to either of the groups described above, is the sis proto-oncogene. Its translation product is highly similar to the B-chain of the platelet-derived growth factor (PDGF) (17).

In a recent paper (12), we have described proto-oncogene expression patterns in primary human renal cell carcinomas. We found that c-ras and c-myc were clearly detectable in most of the tumors. Furthermore, expression of c-fes was observed in a small percentage of these tumors. Since RNA isolated from primary human renal cell carcinomas was often of low quality probably due to necrosis in the tumor, we wanted to test xenograft tumor lines of renal cell carcinomas in nu/nu mice as a suitable alternative. The nu/nu mouse, described by Flanagan (1966) (7) and later found to be thymus deficient and lacking T-cell mediated immune response by Pantelouris (1968) (18), was chosen as an animal host for this type of transplantation experiments, because of its usefulness for a wide range of human tumors (8). We already described that, histologically and ultrastructurally, xenografts of human renal cell carcinomas in nu/nu mice resemble primary renal tumors (13).

In the present study, we have studied expression of c-Ha-ras, c-Ki-ras, N-ras, c-myc, c-myb, c-abl, c-fes, c-sis and the p53 gene by Northern blot analysis in xenografts of five different human renal cell carcinomas and an established kidney cell line of a monkey (Vero).

MATERIALS AND METHODS

Tumor materials

Xenografts of human renal cell carcinomas in *nu/nu* mice were obtained from the Department of Urology, Dijkzigt Hospital, Rotterdam. All xenografts were derived from primary tumors of patients with apparent metastases at the time of diagnosis. Xenografts showed no dedifferentiation compared to the original tumors (14). Upon removal, tumor material was immediately frozen in liquid nitrogen. The established kidney cell line (Vero), derived from an African green monkey, was also included in this study.

RNA isolation and Northern blot analysis

Total cellular RNA was isolated according to the procedure described by Auffray and Rougeon (1). Upon poly(A) selection by oligo(dT)-cellulose chromatography, RNA was dissolved in sodium phosphate buffer (10 mM, pH 7.0), which contained DMSO (50%) and glyoxal (1 M), and heated at 50 °C for 1 hr. RNA was size fractionated by agarose gel electrophoresis (1.0% agarose gel) and transferred to nitrocellulose or Hybond-N membrane as described before (22).

Preparation of molecular probes

The characteristics of the molecular probes used in this study are summarized in Table I. The probes were prepared as described before (14). Radioactive labeling of the probes was performed according to Van den Ouweland *et al.* (17).

Hybridization and dehybridization

Hybridization was performed under conditions as described by Church and Gilbert (5) and dehybridization according to the procedures as recommended by Amersham.

Table I: DNA fragments used as molecular probes.

Proto-oncogene	Fragment used as probe		Reference
N-ras	SalI/EcoRI	0.65 kbp	(9)
v-Ki-ras	EcoRI/EcoRI	1.15 kbp	(25)
c-Ha-ras	SmaI/SmaI	0.65 kbp	(25)
c-myc	SacI/SacI	1.20 kbp	(6)
c-myb	BamHI/XbaI	0.80 kbp	(19)
p53 gene	PstI/PstI	0.55 kbp	(11)
v-abl	PstI/PstI	0.70 kbp	(24)
c-fes	EcoRI/EcoRI	0.95 kb	(22)
c-sis	BamHI/BamHI	1.80 kbp	(17)

RESULTS

Expression patterns of proto-oncogenes from the ras gene family are well documented. The human c-Ha-ras mRNA is 1.4 kb (25, 28), the human N-ras-specific transcript is 2.2 kb (9) and transcription of the human c-Ki-ras gene results in a 4.6 kb mRNA (25). A complicating factor in the interpretation of results concerning ras expression is the fact that the ras genes are strongly homologous, therefore crosshybridization is often observed in Northern blot analysis. This is illustrated in Fig. 1 (lanes A to D), in which typical ras hybridization patterns are shown, using an N-ras (lane A-B), c-Ki-ras (lane C) and a c-Ha-ras (lane D) probe. In lane A (Fig. 1), it is clearly shown that the N-ras probe in addition the N-ras-specific mRNAs (doublet at 2.2 kb) also detects the c-Ki-ras (4.6 kb) and the c-Ha-ras transcript (1.4 kb). In the interpretation of the results described in this report, however, only specific transcripts are considered, namely 1.4 kb for c-Ha-ras, 2.2. kb for N-ras and 4.6 kb for c-Ki-ras. It is evident that because of crosshybridization ras expression cannot be studied unambiguously by a dot blot assay, but requires Northern blot analysis.

The results of the ras gene expression studies are summarized in table II. All tumors except RC21 showed detectable levels of N-ras transcripts. It appeared that both RC2 and RC43 contained the highest level of c-Ki-ras mRNA. It should be noticed that in these two tumor lines also a second mRNA at approximately 5.3 kb was visualized using the v-Ki-ras probe. c-Ha-ras expression was the highest in RC21 and was not found in RC2 and NC65. From these data it appeared that in each of the tumors at least one of the ras genes was expressed.

Expression of c-myc, c-myb and P53

The cellular localization of proto-oncogene products is indicative for their mode of action and for the way they play a role in carcinogenesis. The c-myc, c-myb and p53 gene products are all localized in the cell nucleus. Expression of these oncogenes was also studied by a Northern blot analysis and the results are summarized in table II. In none of the xenografts detectable levels of c-myb were

Fig. 1: Northern blot analysis of xenografts of human renal cell carcinomas. Poly-A selected RNA from RC2 (lane E, F and H), RC14 (lane B and D) RC43 (lane C and G) and Vero (lane A) was size fractioned by agarose gel electrophoresis and analyzed by Northern blot technique. Molecular probes used were N-*ras* (lane A and B), v-Ki-*ras* (lane C), c-Ha-*ras* (lane D), c-*myc* (lane E), p53 (lane F), c-*fes* (lane G) and v-*abl* (lane H).
Molecular weight markers included are single strand HindIII digested lambda DNA molecules.

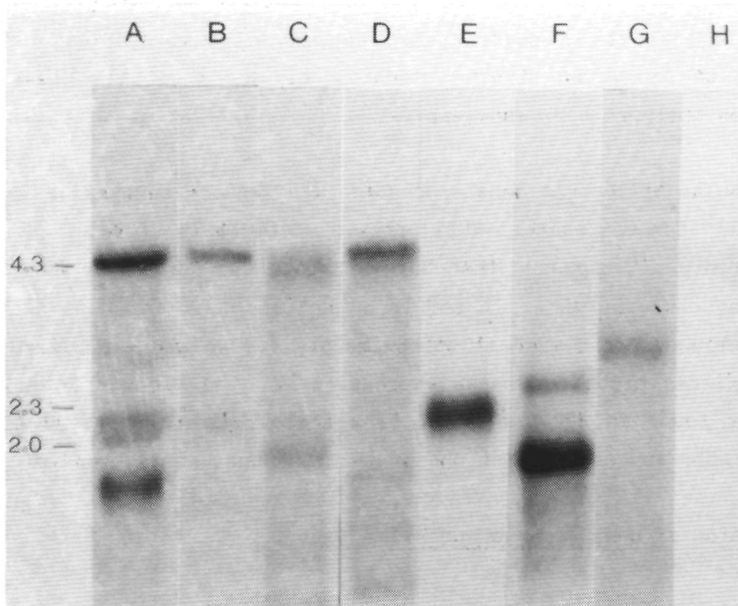


Table II: Summary of proto-oncogene expression in xenografts of human renal cell carcinomas and the Vero cell line.

probe	mRNA	RC2	RC14	RC21	RC43	NC65	Vero
N-ras	2.2 kb	+	+	0	+	+	++
c-Ki-ras	4.6 kb	++	+	+	++	+	NT
c-Ha-ras	1.4 kb	0	+	++	+	0	NT
c-myc	2.3 kb	+++	++	0	+++	++	NT
c-myb	5.0 kb	0	0	0	0	0	0
p53	3.0 kb	++	0	0	+	0	++
c-fes	3.0 kb	++	+	+	++	+	0
v-abl	6.6 kb	0	0	0	0	0	++
	4.8 kb	+	+	+	+	0	+++
	2.9 kb	0	+	0	0	0	++
	0.8 kb	0	0	0	0	0	NT
v-sis	3.5 kb	0	0	0	0	0	NT

Symbols: 0: no expression detected; + to +++: detectable to high levels of expression; NT: not tested.

present, which is in agreement with the fact that c-*myb* expression is predominantly confined to hematopoetic malignancies (19). High levels of c-*myc* expression were found in all xenografts, except in RC21. The possibility that in RC21 another member of the *myc* gene family (N-*myc* or L-*myc*) was expressed was not tested and can therefore not be excluded.

The human p53 specific transcript was found at elevated levels in RC2 and RC43. These data indicate that in most of the xenografts tested, detectable levels of mRNA of a nuclear proto-oncogene were present.

Expression of c-abl and c-fes/fps

The c-*abl* and c-*fes* proto-oncogenes both contain a domain that encodes a tyrosine-specific protein kinase (21). The gene products of these two genes are localized in the cytoplasm and at least a fraction of them seems associated with membranes. Unlike *ras* and *myc*/p53, elevated expression of these genes is not common in human malignancies (25). Detectable levels of *fes* transcripts are sometimes found in haematopoetic malignancies (25), lung tumors (25), breast carcinomas and renal cell carcinomas (12, 25). Expression of c-*abl* in primary tumors is only reported in the case of chronic myelogenous leukemia (25). Northern blot analysis of RNA of the xenografts revealed that 4 out of 5 tumors exhibited *abl* expression. They included RC2, RC43, RC4 and RC21. The Vero kidney cell line also expressed the *abl* proto-oncogene. In contrast to the xenografts, a number of different *abl* transcripts were found in the cell line. As far as the expression of the *fes*/*fps* proto-oncogene was concerned, in all xenografts a 3.0 kb *fes*/*fps* specific mRNA was found (Fig. 2). Some fluctuation in the levels of the transcripts was observed. No *fes*/*fps* transcript could be detected in the Vero kidney cell line. The fact that c-*fes*/*fps* expression was found in xenografts of human renal cell carcinomas is in agreement with previous studies on primary renal tumors (12, 25).

Expression of c-sis

The c-*sis* proto-oncogene contains sequences that could encode a protein similar to the B-chain of platelet-derived-growth factor

(PDGF). A concensus is emerging that in fact the c-sis locus encodes the B-chain of PDGF. It was of interest to see whether in renal cell carcinomas autocrine growth stimulation as a consequence of c-sis activity was involved. Northern blot analysis revealed no c-sis transcripts and, therefore autocrine growth stimulation as a consequence of c-sis activation can be ruled out.

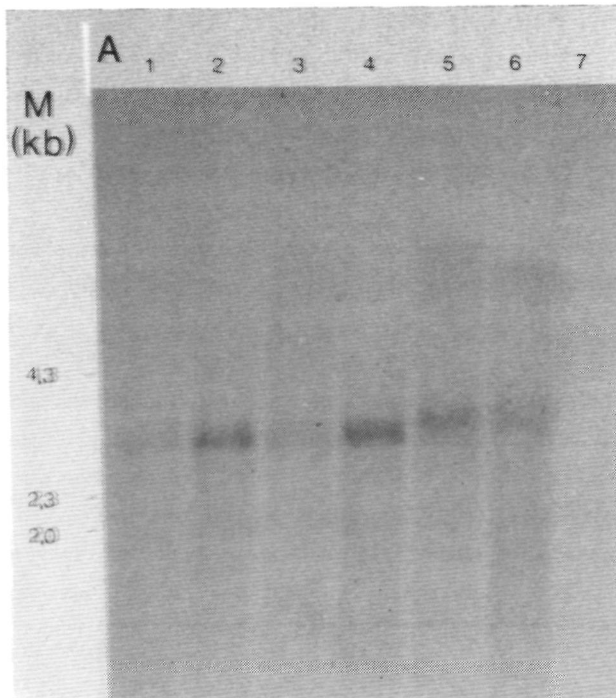


Fig. 2: Comparative Northern blot analysis of c-fes mRNA in xenografts RC2 (lane 2), RC8 (lane 3), RC14 (lane 5), RC21 (lane 6), RC43 (lane 4) and NC65 (lane 1). The c-fes EcoR1EcoR1 0.95 kb DNA fragment was used as a molecular probe. Molecular weight markers included are single strand HindIII digested lambda DNA molecules.

DISCUSSION

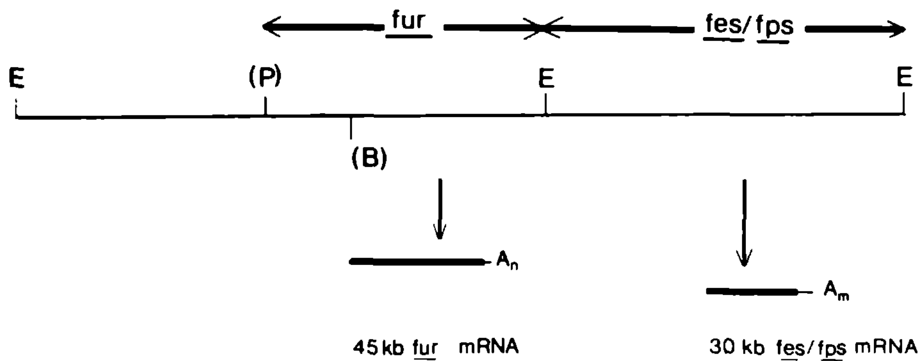
Proto-oncogene expression patterns in xenografts of human renal cell carcinomas were studied to determine whether xenografts could be used as an alternative for primary renal cell carcinomas. A major problem in studying mRNA from primary human renal cell carcinomas is degradation of mRNA. In contrast to primary tumors high amounts of intact mRNA could be isolated routinely from xenografts of human renal cell carcinomas. This was probably due to the fact that the time period elapsing between excision of the primary tumor and its freezing in liquid nitrogen was much longer in the clincal setting than under condition of a laboratory experiment. It is well established that tumor devitalization already starts when the renal vascular pedicle in radical nephrectomy is clamped. The better presentation of tumor tissue in xenografts was confirmed by microscopical examination of the xenografts revealing homogenous tumor structures and almost no necrosis.

Our results indicated that as far as proto-oncogene expression patterns are concerned, xenografts of human renal cell carcinomas provide a good alternative for primary tumors. Expression of all three members of the ras gene family was found in the xenografts. This is in accordance with earlier observations in primary renal tumors in our laboratory (Schalken, unpublished observation) and in agreement with results from other groups (25).

Similarly, expression of c-myc in xenografts is also in good agreement with studies on primary tumors. The fact that in one of the xenograft tumor lines no c-myc transcripts could be found does not necessarily mean that this tumor does not contain a transcript that is related to c-myc. In recent studies (16), the discovery of N-myc and L-myc genes were described and they are related to the c-myc gene. However, limited nucleotide sequence homology could explain the failure to detect transcripts of them with a c-myc probe, especially under the hybridization condition of high stringency that were used.

Expression of the c-fes/fps proto-oncogene in renal cell tumors is a matter of interest. In contrast to primary human renal cell carcinomas

Fig. 3: Schematic representation of the human genomic region containing the fur gene and the fes/fps proto-oncogene. The putative promotor region is situated between the BamHI and PstI site. E= EcoRI; B= BamHI; P= PstI. The restriction endonucleases placed between brackets indicate that not all the cleavage sites for this enzyme are depicted.



in which c-fes/fps expression was found in only 10% of the cases, the xenografts exhibited in all cases tested a clearly elevated level of fes/fps expression. At the moment it is not clear how to explain this observation. It is well established that in normal cells expression of c-fes/fps is restricted to cells of hemopoietic origin and mainly to those of the myeloid lineage (28). The possibility that many myeloid cells were present in the xenografts and that the observed fes/fps expression was due to such cells in the tumor specimens could be ruled out on the basis of histopathological analysis. The fact that all xenografts exhibited fes/fps expression suggests that xenograft-specific factors are involved and may be selected for, during in vivo propagation of the tumor cells. In situ hybridization or immunofluorescence analysis could shed light upon this matter by identifying in xenografts as well as in primary tumors the cell types that express the proto-oncogene.

The high levels of fes/fps expression in xenografts of renal cell carcinomas is also remarkable. In specimens of normal kidney, the fes/fps DNA region seems transcriptionally silent. The genetic region immediately upstream of the proto-oncogene, however, is transcriptionally active in tissue specimens of normal kidney (22). This region, which we have designated fur (for fes/fps upstream region) contains a gene, which is located very closely (less than 1 kbp) to the proto-oncogene (22). For a schematic representation of this genetic region in the human genome, see Fig. 3. The fur gene, which seems to encode a membrane associated protein with a recognition function, has a relatively strong promoter (23). The promoter of the proto-oncogene remains to be identified. It is tempting to speculate that in renal cell carcinomas the promoter region of the fur gene is involved in expression of the proto-oncogene. The fact that xenografts of human renal cell carcinomas resemble primary renal cell carcinomas and that mRNA of high quality can be isolated from them enables characterization of the fes/fps transcription unit in these tumor cells by cDNA analysis and S1 nuclease protection experiments. By this approach, the hypothesis mentioned above can be tested now.

ACKNOWLEDGEMENTS

We wish to thank Dr. J.C. Romijn of the Department of Urology, Dijzigt Hospital, Rotterdam, for providing the tumor material.

We acknowledge the excellent technical assistance of M. de Jong.

This work was supported by the Dutch Kidney Foundation, contractno. C85.531.

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CHAPTER V

TISSUE SPECIFIC MARKERS IN FLOW CYTOMETRY OF UROLOGICAL CANCERS: (II) CYTOKERATIN AND VIMENTIN IN RENAL CELL TUMORS

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ABSTRACT

Nine primary human renal cell tumors (RCT), one lymphnode metastasis, 4 human xenografts of a RCT in nude mice and a rat RCT line were analysed by flow cytometry (FCM) using propidium iodide for DNA analysis and antibodies to cytokeratin and vimentin in the indirect immunofluorescence technique for labelling of specific tumor cell populations. By means of 2-dimensional FCM analysis, vimentin- and cytokeratin-positive (tumor) cells were compared and their DNA content and proliferative fraction could be analysed separately from those of cytokeratin-negative stromal and inflammatory cells. In primary human RCT, 2 subpopulations of cells were detected and analysed separately. Small numbers of tumor cells with an abnormal DNA stemline were also detected. In addition, co-expression of intermediate filament proteins of both the cytokeratin and the vimentin types was detected in the aneuploid cell population. Comparison of 2 model systems of RCT with primary human RCT revealed a similar pattern of tumor cell subfractions within these tumors. The 2-parameter FCM analysis permits the detection of subpopulations in complex cell suspensions and the quantification of these fractions, as well as analysis of their cellular DNA content.

INTRODUCTION

In a recent report (Feitz et al., 1985) we have evaluated the usefulness of DNA flow cytometry (FCM) in combination with staining of the cytoplasmic intermediate filament proteins in bladder carcinomas. These tissue-specific proteins can be detected immunocytochemically by means of monoclonal and polyclonal antibodies and can be used in the FCM analysis of complex human tumors (Ramaekers et al., 1984a, b, 1986). In particular, antibodies to cytokeratins, the intermediate filament proteins specific for epithelial cells, enable carcinoma cells to be analyzed separately from the non-epithelial stromal and inflammatory cell components that are present in most tumors.

Investigation of human primary renal cell tumors (RCT) showed that these neoplasms contain several tumor cell populations, each with a

different intermediate filament staining pattern. Cytokeratin was found in many tumors, but nearly all túmors co-expressed the mesenchymal type of intermediate filament protein vimentin. However, primary RCT and lymphnode metastases of RCT also contained either cytokeratin or vimentin only (Herman et al., 1983a). Therefore, we suspect that biological differences in the subfractions of RCT may give rise to differences in behaviour of tumor metastases, and undoubtedly have consequences of chemotherapy.

The development of model systems for further cell biological and experimental investigations of RCT has led to the establishment of 4 human renal cell xenografts in nude mice (Kurth et al., 1984) and of a tumor line from a rat RCT in Wistar Lewis rats (deVere White et al., 1980). In this study, we present findings on the 2-parameter FCM analysis of cellular DNA and intermediate filament content of these RCT models in comparison with primary human kidney tumors. We have tried to answer the following questions:
(1) Does application of this 2-parameter analysis to RCT allow separation of the tumor cells from inflammatory and stromal cells, making it possible to estimate ploidy and proliferative fractions more accurately, as in the case of bladder carcinoma (Feitz et al., 1985)?
(2) Do aneuploid (tumor) cells contain both cytokeratin and vimentin?
(3) How can RCT animal model systems be compared with primary human RCT?

MATERIAL AND METHODS

Nine primary renal cell tumors (RCT) and one RCT metastasis (number 1 to 10 in Table I), 4 human RCT xenografts in nude mice (designated RC2, RC14, RC43, NC65) and a rat RCT line (designated rRCT) continuously retransplanted in Wistar Lewis rats (deVere White et al., 1980) were used in this study (Table I). Representative parts of tumor tissue were chosen which included all macroscopically identifiable histological patterns of the tumor. Necrotic and hemorrhagic areas were excluded. The material was divided into 3 parts: one part was fixed in formalin and embedded in paraffin for routine histology, another was snap-frozen in liquid nitrogen for immunohistochemical

Table I: DNA index of RCT obtained after the one- (DNA index) and 2-parameter FCM analysis (DNA index pKer and DNA pVim). In the cases in which an aneuploid tumor cell fraction was present, the DNA index of this latter fraction is given only

NUMBER	DNA index	DNA index (pKer)	DNA index (pVim)
1	0.97	0.97	0.97
2	0.80[2]	0.80[2]	1.05[2]
3	1.40	1.36	1.35
4	0.98	1.30[1]	1.35[1]
5	0.94	0.94	0.94
6	0.96	1.60[1]	1.60[1]
7*	0.95	1.54[1]	- [3]
8	1.19	1.30	1.17
9	0.93	0.96	0.97
10	1.66	1.66	1.66
RC2	1.40	1.39	1.40
RC14	1.87	1.84	1.77
RC43	2.24	2.29	2.35
NC65	0.97	1.88[1]	1.92[1]
rRCT	0.98	1.88[1]	1.87[1]

[1] In these cases labelling with either cytokeratin or vimentin antibodies resulted in the detection of an aneuploid peak not seen without labelling.

[2] Hypoploid tumor cell fraction present in the suspension (cf. Fig. 2c, d).

[3] Some aneuploid cells.

Human RCT (1 to 10, 7* is a lymphnode metastasis from 6), RCT xenografts (RC2, RC14, RC43, NC65) and a rat RCT (rRCT).

staining reactions, and a third was used for preparation of a single cell suspension for FCM.

Routine histological classification was done from hematoxylin-eosin-stained paraffin sections (Bennington and Beckwith, 1975).

Cell suspensions were prepared from the fresh tissues as described before (Herman et al., 1983b; Feitz et al., 1985). From solid tumor material single cell suspensions were prepared by mechanical and enzymatic disaggregation using a mixture of collagenase Type II (1176 U/ml, Worthington, Freehold, NY) and DNase I (200 U/ml, Sigma, St. Louis, MO) at 37 °C for 2 hr. After 3 subsequent filtration steps (300-µ metal sieve, 25-gauge needle and 50-µ pore size nylon filter) a single cell suspension was obtained and the cells were fixed in 70% ethanol (-20 °C) and stored at -20 °C to -40 °C until further processing.

Immunohistochemical staining procedures and antibodies

Frozen sections of normal kidney tissue, primary RCT and a metastasis, as well as 4 xenografts of RCT and a rat RCT, were incubated with intermediate filament antibodies by the indirect immunofluorescence technique (Ramaekers et al., 1983b). Antibodies used in this study were: (1) An affinity purified polyclonal rabbit antiserum to human skin keratins (pKer) which reacts with virtually all epithelial tissues, but not with non-epithelial tissues. The antiserum was diluted 1:5 in PBS for staining of cells in suspension and 1:25 for staining of frozen sections. (2) An antiserum to calf lens vimentin (pVim), raised in a rabbit. Preparation and specificity of this antiserum have been described (Ramaekers et al., 1983b).

Ethanol fixed cell suspensions from RCT were split into 4 fractions. One sample was stained with propidium iodide (PI) as described below and used for cell kinetic studies. A second sample was used for calculation of the DNA index, using chicken red blood cells (CRBC) as internal standard (Hamilton et al., 1980; Tannenbaum et al., 1978). The other 2 samples were stained separately with the antibodies pKer and pVim (Feitz et al., 1985; Oud et al., 1985; Ramaekers et al., 1984a, b, 1986). In brief, cells were incubated for 30 min with the antibody directed against the specific intermediate filament proteins,

washed several times, and incubated with an appropriate FITC-conjugated second antibody for 30 min. Thereafter, DNA staining was performed using propidium iodide (PI) on RNase treated cells.

FCM analysis

Cell analyses were performed using a Cytofluorograph 50H (Ortho Instruments, Westwood, MA) as described before (Feitz et al., 1985). All data were stored in correlated (list) mode on a PDP 11/34 computer (Digital, Marlboro, MA) for subsequent data analysis. The normal human G_1 DNA-index in our laboratory is 2.65 to 2.75 times the fluorescence intensity of CRBC (Hiddeman et al., 1984). Cell cycle analysis was done as described by Baisch et al. (1975). As a control, cells incubated only with an FITC-conjugated second antibody were analysed and in this control all cells were negative.

RESULTS

Immunohistochemistry

Frozen sections of normal kidney, primary RCT and a metastasis, human xenografts of RCT in nude mice and a rat RCT were incubated with the antibodies pKer and pVim (Fig. 1). In normal kidney tissue, epithelial structures such as collecting ducts and parietal glomerular epithelial cells are stained with the cytokeratin antibody. Staining with the vimentin antibody is found only in endothelial and peritubular cells, in vascular walls and glomerular cells (Fig. 1a, b). Primary human RCT and xenografts of these tumors as well as a rat RCT were found to react with both the cytokeratin and the vimentin antibodies (Fig. 1c-h). In double labelling studies it was seen that vimentin positive cells were also positive for cytokeratin (see also Herman et al., 1983a).

Labelling of RCT in single cell suspensions and FCM analysis

Ethanol fixed tumor cells in a single cell suspension were incubated with the antibodies pKer and pVim and thereafter with the

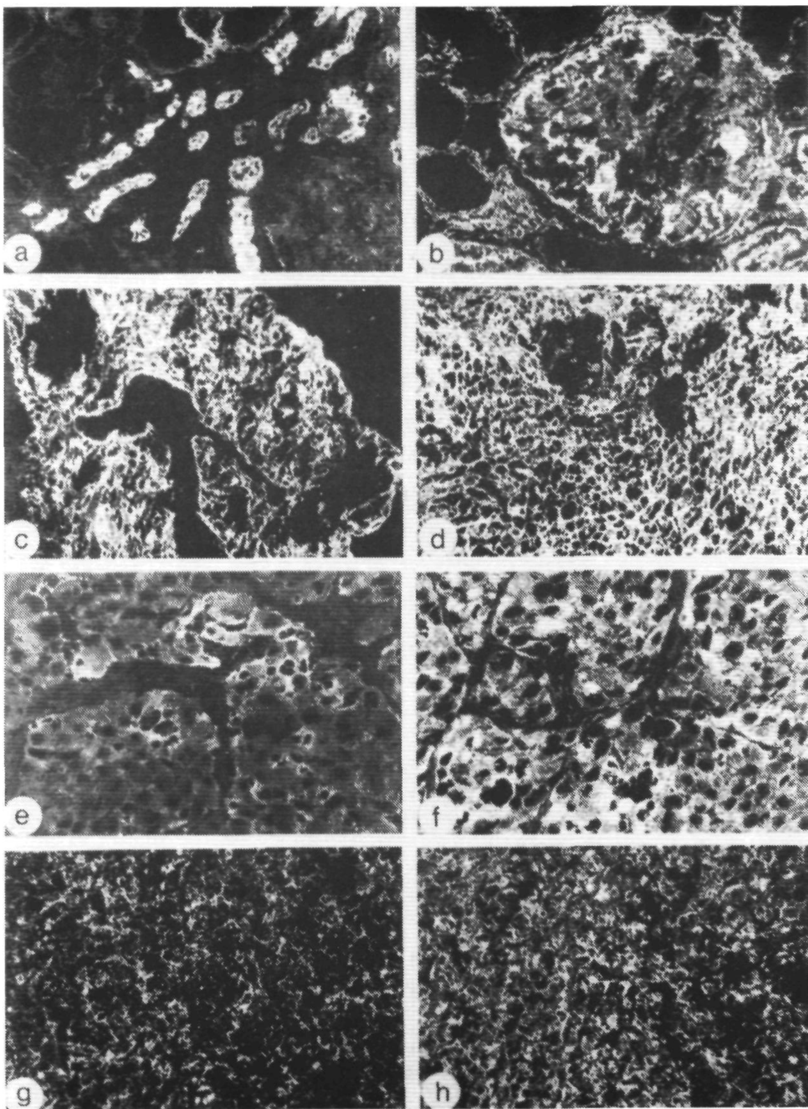


Fig. 1: Immunofluorescence micrographs of normal kidney (a, b), a primary human renal cell tumor (c, d), a xenograft of a human renal cell tumor in a nude mouse (NC65) (e, f), and a rat renal cell tumor in Wistar Lewis rats (rRCT) (g, h), stained with the cytokeratin antiserum pKer (a, c, e, g) or with the vimentin antiserum pVim (b, d, f, h). Note the co-expression of both types of intermediate filament proteins in tumor cells. a-h, x150.

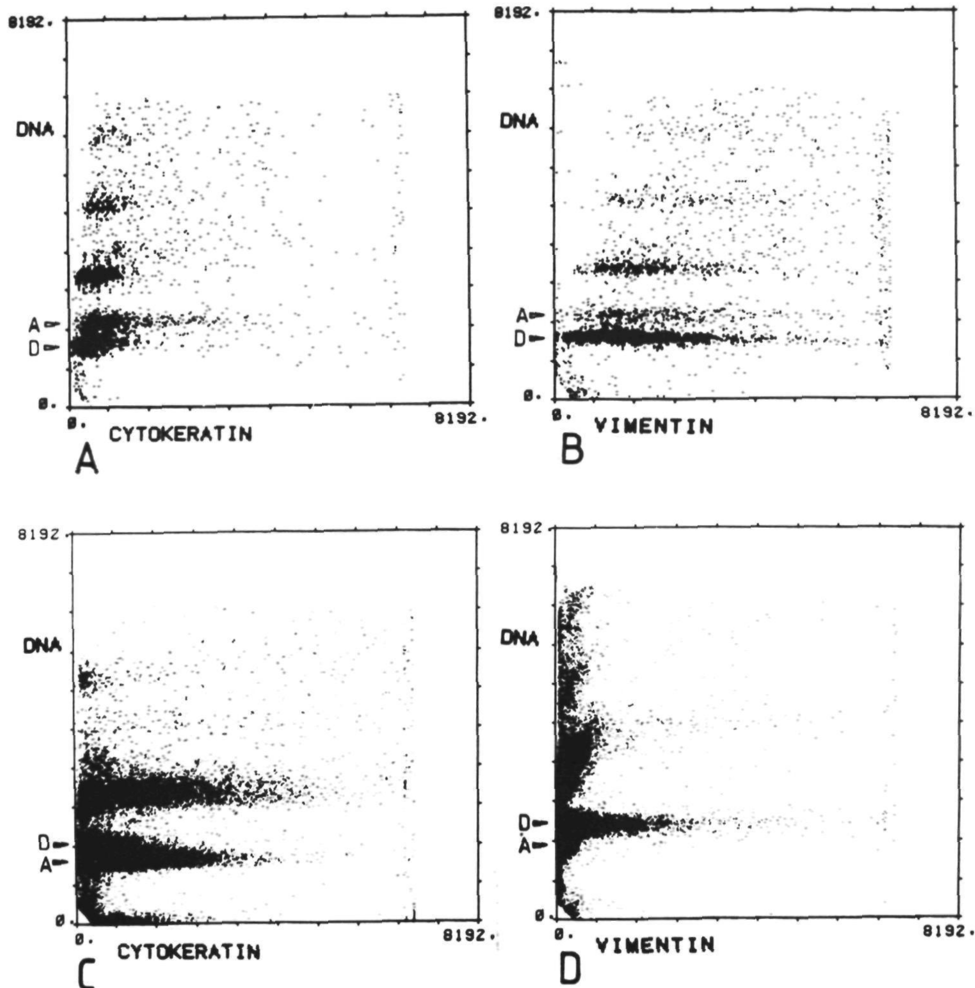


Fig. 2: Example of a 2-parameter FCM analysis of a primary human RCT (no. 3) (a, b) containing an aneuploid tumor cell population positive for cytokeratin (a) and vimentin (b) in addition to a diploid peak containing stromal and inflammatory cells (A= aneuploid, D= diploid DNA index). Note that in the tumor cell suspension containing a hypoploid fraction (no. 2) (c, d) no tumor cells positive for vimentin are seen (d).

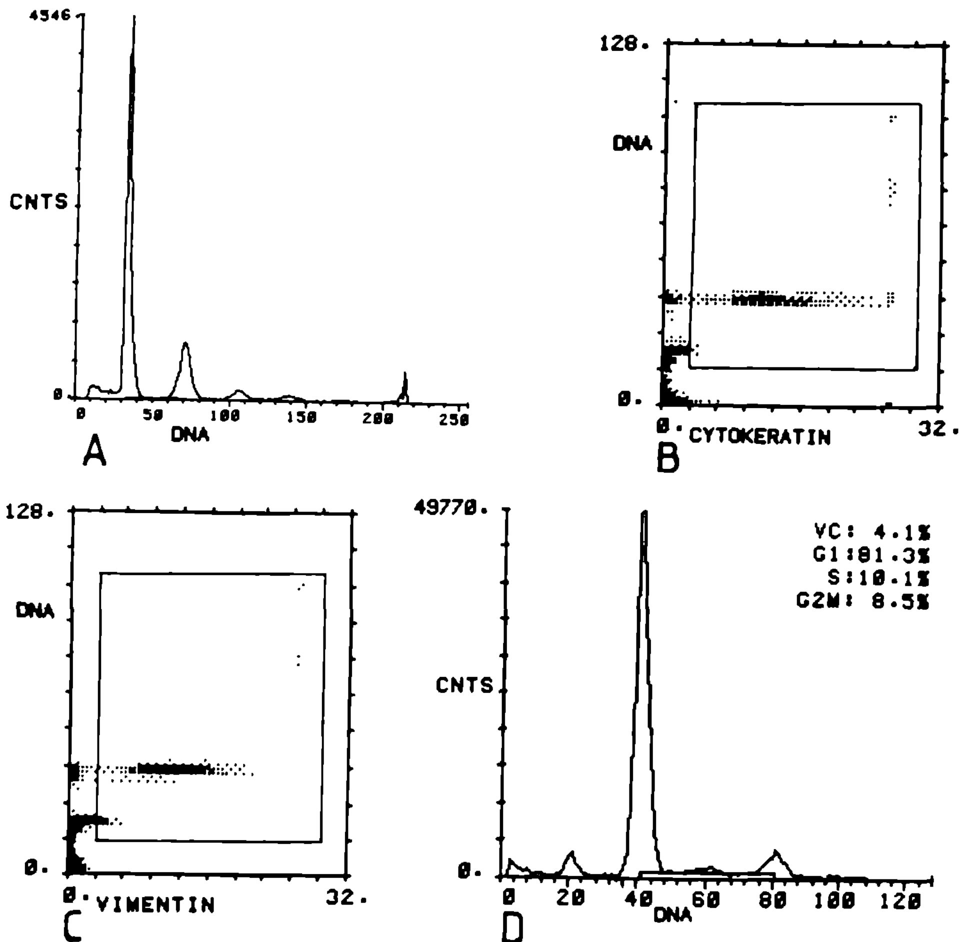
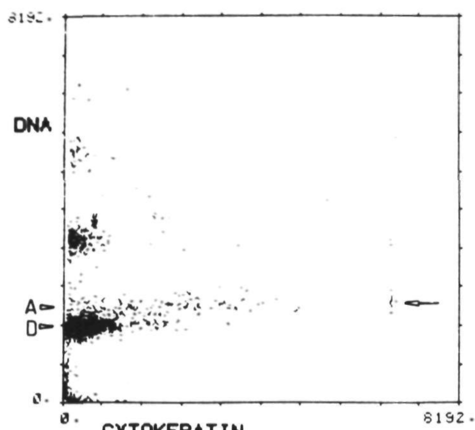
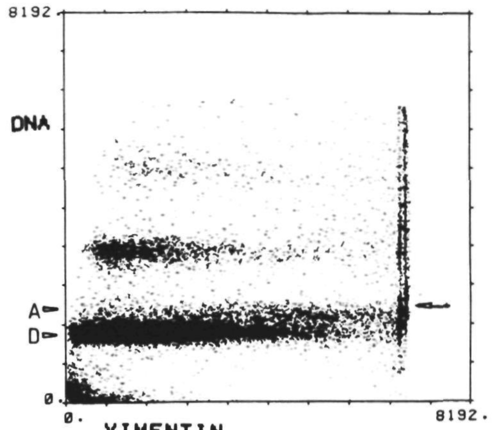
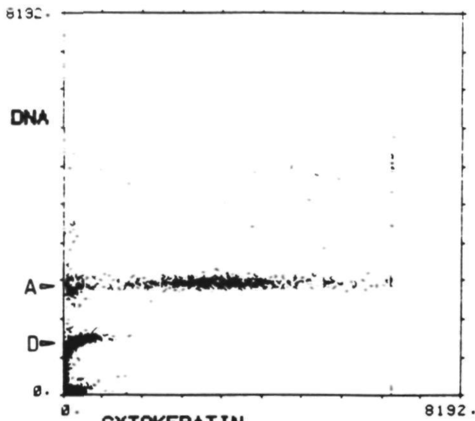
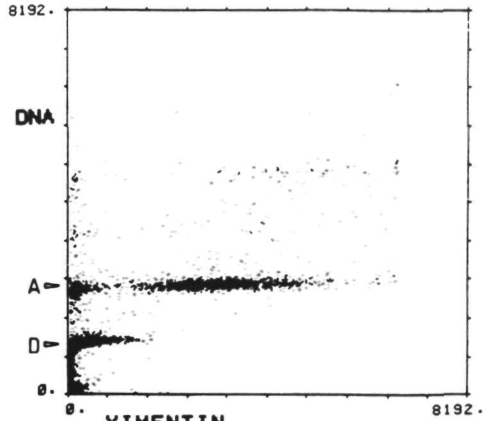


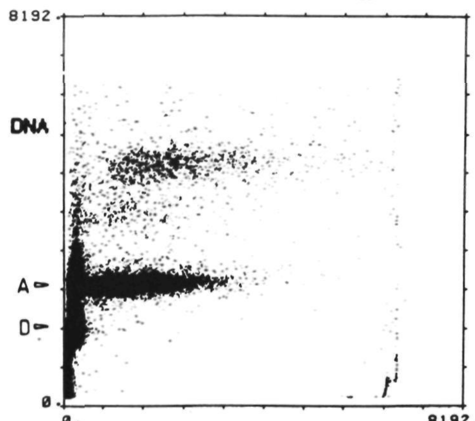
Fig. 3: Example of a 2-parameter FCM analysis of a human RCT xenograft in a nude mouse (NC65) using the cytokeratin antibody pKer and the vimentin antibody pVim in addition to DNA staining with PI. (a) One-dimensional FCM DNA pattern of the cell suspension stained only with PI. (b) A 2-dimensional projection of the DNA-content (as PI fluorescence) and the cytokeratin content (as FITC fluorescence) from the same cell suspension as shown in (a). (c) A 2-dimensional projection of the DNA content and the vimentin content of the same tumor. Note that the tumor fraction is cytokeratin- and vimentin-positive. (d) DNA histogram of the cytokeratin-positive cells present in the cell suspension. The cell cycle parameters are indicated in the upper right corner.











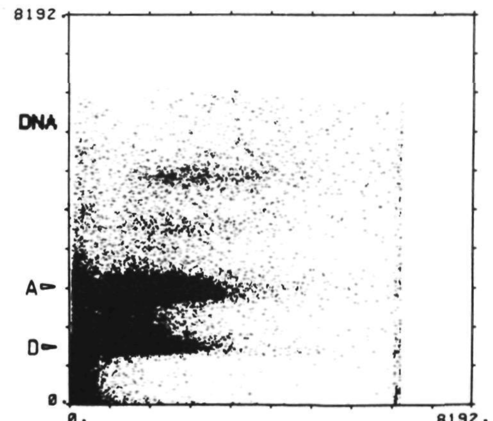


Fig. 4

Fig. 4: Comparison of the 2-parameter FCM analysis of a primary human renal cell tumor (no. 4) (a, b) with 2 tumor model systems, one xenograft of a human RCT in a nude mouse (NC65) (c, d) and a rat RCT (rRCT) (e, f). (a, b) Two-dimensional projections of the DNA content and the cytokeratin-positive (a) and vimentin-positive (b) cell fractions of a primary human renal cell tumor. (c, d) Projections of DNA content and cytokeratin (c) and vimentin (d) content of a xenograft of the human renal cell tumor, and (e, f) a rat renal cell tumor (cytokeratin in e and vimentin in f). Note that in all cases the tumor fraction is cytokeratin- and vimentin-positive next to vimentin-positive stromal cells (A= aneuploid, D= diploid DNA index).

FITC-conjugated second antibody and PI. In the 9 primary human RCT and the lymphnode metastasis, analysed in this way, the vimentin-positive cell population contains stromal cells and inflammatory cells (Fig. 2d) next to the tumor cells, which become clearly visible as an aneuploid peak in 7/10 cases (see Table I and Fig. 2b). In these cases nearly all tumor cells are also cytokeratin-positive (Fig. 2a, c). The cytokeratin-negative cell population is represented by the stromal and inflammatory cells of mesenchymal origin which are vimentin-positive. Table I summarizes the FCM data obtained for these human RCT before and after immunolabelling. This immunolabelling procedure made it possible to detect an abnormal DNA stemline in RCT in 7/10 instead of 4/10 samples.

An example of analysis of a tumor cell suspension from a RCT xenograft in nude mice is shown in Figure 3. Figure 3a shows the normal DNA histogram before labelling with intermediate filament antibodies. After labelling with the cytokeratin antibodies, epithelial (tumor) cells can be identified and their DNA content and cell cycle parameters analysed (Fig. 3b, d). As expected from our immunohistochemical findings, tumor cells with an abnormal DNA stemline are also found in the vimentin-positive fraction (Fig. 3c). These can also be analysed for their DNA content and proliferative fraction by placing a window around the vimentin-positive cells. Both the cytokeratin and vimentin-positive cell fractions show a similar or

identical DNA index, indicating co-expression of both intermediate filament proteins in a certain number of the tumor cells. Similar data have been obtained for the rat RCT model system. Fig. 4 compares 2-parameter DNA analysis of a primary human RCT (Fig. 4a, b) with that of a human RCT xenograft in a nude mouse (Fig. 4c, d) and that of the rat RCT (Fig. 4e, f). All 4 cases of human RCT xenografts in nude mice contained an abnormal DNA stemline. Tumor cells expressing cytokeratins were found in all 4 tumors while a positive staining reaction was also found with the vimentin antibodies in the tumor cell population (Fig. 4d), although with varying intensity. The DNA content of tumor cells in the xenografts, however, is higher than that of the primary human RCT (see Table I and compare Fig. 4a, b with Fig. 4c, d). The rat RCT, analysed with the 2-parameter FCM analysis, also contained an abnormal DNA stemline with a DNA index of 1.88. The tumor fraction contained aneuploid cells expressing both cytokeratin (Fig. 4e) and vimentin (Fig. 4f).

DISCUSSION

Flow cytometric analysis of renal cell tumors offers a new diagnostic tool for the clinician and has additional prognostic value (Baisch et al., 1982; Herman et al., 1984). Tumors can be further subclassified on the basis of this technique and selection of patients at risk is made possible by detection of biologically important cell fractions (Ljungberg et al., 1985; Lovett et al., 1984; Otto et al., 1984; Schwabe et al., 1983; Verheyen et al., 1985). The use of tissue specific markers, such as cytokeratins, in combination with DNA analysis in flow cytometry permits better analysis of tumor fractions within cell suspensions and may provide further information about subfractions in complex tumors (Feitz et al., 1985). When polyclonal and monoclonal antibodies to cytokeratin and vimentin are used, cell subpopulations can be separately analysed by the indirect immunofluorescence technique. These antibodies are already widely used as tissue specific markers in immunohistopathology and cytopathology (Ramaekers et al., 1983b). The use of these tissue specific markers in flow cytometric analysis of complex tumors has been described for some tumor types (Feitz et al., 1985; Oud et al., 1985; Ramaekers et al.,

1986). In this study the advantages of application of the cytokeratin and vimentin antibodies in 2-parameter FCM analysis of renal cell tumors have been demonstrated. First, by means of 2-parameter FCM analysis the cytokeratin-positive tumor cell population can be separated and the DNA content of the tumor cells calculated more precisely than without labelling. Malignant cells from RCT can be analysed for their DNA content, separately from stromal and inflammatory cells with antibodies to cytokeratin for specific labelling. As in the case of bladder carcinomas (Feitz et al., 1985), in RCT cell suspensions containing only a low percentage of tumor cells the 2-parameter FCM method makes it possible to detect this malignant fraction and to determine its ploidy and proliferative capacity. The detection of tumor cells with an abnormal DNA stemline therefore becomes more sensitive. Second, in several cases of RCT in which an abnormal DNA stemline was present, a positive staining pattern of the tumor cells with both cytokeratin and vimentin was seen. Co-expression of these proteins in RCT cells is to be expected. The FCM data presented here thus confirm our earlier immunohistochemical findings (Herman et al., 1983a). They clearly show that aneuploid cells (the tumor cells) of RCT contain both cytokeratin and vimentin. Thirdly, quantification of the different biological subfractions in RCT becomes possible. It remains to be evaluated whether or not this refinement in the classification and quantification of subpopulations gives more information and better correlation with the prognosis of the patients.

In a comparison of 2 RCT model systems with some of the human RCT obtained from patients, comparable biological properties of tumor cells were found. First, in all xenografts as well as in the rat tumor model an abnormal DNA stemline was found. This tumor fraction could be analyzed separately from animal stromal cell components and quantified by the 2-parameter FCM analysis. The intermediate filament staining pattern found in these model tumors is therefore comparable to the pattern found in most primary human RCT. Co-expression of cytokeratin and vimentin was seen in these tumors, since aneuploid cells were found in both fractions and all cells which were cytokeratin- and vimentin-positive had about the same DNA index.

Therefore, the use of a combination of immunohistochemical and flow cytometric techniques in the investigation of complex tumors may provide additional information about subpopulation characteristics and biological potentials, independent of histological parameters. Whether this information can be translated into more effective prognostic criteria and patient management remains to be demonstrated.

ACKNOWLEDGEMENTS

We thank Mr. J. Boezeman for help with the computer programs, Mrs. A. Huysmans and Mr. O. Moesker for immunohistochemical staining procedures, Mr. B.Th. Hendriks, Mr. W.P. Peelen, Mrs. C. Verrijp and Mrs. J. Vedder for preparing the cell suspensions and the members of the Urological Clinic, University of Nijmegen, for providing the tumor material. We are grateful to Dr. C.J. Herman (SSDZ, Delft) for stimulating discussions and critical reading of the manuscript.

This study was supported by grants from the Queen Wilhelmina Fund, The Netherlands Cancer Foundation.

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EXPRESSION OF THE HUMAN FES CELLULAR ONCOGENE IN RENAL CELL TUMORS

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SUMMARY

Renal cell tumors were screened for expression of the cellular oncogenes c-*abl*, c-*fes*, c-*fms*, c-*myc*, c-*ras* and c-*sis* in dot blot hybridization analysis. Expression of c-*ras* and c-*myc* was clearly detectable in most of the 15 tumors that were studied. The c-*fes* oncogene appeared to be expressed in only two of them. Comparative Southern blot analysis of molecularly cloned human c-*fes* DNA and genomic DNA of the 15 renal tumors revealed no major genetic differences. Northern blot analysis of poly(A)-selected RNA from the *fes*-positive tumors with the complete viral v-*fes* oncogene of the Gardner-Arnstein strain of feline sarcoma virus as a molecular probe revealed hybridization of RNA species of 3.0 and 4.5 kb, respectively. The 3.0 kb c-*fes* transcript has also been reported in RNA from patients suffering from acute myelogenous leukemia (13). The 4.5 kb transcript, however, has not been described before and represents either a c-*fes*-related splicing intermediate or, more likely, a completely processed transcript. The results of this study could imply that human c-*fes* coding sequences are more extensive than were previously assumed.

KEY WORDS

Renal cell tumors, Oncogene expression, c-*fes*.

INTRODUCTION

Based upon morphological and histochemical features, renal cell tumors are thought to be adenocarcinomas and to originate from the proximal convoluted tubule (2). In a recent study, Herman et al. (10) reported expression of vimentin and keratin within the same cell. These intermediate filament types are generally biological markers of both sarcomatous and carcinomatous tumors. Additional biological markers are required to enable further characterization of renal tumors. Potentially useful in this respect are the cellular oncogenes. These genes have been implicated in the induction of malignant

transformation in general and their elevated levels of expression in a number of human tumor cell lines as well as fresh human tumors is documented (13). Sometimes, malignant activation of cellular oncogenes seems to involve point mutations or more extensive genetic changes such as chromosomal rearrangements or translocations (21). In many tumors, cellular oncogenes such as c-H-*ras*, c-K-*ras*, c-*myc* and c-*fos* seem to be involved simultaneously (13). Other cellular oncogenes, such as for example the *fes* cellular oncogene, show a more restricted expression pattern. Expression of this cellular oncogene was mainly found in lung and hematopoietic malignancies and, sporadically, in renal tumors (13).

To test the relevance of expression patterns of cellular oncogenes as markers in renal tumors, we set up a dot blot screening assay to determine the expression of a number of cellular oncogenes in a single experiment. To assess the potential implications of the expression of the cellular *fes* oncogene in renal tumors, we studied its genetic organization by Southern blot analysis and analyzed *fes*-related RNA transcripts by Northern blot analysis. The discovery of a new c-*fes*-related transcript is discussed.

MATERIALS AND METHODS

Renal Cell Tumors

Renal cell tumors were obtained from the Department of Urology of the St. Radboud University Hospital. Preoperative diagnostic procedures including intravenous urography, selective renal arteriography, cavography, lung tomography, computer scanning tomography and bone scanning were performed. By this means, a valid T.N.M. classification of the tumors could be made. The renal tumors from patients including those with positive lymphnodes (N^+) and those with metastases (M^+) were removed by a paramedian transabdominal radical nephrectomy. Tumor material was frozen in liquid nitrogen for biochemical analysis.

Gel Electrophoresis and Hybridization

Restriction endonucleases were purchased from either New England Biolabs or Bethesda Research Laboratories (BRL) and used according to the specification of the manufacturers. High molecular weight DNA was isolated from tumor samples frozen in liquid nitrogen. Molecularly cloned human v-*fes* homologous sequences were isolated from bacterial strain HB101 transformed with recombinant plasmid pPH2. pPH2 is derivative of pSP64 containing a 12 kb EcoRI DNA insert that includes all human v-*fes* homologous sequences detectable by Southern blot analysis. Analysis of restriction endonuclease digested high molecular weight DNA from renal cell tumors was essentially as described by Southern (14). DNA fragments were size fractionated by electrophoresis through 0.8% agarose (Seakem) gels, transferred to Gene Screen Plus (New England Nuclear) and hybridized to ^{32}P-nick-translated DNA probes. Molecular probes were nick-translated to specific activities of about 2-5 x 10^8 cpm/µg. Hybridization analysis was performed under conditions of low stringency at 42 °C for appropriate periods of time in 40% formamide (deionized, Mixed Bed Resin AG501-X8D, Biorad), 1% SDS, 1 M NaCl, 10% dextran sulphate and 2-5 x 10^5 cpm/ml nick-translated DNA. DNA probes were labeled according to the method of Rigby et al. (12) using ^{32}P-dCTP (3,000 Ci/mmole) from Amersham, DNAse I from Sigma and E. coli DNA polymerase I from Boehringer. cDNA was labeled to high specific activity by reverse transcription of poly(A)-selected RNA (25 µg/ml) in 100 mM Tris-HCl (pH 8.3), $MgCl_2$ (8 mM), NaCl (20 mM), dithiothreitol (10 mM), a mixture of dATP, dCTP, dGTP and dTTP (5 µM each), especially treated calf thymus DNA (15) (0.25 µg/ml) and reverse transcriptase (20 U) for 20 min at 42 °C. Using ^{32}P-dCTP, specific activities of the cDNA probes were about 1-2 x 10^8 cpm/µg. Upon hybridization, filters were washed twice in 2 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M Na-citrate at room temperature for 5 min and twice in 2 x SSC and 1% SDS at 50 °C for 30 min and autoradiography was performed by exposing XAR-2 film (Kodak) for up to four days at -70 °C with Dupont lightning plus intensifying screens.

Preparation of DNA Probes

Characteristics of the molecular probes used in this study are

summarized in Table 1. DNA probes were prepared by digestion of 50-100 µg of DNA with appropriate restriction endonucleases followed by electrophoresis through low melting point agarose (BRL). The desired bands were excised from gels and, upon melting by heating at 65 °C for 15 min as described (20), agarose was removed by two extractions with phenol, equilibrated with 10 mM Tris-HCl (pH 7.0) and 0.1 mM EDTA and one extraction with phenol/chloroform/isoamylalcohol (25:24:1). After concentration using butanol-2, DNA was precipitated in ethanol and 0.1 M NaOAc (pH 5.2).

Table 1: DNA fragments used as molecular probes

Source DNA fragments	Fragment used as probe	Reference
c-*abl* (mouse)	1.2 kb PstI-PstI (cDNA)	19
c-*fes* (human)	1.3 kb EcoRI-BglII	8
v-GA-*fes* (feline)	3.8 kb BGlII-HindIII	9
v-*fms* (feline)	1.45 kb PstI-PstI	5
c-*myc* (human)	1.2 kb EcoRI-ClaI	3
c-H-*ras*-1	5.2 kb BamHI-BamHI	7
v-H-*ras* (rat)	0.96 kb HindIII-HindIII	6
c-*sis* (human	1.7 kb BamHI-BamHI	18
actin (hamster)	1.2 kb PstI-PstI (cDNA)	4
feline leukemia virus proviral DNA	5.0 kb XhoI-XhoI	11
vimentin (hamster)	0.5 kb PstI-PstI (cDNA)	4

RNA Isolation and Northern Blotting

Total cellular RNA was isolated according to the lithium-urea method described by Auffray and Rougeon (1). Upon poly(A)-selection by oligo-dT-cellulose chromatography, 10 µg RNA was dissolved in sodium phosphate buffer (10 mM, pH 7.0), which contained DMSO (50%) and glyoxal (1 M), and heated to 50 °C for one hour. RNA was size fractionated by agarose gel electrophoresis (1.0% agarose gel) and

transferred to Gene Screen Plus for hybridization analysis as described above.

RESULTS

To detect expression of the cellular oncogenes abl, fes, fms, myc, ras and sis in a single experiment, a dot blot hybridisation assay was performed in which increasing amounts of DNA homologous to those cellular oncogenes were bound to Gene Screen Plus paper. Upon denaturation of the DNA, hybridization was performed with a ^{32}P-cDNA probe synthesized in reverse transcriptase reactions with poly(A)-selected RNA isolated from renal cell tumors as templates. Synthesis of ^{32}P-cDNA was performed with random oligodeoxynucleotides from DNAse-digested calf thymus DNA as primer (see under Materials and Methods). A typical example of such a dot blot hybridization analysis is shown in Fig. 1. A clear hybridization signal is observed in places where DNA is spotted homologous to v-H-ras, c-H-ras-1 and c-myc (human), indicating the presence of relatively high levels of related transcripts in this particular tumor. A weaker hybridization signal is observed in places where v-Ga-fes and c-fes (human) is spotted. It should be noted that in only 2/15 of the tumors that were tested expression of the cellular fes oncogene could be detected in this way. Expression of c-myc and c-H-ras was found in most of the 15 renal cell tumors that were analyzed. Transcripts homologous to the cellular oncogenes abl, fms and sis were not detectable in the tumors by this procedure. To control the assay conditions and the quality of the RNA preparations, actin and vimentin genes were included in the experiments (Fig. 1). It should be noted that the experimental approach does not allow the identification of the cell types that express the various cellular oncogenes.

The renal cell tumor whose expression pattern of some cellular oncogenes is shown in Fig. 1, was obtained from a 41 year old male patient. The tumor was found in the left kidney following an intravenous urogram indicated in the investigation of renal stone disease. The tumor was staged $T_2N_0M_0$ (clear cell). However, it should be noted that histological analysis revealed papillary structures in

the tumor as well as multiple intracytoplasmatic inclusions. The inclusions were identified by electronmicroscopy as crystalline protein bodies. Expression of vimentin intermediate filament protein was observed in an immunofluorescence study (Ramaekers, personal communication) which was in agreement with the hybridization data. Furthermore, the observation that in some cells of a renal tumor both vimentin and keratin were expressed, was also made in this particular tumor (data not shown). In a soft agar assay, the renal tumor cells showed no colony formation.

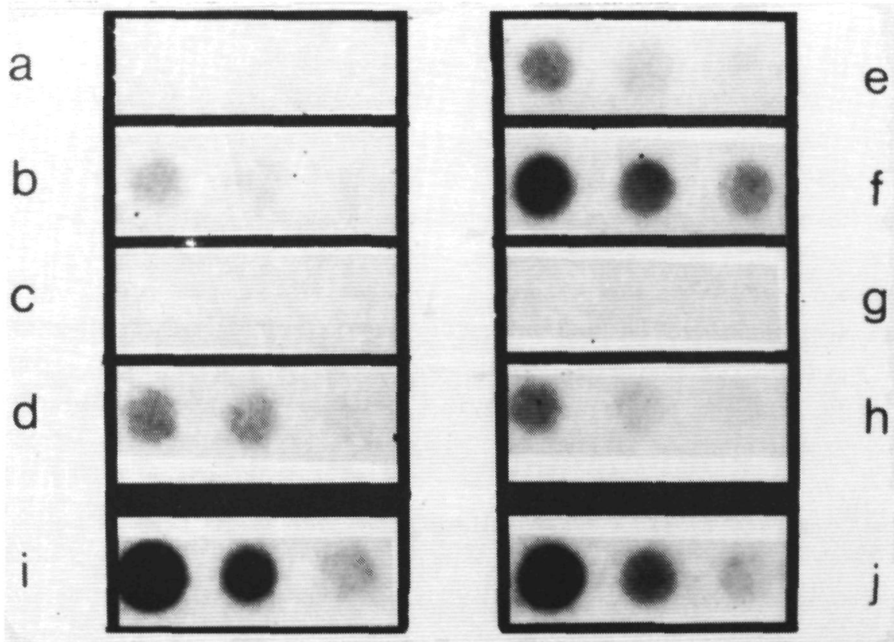

Fig. 1: Analysis of expression of cellular oncogenes in renal cell tumor # 63 by dot blot hybridization analysis. Increasing amounts of DNA samples (8, 40 and 200 ng) representing molecularly cloned sequences of viral or cellular oncogenes were spotted onto Gene Screen Plus and analyzed as described in the text. DNA samples included c-abl (mouse) (A), c-fes (human) (B), v-fms (feline) (C), c-myc (human) (D), v-H-ras (rat) (E), c-H-ras-1 (human) (F), c-sis (human) (G) and v-GA-fes (feline) (H). Actin (hamster) (I) and vimentin (hamster) (J) were included as controls. As a molecular probe, ^{32}P-cDNA synthesized with poly(A)-selected RNA from tumor # as template was used.

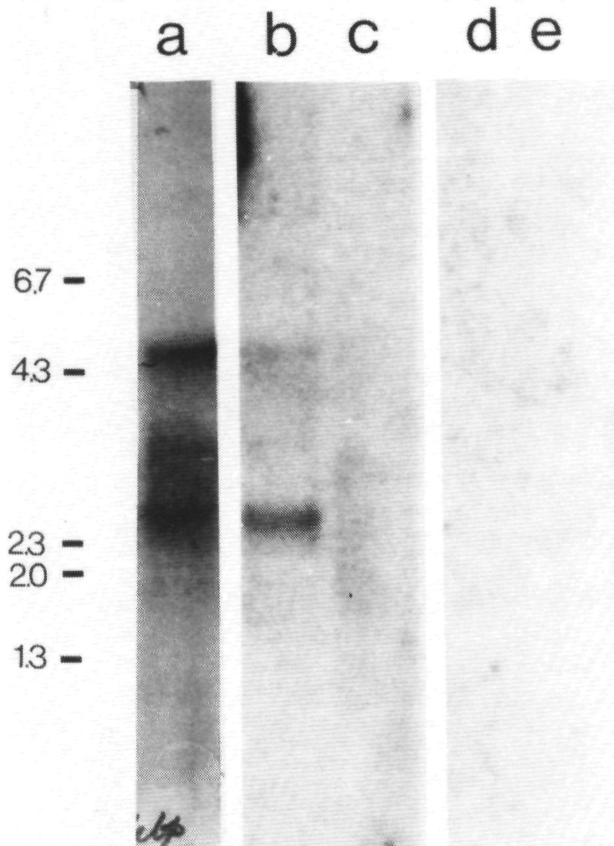

Fig. 2: Northern blot analysis of expression of the *fes* cellular oncogene in renal cell tumors. Poly(A)-selected RNA from tumor # 160 (lane A), tumor # 63 (lane B), normal kidney (lane C), tumor # 87 (lane D) and tumor # 148 (lane E) were size fractionated by agarose gel electrophoresis and analyzed by Northern blot analysis. The complete viral oncogene v-GA-*fes* was used as a molecular probe. Molecular weight markers included are single strand HindIII-digested λ DNA fragments.

In a further analysis of the *fes* expression in renal tumors, we studied the *fes* transcripts by Northern blot analysis. As a molecular probe, the complete viral oncogene of the Gardner-Arnstein strain of feline sarcoma virus was used. Two *fes*-related RNA species were detected in a poly(A)-selected RNA sample from the two renal tumors. Their lengths were 4.5 and 3.0 kb (Fig. 2, lanes A and B). A difference in the relative amounts of the two RNA species was observed. No *fes*-related RNA transcripts were detected in poly(A)-selected RNA samples of two other renal tumors (Fig. 2, lanes D and E) or from normal kidney (Fig. 2, lane C). Since the DNA probe contained some genetic sequences of feline leukemia virus (FeLV), hybridization analysis was also performed with a large portion of the

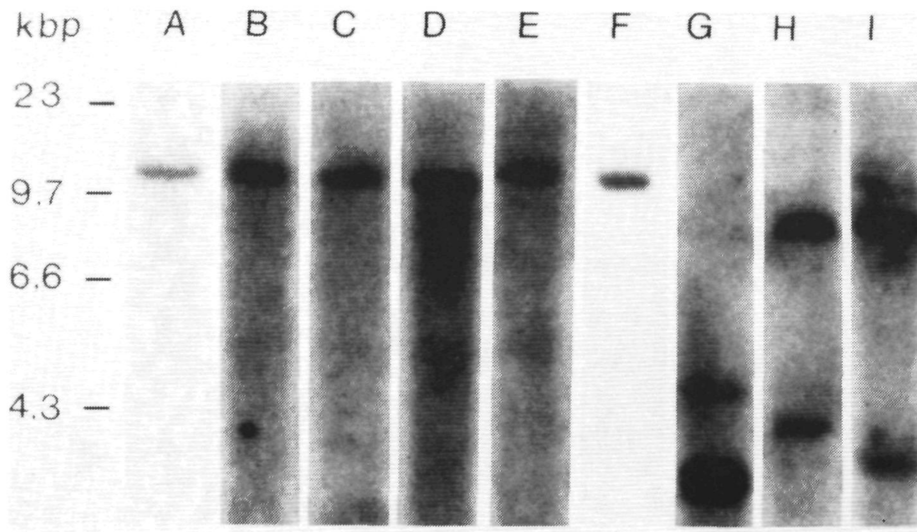

Fig. 3: Comparative Southern blot analysis of the c-*fes* locus in human renal cell tumors and molecularly cloned human c-*fes* sequences. Ten ug of high molecular weight DNA of normal human liver (lane A), renal cell tumor # 63 (lane B), tumor # 87 (lane C), tumor # 148 (lane D), tumor # 160 (lane E) and 0.5 ng of pPH2 DNA (lane F) or mixtures of DNA from tumor # 63 (10 ug) and pPH2 DNA (0.5 ng) (lanes G, H and I) were digested with restriction endonuclease EcoRI (lanes A, B, C and D) or combinations of EcoRI and BamHI (lane G), EcoRI and HindIII (lane H) or EcoRI and XhoI (lane I), transferred to Gene Screen Plus and hybridized to the same molecular probe as described in the legend to Fig. 2. Molecular weight markers included are HindIII-digested λ DNA fragments. In the lower part of the figure, a schematic restriction map of the human c-*fes* 12 kb EcoRI DNA fragment is depicted. B, BamHI; H, HindIII; E, EcoRI; X, XhoI.

proviral DNA of FeLV as a molecular probe but not hybridization was observed (data not shown).

A 3.0 kb _fes_-related RNA species was found in RNA isolated from myeloblasts of patients suffering from acute myelogenous leukemia (13). Our observations suggest that expression of the c-_fes_ locus in renal cell tumors differs from that in acute myelogenous leukemia. To investigate whether or not this could be explained by chromosomal rearrangement, we analyzed the genetic organization of the c-_fes_ locus in renal cell tumors by Southern blot analysis and compared the data with those obtained with molecularly cloned human c-_fes_ DNA (Fig. 3). With the complete v-GA-_fes_ oncogene as a molecular probe, all human v-GA-_fes_ homologous sequences were found in a 12 kb DNA fragment in control human genomic DNA digested with restriction endonuclease EcoRI (Fig. 3, lane A). Similar analysis of genomic DNA isolated from the 15 renal tumors revealed the same 12 kb EcoRI DNA fragment in each case (Fig. 3). The results of only four tumors are shown (Fig. 3, lanes B, C, D and E). Southern blot analysis of mixtures of genomic DNA of a _fes_-positive renal tumor (tumor # 63) and cloned human c-_fes_ DNA digested with combinations of restriction endonuclease EcoRI with BamHI, HindIII or XhoI (Fig. 3, lanes G, H and I, respectively) did not reveal any differences. These results indicate that there are no major genetic changes in the parts of the c-_fes_ loci in the renal tumors that can be detected with the complete viral v-_fes_ oncogene as a probe.

DISCUSSION

The present study indicates that expression of the _fes_ cellular oncogene occurs in some renal cell tumors and that it involves two RNA species of different sizes. The 3.0 kb RNA has a similar molecular weight as the transcript found in myeloblasts of patients with acute myelogenous leukemia (AML) (13). The other transcript is 4.5 kb. The novel appearance of a 4.5 kb _fes_ transcription product in some renal tumors could be explained in different ways. The 4.5 kb RNA species is probably not the result of a chromosome translocation nor of other major chromosomal changes in the v-_fes_ homologous region of renal cell

tumors since no abnormalities were seen in restriction endonuclease analyses of tumor DNA. A minor genetic change, on the other hand, such as a point mutation, a small deletion or insertion is usually not detected by the Southern blot analysis. It could be assumed that such a minor genetic change would affect the processing of c-fes pre-mRNA in such a way that two different products are being formed from one gene. In that case, it may be that one particular step in the splicing process is limited in renal tumors since no other fes-related splicing intermediates were detected. Differential gene splicing is a well documented phenomenon in tumor viruses. There is no reason why this mechanism might be limited to viral genes.

Alternatively, the new transcript could be due to a secondary mutation in a tumor cell which grew out to a subpopulation of cells within the tumor. Finally, the possibility that both RNAs constitute independent and naturally occurring transcripts cannot be excluded. Clearly, more experimental work is needed to discriminate between these explanations.

While the 3.0 kb transcript is approximately of the same size as the viral gene, v-fes, used as a molecular probe in these studies, the newly discovered occurrence of a 4.5 kb transcript in two different tumors raises the possibility that the human c-fes locus is larger than the 12 kb EcoRI restriction endonuclease fragment that contains all sequences homologous to v-fes. It is accepted that the retroviral oncogenes which have been generated in a process in which RNA tumor viruses have captured cellular genetic sequences from their natural hosts, often represent only a portion of the cellular locus (9, 19). It is , therefore, possible that the v-fes oncogene of the Gardner-Arnstein strain of feline sarcoma virus encodes only the tyrosine-specific protein kinase domain of a more complex protein and that parts of the cellular locus involved are not represented in any of the other fes-related viral oncogenes. The epidermal growth factor receptor (17) and the insulin receptor (16) are examples of such complex proteins with a tyrosine-specific protein kinase domain as an intrinsic structural component. The 4.5 kb fes-related RNA species could represent a transcript of a larger yet to be defined c-fes locus. Further studies are required to resolve this matter.

ACKNOWLEDGEMENTS

This work was supported by The Netherlands' Kidney Foundation, contractno. 85.531.

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SUMMARY AND CONCLUSIONS

Chapter I represents a review of literature on renal cell carcinoma to put the issues of this thesis in the right context. Up until this moment no consistant therapeutical approach exists for metastasized renal cell carcinoma in man. One of the methods to achieve a progression in the treatment of this disease is to obtain a better understanding of biological and biochemical aspects of the tumor. For this purpose a spontaneous rat renal cell carcinoma, several human renal cell carcinoma xenografts in nude mice and primary human renal cell tumors were studied and compared. In order to give an introduction on renal cell carcinoma an inventory is made on epidemiology, clinical presentation and metastatic behaviour. Also features of possible prognostic value as identifiable by macroscopic, microscopic and ultrastructural evaluation are surveyed. Eventually recent developments in the characterization of tumor cells, applied and studied in this thesis, are described as there are intermediate filament studies, flow cytometric cellular DNA content measurement and activated oncogene determination.

In chapter II a tumor model system, derived from a spontaneously arosen renal cell carcinoma of a Whistar Lewis rat is characterized in a multidisciplinary way and compared to human renal cell carcinoma. It is shown that a great resemblance of histological and ultrastructural features exist. Also the expression of intermediate filaments (i.e. coexpression of vimentin and cytokeratin) and results of DNA measurements in flow cytometric double labeling experiments are comparable to what is encountered in primary human renal cell carcinomas. In addition, increased levels of vimentin mRNA were found. A positive growth capacity in the double layer soft agar human tumor cloning system was evident. It is concluded that this tumor model has several histological and biological properties in common with the human renal cell tumor and that it is a suitable model for studying new cytotoxic drugs.

Chapter III describes an analogous study performed on four human renal cell carcinoma xenografts maintained in nude mice. The results showed that these four tumor lines contained a variety of histological,

histochemical and biochemical properties that also occur in the primary human tumor. It became clear, however, that also marked differences in character between the different xenografts existed, demonstrating the heterogeneity of renal cell carcinoma. Such xenografts, representing different subtypes of renal cell carcinoma, may prove to be of great value in *in vitro* screening of effects of new chemotherapeutics.

In chapter IV the activated oncogene expression in several xenografts and a monkey kidney cell line is studied. The oncogene analysis was performed by Northern blot technique. It was found that all xenografts expressed c-*ras*. Expression of c-*myc* or the p53 gene was found in all xenograts but one. Interestingly c-*fes*/*fps* mRNA was elevated in all tumors in contrast to what is seen in primary human renal cell carcinomas, where c-*fes*/*fps* expression is only present in 10-20% of the cases. The possible explanations were discussed. Furthermore it was concluded that the xenograft tumor model system is very useful in oncogene expression studies as it is capable to provide consistant high amounts of mRNA. As far as molecular oncology is concerned, the xenografts are easy to handle and oncogene expression is comparable to what is reported in literature about human renal cell carcinomas and to our own results in primary renal cell tumor oncogene analysis (chapter VI).

Chapter V describes the use of cytokeratin and vimentin as tissue specific markers in histochemical and flow cytometric double labeling studies of primary renal cell carcinomas, a tumor metastasis and several tumor model systems. The different cell populations that could be identified by immunohistochemical staining, could also separately be analysed for their DNA content. This was done by a double labeling flow cytometric technique. With this method also a coexpression of vimentin and cytokeratin was detected in the aneuploid tumor cell population. It was shown that renal cell tumor model systems could, in this regard, be compared to primary renal cell tumors.

In chapter VI an analysis of the oncogene expression in 15 primary renal cell carcinomas with dot blot technique is described. An expression of c-*ras* and c-*myc* was clearly detectable in most of these

tumors. In two cases an additional oncogene, c-fes, which is known to have a tyrosine specific kinase activity, was found to be expressed. Northern blot analysis revealed two mRNA species, one of 3.0 kb and one of 4.5 kb. Southern blot techniques were performed to check the structure of the c-fes locus but no major changes could be found in its genetic organization.

Reviewing the experimental chapters of this thesis, two questions arise. Firstly, what is the value of these tumor model systems in relation to the research for more successfull approaches in the treatment of renal cell carcinoma? Secondly, which direction the further development of the research needs to follow.

As far as the tumor model systems concerned, a division is to be made between the rat renal cell tumor and the xenografts. The rat tumor line is the more easy evaluable model system. It is easily maintained in different generations of rats and appears to be histologically and biologically stable. As a result of this, one is able to conduct well designed and reproducable studies. However, as it concerns a pure animal model, there remains uncertainty about the direct applicability of the experimental results to the human renal cell carcinoma, taking in account that the renal cell carcinoma is a very complex tumor, presenting a great variability of characteristics and behaviour (chapter I).

The xenografts offer a different problem. In these cases it concerns original human tumors, from which a stable tumor line was established in the nude mouse. These xenografts often appear to have a very specific character. Also one has to realize that a certain xenograft is developed from only a small part of the primary tumor, which is the part of the tumor that possesses the potential of growth in a host animal. In addition it is very well possible, that cells with certain specific biochemical features are furthermore selected by the process of transplantation. The latter is suggested by the results of the oncogene analyses of several xenografts (chapter IV). In the interpretation of results obtained in xenografts one should always keep in mind that this xenograft only represents a part of the original tumor and also the suggested positive selection for new

characteristics during further transplantations may have a decisive influence on the outcome of experiments. Furthermore it became clear from the study of xenografts that certain features suggesting an aggressive tumor character in vivo, as there are histological dedifferentiation and a high degree of aneuploidy, do not necessarily lead to a greater growth potential in vitro (RC43, chapter III). Finally one should consider the fact that in this model a human tumor is maintained against a totally different biological background compared to the original tumor. The nude mouse is not only immunologically deficient, but also all species specific interactions between tumor and host are not present.

If one takes the special demands for an animal laboratory in account necessary for maintaining nude mice, one must come to the conclusion that establishing xenografts is a relatively expensive method, in which a model system is obtained, undoubtedly superior to an animal model, but with certain clear restrictions.

So, the strategy has to be, that one uses the model for its better experimental accessibility compared to the primary tumor, but that the relevance of the results always should be checked on the original carcinoma.

In answer to the question in which direction the research of the renal cell carcinoma should be pointed, it is obvious that the answer has to be found in the solution of the structure and functional mechanisms of the gene systems involved in the origin of this form of cancer.

The molecular biological results described in this thesis must be seen in the light of a breakthrough in genetics at this moment, as a result of the DNA recombination technology. Nowadays a great variety of DNA research is performed, both with a scientific and with a commercial goal. Many smaller or bigger progressions are made in this field, resulting in new biological concepts, for instance in carcinogenesis.

In this light our data must be regarded as a small part of a hughe molecular oncological research program. Also one has to realize that the renal cell carcinoma is only one of the many malignancies, that as

a result of its specific characteristics (as any other tumor) can only contribute modestly to the total understanding of carcinogenesis. There are some hundred different tumors identified in humans (the exact amount depends of course on the method used for their catalogization). They all have their individual advantages and disadvantages in experimental research.

The renal cell carcinoma generally concerns a tumor well fit for DNA and RNA studies because of its generally large volume, which makes it possible to retrieve a sufficient amount of tissue without endangering the histological diagnosis. A disadvantage of the primary renal cell tumor as a molecular biological study object, is that as a result of the relatively long time elapsing between devitalisation of the tumor by clamping the renal vessels during the operation and the moment of fixation by freezing it in liquid nitrogen, the quality of RNA worsens quickly.

Another difficulty is that in certain cases the results of the analyses can be influenced by the fact that the tumor is not homogenous as a result of marked preexistent necrosis.

As mentioned in the introduction of this thesis there are two mechanisms by which oncogenes can contribute to the deregulation of the cell. Besides a raised transcription activity of a basically normal proto-oncogene, also a structural change in the gene can lead to a modified gene product, resulting in a changed cellular fenotype. Also our molecular biological analyses reveal twofold results.

Firstly an insight is obtained in which of the oncogenes and groups of oncogenes are activated in the renal cell carcinoma and some of its model systems. The findings appear to support the hypothesis of the multiple step principle in carcinogenesis and also show that the renal cell carcinoma is grossly comparable to many other tumors in this respect.

Secondly, the expression of a specific oncogen, c-*fes*, was studied. Abnormal *fes* mRNA species were found in the renal cell carcinoma and in addition c-*fes* appears to be expressed in almost all xenografts, in

contrast to what is found in primary renal cell carcinoma (chapter IV and VI). These findings have already led to a further research program on c-*fes*.

In conclusion, the experimental results of this thesis constitute a first step towards an extensive research program, which can eventually, by a team of researchers during many years, lead to a substantial contribution to the knowledge of the origin and development of cancer.

SAMENVATTING EN CONCLUSIES

In hoofdstuk I wordt een overzicht van de literatuur over het niercelcarcinoom gegeven met het doel de onderzoekingen, zoals deze worden beschreven in dit proefschrift, in hun juiste context te plaatsen. Tot op dit moment bestaat er geen eenduidige therapeutische benadering voor het gemetastaseerd niercelcarcinoom in de mens. Een van de wijzen om vooruitgang te boeken in de behandeling van deze ziekte, is te trachten een beter inzicht te verkrijgen in de biologische en biochemische aspecten van deze tumor. Met dit doel voor ogen worden een spontaan in een rat ontstane niertumor, verschillende xenografts van het humane niercarcinoom in de naakte muis naast primaire menselijke niertumoren bestudeerd en vergeleken. Hoofdstuk I geeft een introductie omtrent het niercarcinoom aan de hand van een overzicht van de epidemiologie en het klinische gedrag. Verder worden mogelijke prognostische factoren besproken. Tenslotte worden nieuwe onderzoeksmethoden waaronder intermediaire filamentenstudies, DNA-metingen met behulp van flowcytometrie en studies van geactiveerde oncogenen nader toegelicht.

In hoofdstuk II wordt in een multidisciplinaire benadering een tumormodelsysteem, dat afkomstig is van een spontaan ontstaan niercelcarcinoom in een Wistar Lewis rat gekarakteriseerd en vergeleken met het primaire menselijke niercelcarcinoom. Er wordt aangetoond dat een grote gelijkenis voorhanden is op zowel histologisch als electronenmicroscopisch gebied. Ook de expressie van intermediaire filamenten (i.e. co-expressie van vimentine en cytokeratine) en de resultaten van DNA-metingen in flowcytometrische dubbellabeling experimenten blijken vergelijkbaar te zijn met hetgeen men vindt in de primaire menselijke niercelcarcinomen. Daarenboven wordt een verhoogd expressie niveau van vimentine mRNA gevonden. Een positieve groeicapaciteit in het HTCS (human tumor cloning system) is duidelijk. De conclusie luidt dat dit tumormodel verschillende histologische en biologische eigenschappen gemeen heeft met het humane niercelcarcinoom en dat het een geschikt model is voor onder andere de studie van nieuwe cytotoxische medicamenten.

Hoofdstuk III beschrijft een gelijkaardige studie, uitgevoerd aan de

hand van vier xenografts van primaire humane niercelcarcinomen. De resultaten geven aan dat deze vier tumorlijnen een onderling zeer gevarieerd profiel ten aanzien van histologische, histochemische en biochemische eigenschappen ten toon spreiden, waarvan de diverse elementen ook in het primaire niercarcinoom voorkomen. Het is duidelijk dat er ook een aanzienlijk verschil in karakter tussen de onderlinge xenografts bestaat, waarmee tevens de heterogeniciteit van het niercelcarcinoom wordt aangetoond. Als zodanig kunnen xenografts, wanneer zij beschouwd worden als vertegenwoordigers van de verschillende subtypes van het niercelcarcinoom, van grote waarde blijken te zijn bij in vitro screening van de effecten van nieuwe chemotherapeutica.

In hoofdstuk IV wordt de geactiveerde oncogen expressie van verschillende xenografts en een apeniercellijn bestudeerd. De oncogeenanalyse wordt uitgevoerd aan de hand van Northern blot techniek. Als resultaat wordt gevonden dat alle xenografts c-ras expressie vertonen. Expressie van c-myc of van het p53 gen werd gevonden in alle xenografts op één na. Interessant is het gegeven dat c-fes/fps mRNA verhoogd was in alle tumoren, in tegenstelling tot wat bij primaire humane niercelcarcinomen wordt gevonden, waar c-fes/fps expressie slechts aanwezig is in 10-20% van de gevallen. De mogelijke verklaring hebben wij bediscussieerd. Verder wordt geconcludeerd dat de xenograft tumor model systemen zeer nuttig zijn bij oncogen expressiestudies van het niercarcinoom, aangezien het door de homogeniteit van de tumor mogelijk is aanzienlijke hoeveelheden mRNA te isoleren. In het kader van de moleculaire oncologie zijn de xenografts goed te bewerken tumorsystemen en de oncogen expressie lijkt vergelijkbaar met hetgeen bekend is over het primaire menselijke niercelcarcinoom.

Hoofdstuk V beschrijft het gebruik van cytokeratine en vimentine als weefselspecifieke markers bij histochemische en flowcytometrische studies van het primaire niercarcinoom, een tumormetastase en verschillende tumormodelsystemen. De verschillende celpopulaties die kunnen worden geindentificeerd door immunohistochemische kleuring, kunnen ook afzonderlijk worden geanalyseerd wat betreft hun DNA-inhoud aan de hand van de flowcytometrie met het gebruik van twee

verschillende kleurlabels. Met behulp van deze methode werd ook een co-expressie van vimentine en cytokeratine aangetoond in aneuploide tumorcelpopulaties. Het was duidelijk dat niercel tumor model systemen in dit opzicht vergeleken kunnen worden met het primaire niercelcarcinoom.

In hoofdstuk VI wordt de analyse beschreven van de oncogen expressie in 15 primaire niercelcarcinomen aan de hand van dot blot techniek. Een expressie van c-*ras* en c-*myc* is duidelijk aantoonbaar in de meeste van deze tumoren. In twee gevallen blijkt een additioneel oncogeen, c-*fes*, waarvan bekend is dat het een tyrosine specifieke kinase activiteit heeft, tot expressie te komen. Northern blot analyse bracht twee mRNA soorten aan het licht, een van 3.0 kb en een van 4.5 kb. Southern blot technieken werden uitgevoerd om de structuur van het c-*fes* locus te analyseren. Hierbij konden geen veranderingen van enig belang in de genetische organisatie gevonden worden.

Wanneer men de experimentele hoofdstukken van dit proefschrift overziet, dringen zich twee vragen op. Ten eerste, wat is de waarde van deze tumormodelsystemen bij het onderzoek van het menselijk niercarcinoom. En ten tweede, in welke richting moet de ontwikkeling van de research t.a.v. de niertumor verder gaan.

Ten aanzien van de tumormodelsystemen dient een onderscheid gemaakt te worden tussen het rattemodel en de xenografts. De rattetumorlijn is het meest eenvoudig te bestuderen modelsysteem. Het is vrij gemakkelijk te handhaven in opeenvolgende generaties ratten en blijkt daarbij histologisch en biologisch stabiel te zijn. Door dit gegeven wordt men in staat gesteld weloverwogen en reproduceerbare experimenten uit te voeren. Echter, omdat dit model een zuiver diermodel is, bestaat er onzekerheid in hoeverre men de experimentele resultaten mag projecteren op het menselijk niercarcinoom. Te meer omdat het niercarcinoom bij de mens een zeer complexe tumor is met een zeer variabel beloop (hoofdstuk I).

Enigszins anders ligt het met betrekking tot de xenografts. Hierbij betreft het oorspronkelijke menselijke tumoren, waaruit een stabiele tumorlijn in naakte muizen tot stand is gebracht. Deze xenografts

blijken vaak een geheel eigen karakter te bezitten, dat onderling nogal kan verschillen. Daarnaast dient men zich te realiseren dat een dergelijke xenograft ontstaat uit slechts een deel van de primaire tumoren, en wel dat deel dat de potentiële eigenschap van groei in een gastheer bezit. Daarbij is het heel goed mogelijk, dat er een verdere selectie ontstaat voor cellen met bepaalde specifieke biochemische karakteristieken, die bijdragen tot een groter percentage succesvol overgezette cellen tijdens verdere transplantaties. Dit aspect wordt althans gesuggereerd door de resultaten van oncogeenanalyses van de diverse xenografts (hoofdstuk IV). Men mag bij de interpretatie van resultaten niet verhelen, dat een xenograft waarschijnlijk slechts een deel van de oorspronkelijke tumor vertegenwoordigt. Daarenboven draagt de veronderstelde positieve selectie voor nieuwe eigenschappen tijdens verdere transplantaties er toe bij dat de resultaten van experimenten wezenlijk beïnvloed kunnen worden. Ook is uit de bestudering van xenografts duidelijk geworden, dat kenmerken van een tumor, die wijzen op een agressief gedrag in vivo, zoals bijvoorbeeld histologische dedifferentiatie van cellen of een hoge mate van aneuploïdie, geenszins hoeven te leiden tot een groter groeipotentieel in vitro (RC43, hoofdstuk III). Tenslotte moet nog genoemd worden de vanzelfsprekende beperking, dat in dit modelsysteem sprake is van de groei van een menselijke tumor in een totaal verschillend biologisch milieu in vergelijking met de primaire tumor. Niet alleen is de naakte muis immunologisch kreupel, maar ook alle species-specifieke interacties tussen de gastheer en de tumor ontbreken.

Wanneer men in overweging neemt dat er speciale eisen dienen te worden gesteld aan een dierenlaboratorium alleen al om met naakte muizen te kunnen werken, is de studie van xenografts een naar verhouding dure methodiek. Men verkrijgt echter een modelsysteem dat ondanks de genoemde beperkingen, ongetwijfeld superieur is aan een diermodel. De strategie moet dus zijn dit model te gebruiken voor een betere experimentele toegankelijkheid dan de primaire tumor, maar de relevantie van de gevonden resultaten dient steeds aan het authentieke carcinoom getoetst te worden.

Op de vraag in welke richting de research van het niercarcinoom en van de carcinogenese in het algemeen zich zou moeten ontwikkelen, is het

voor de hand liggend dat het antwoord gezocht moet worden in de opheldering van de structuur en het functionele mechanisme van de gensystemen betrokken bij het ontstaan van deze vorm van kanker.

De bevindingen in dit proefschrift op moleculair oncologisch gebied moeten gezien worden in het licht van een doorbraak in de genetica op dit moment, als gevolg van de recombinant DNA technologie. Hedentendage wordt op velerlei manieren aan DNA onderzoek gedaan, zowel met een wetenschappelijk als een commercieel oogmerk. Op vele fronten tegelijk wordt vooruitgang geboekt, die bijdraagt tot nieuwe biologische concepten, o.a. op het terrein van de oncologie.

Onze waarnemingen kunnen dan ook niet anders gezien worden dan als een klein onderdeel van een omvangrijk moleculair oncologisch onderzoek. Ook moet men zich realiseren dat het niercarcinoom slechts een van de vele tumoren is, dat door zijn specifiek karakter evenals elke andere tumor, maar een klein deel zal kunnen bijdragen tot het totaal begrip van het ontstaan van kanker.

Er zijn ongeveer honderd verschillende tumoren bekend bij de mens (het precieze aantal is uiteraard afhankelijk van de indeling die men maakt). Zij hebben allen hun voor- en nadelen bij experimenteel onderzoek. Het niercarcioom betreft een tumor, die door zijn over het algemeen aanzienlijk omvang geschikt is voor het aanleveren van voldoende weefsel voor DNA en RNA bewerking, zonder dat hierdoor de histologische diagnostiek gevaar loopt. Een nadeel van de primaire niertumor als moleculair oncologisch studie-object, is dat door de vrij lange tijdsspanne tussen het moment van devitalisering van de tumor op het moment van afklemmen van de niervaten tijdens de operatie en het moment van invriezen in vloeibare stikstof, de kwaliteit van met name RNA sterk achteruitloopt.

Een tweede moeilijkheid is het feit dat een niertumor, evenals vele andere tumoren, macroscopisch meestal heterogeen is, mede doordat vaak een aanzienlijke necrose in het preparaat aanwezig is. Dit zou in bepaalde gevallen de resultaten van de analyses kunnen vertroebelen, omdat de gevonden gegevens maar op een deel van de totale tumor betrekking hebben.

Zoals in de inleiding van dit proefschrift vermeld wordt, zijn er in grote lijnen twee mechanismen waardoor oncogenen kunnen bijdragen tot de deregulatie van de cel. Naast het feit dat dit kan bestaan uit een verhoogde transcriptie activiteit van een op zichzelf normaal proto-oncogen, kan ook door structurele verandering in het gen een gemodificeerd genproduct ontstaan, met gevolgen voor het fenotype van de cel. Aldus blijkt dat ook onze moleculair biologische analyses tweeërlei resultaten hebben opgeleverd.

Eerstens is er een inzicht verkregen in welke van de belangrijkste oncogenen en oncogencategorieën er geactiveerd zijn in het niercarcinoom en enkele van zijn modelsystemen. De bevindingen blijken daarbij een ondersteuning te vormen voor de hypothese van het meertrapsprincipe van de carcinogenese en geven bovendien aan dat het niercarcinoom in dit opzicht afgezien van enige details in grote lijnen niet afwijkt van vele andere tumoren.

Het tweede aspect van onze analyses betreft de bestudering van de expressie van een specifiek oncogen, namelijk c-*fes*. Er zijn abnormale *fes* mRNA species aangetroffen in het niercarcinoom en daarenboven blijkt c-*fes* in vrijwel alle xenografts tot expressie te komen in tegenstelling tot hetgeen het geval is bij primaire niertumoren, waar deze expressie slechts zelden voorkomt (hoofdstuk IV en VI).

In het licht van deze bevindingen is inmiddels een verder onderzoeksprogramma voor dit c-*fes* oncogen opgezet.

Samenvattend concluderen wij dat de experimentele resultaten van dit werk een eerste stap zijn in een uitvoeriger programma, dat door een team van onderzoekers gedurende een reeks van jaren kan leiden tot een wezenlijke bijdrage in het inzicht in het ontstaan en de ontwikkeling van kanker.

ACKNOWLEDGEMENTS

The cover of this booklet suggests that only one author is responsible for this thesis. Nothing less is the truth.

Most of the people, involved in this thesis are mentioned in the concerning chapters, either in the authors lists or in the acknowledgements.

In addition to their contribution, for which I am very grateful, the special inspiring atmosphere of the urological oncology group of the Radboud University Hospital made this thesis "a must" for me.

Besides this group of people, some persons need to be thankfully mentioned, because of their stimulating attitude and support.

First of all, my father, who designed the cover and who, although it seemed definitely hopeless, never gave up trying to convince me of the necessity to write a thesis.

Secondly, Dr. H.R. de Vries who, also in this respect assisted by Max Lilipaly, guided me with his skills and mature wisdom for which I am profoundly grateful.

Finally, the persons without whom all this really would have been impossible to achieve: Wout Feitz, Jack Schalken and Dorothé Berris.

CURRICULUM VITAE

Herbert F.M. Karthaus was born on November 19, 1950 in Utrecht, The Netherlands. He attended Gymnasium B in Breda and started his medical study at the Medical Faculty of the University of Groningen in 1969. After his graduation in October 1976 he started his surgical training at the St. Annadal Hospital in Maastricht (Head: Prof.Dr. J.M. Greep). In 1980 he proceeded with training in urology, the first year in the St. Annadal Hospital in Maastricht (Head: Dr. M.M. Ausems) and in 1981 and 1982 in the De Weever Hospital in Heerlen (Head: Dr. F.P.H. v.d. Weyer). This stay was interrupted by a three month course in pediatric urology at the London Institute of Urology and Hospital for sick children (Head: P.G. Ransley F.R.C.S.). His registration as an urologist took place in January 1983. From January 1983 until July 1986 he worked as chef de clinique of the Urological Department of the St. Radboud Hospital in Nijmegen (Head: Prof.Dr. F.M.J. Debruyne). Since July 1986 he is attached to the Canisius Wilhelmina Hospital in Nijmegen as consultant urologist in association with R.L.F.M. Corten. He is married to Eleonora A. Schröder and has two children, Frederike and Wouter.

STELLINGEN BEHORENDE BIJ HET PROEFSCHRIFT:

A COMPARATIVE STUDY OF RENAL CELL TUMOR MODEL SYSTEMS.

1. De radicale nefrectomie voor een beperkt niercarcinoom, waarbij men eerst de vaatsteel onderbindt, heeft een gunstig effect op de prognose van de gastheer, maar reduceert de mogelijkheden tot moleculair oncologisch onderzoek van zijn tumor.

2. De benaming niercelcarcinoom roept een beeld van uniformiteit op, dat echter wordt tegengesproken door de gebleken diversiteit van de karakterprofielen van de verschillende xenografts van het niercelcarcinoom in de naakte muis.

3. Succesvolle transplantatie van niercarcinomen in de vorm van een xenograft vraagt aanpassingsvermogen van de tumor.

4. Schematische behandeling van kwaadaardige gezwellen is de belangrijkste bijdrage, die de clinicus kan leveren aan een groter begrip van het gedrag van deze tumor.

5. Hoewel klinische onderzoekstrials veelal als academische liefhebberij worden gezien, dienen perifere specialisten zich te realiseren dat zij door met name hun kwantitatieve inbreng deze trials tot werkelijk zinvolle toetsstenen kunnen verheffen.

6. De belangrijkste bijdragen tot een verbeterde vijfjaars overleving van het coloncarcinoom worden geleverd door de afname van de perioperatieve sterfte en de toename van het percentage tumoren dat gereseceerd wordt.
   (Proefschrift T. Wiggers, Maastricht 1987)

7. De samenwerking tussen clinicus en experimenteel onderzoeker kan slechts dan succesvol zijn, wanneer zij begrip weten op te brengen voor de specifieke problemen van elkaars vakgebied.

8. De onontkoombare prioriteitenkeuze in de academische ziekenhuizen als gevolg van budgetteringsmaatregelen noopt de opleiders van met name de snijdende specialismen tot een kritische beschouwing van de door hen gegeven opleiding.

9. Op basis van immunohistochemische detectie van antilichamen die geassocieerd zijn met normaal darmepitheel, kunnen patienten met een morfologisch identieke colontumor worden ingedeeld in categorieën met een verschillende prognose ten aanzien van deze tumor.
   (Proefschrift T. Wiggers, Maastricht 1987)

10. Bij de cursussen voor zelfafrichting van honden komt men veelal meer aan de weet over het karakter van de baas dan dat van zijn hond.

11. De combinatie van conventionele anaesthesie en acupunctuur vermindert de behoefte aan het peroperatief gebruik van opiaten.

12. Bij de voetjacht op het haas met een meute Bassethonden, heeft lampe juist op het moment dat hij uitgeput dreigt te raken de grootste kans om vrij uit te gaan.