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Monoclonal Antibody against Rat Renal Cell Tumor-associated Antigen as a New Tool for the Analysis of Renal Tumorigenesis

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A monoclonal antibody, NR-2 MAb, against one of the rat renal cell tumor-associated antigens was developed. NR-2 MAb belonged to IgM class and recognized a polypeptide of 81,000 daltons designated as NR-2 antigen, which is of non-glycoprotein nature with a pI of 4.6. NR-2 MAb was employed to probe the histogenesis of renal cell tumors in rats treated with N-ethyl-N-hydroxyethylnitrosamine followed by trisodium nitrilotriacetate monohydrate. Immunohistochemical analysis indicated that NR-2 antigen was expressed in simple hyperplasia, adenomatous hyperplasia and renal cell tumors. Both clear cells and basophilic cells of the simple hyperplasia showed equally strong positive reactions with NR-2 MAb, whereas the vacuolated epithelium was negative. Furthermore, the proximal tubules in nontumorous areas also expressed NR-2 antigen, suggesting that the hyperplastic lesions which eventually lead to renal cell tumors may derive from epithelia of proximal tubules and not directly from vacuolated epithelium. Such NR-2 antigen-positive epithelia of proximal tubules seem to be initiated cells. NR-2 MAb also cross-reacted with preneoplastic liver lesions.

Key words: Monoclonal antibody — Renal cell tumor — Tumorigenesis — Rat

Although many studies dealing with chemically induced renal tumors have been reported,[1–8] the histogenesis of early lesions is still not understood. Analyses by histochemical, immunohistochemical, morphological and biochemical methods have indicated that cytoplasmic vacuolation of the proximal convoluted tubular epithelium might be involved in the histogenesis of renal tumors in NTA-treated rats.[1, 2, 9, 10] However, there has been no convincing evidence derived from these studies. The study of the origin of early lesions during renal tumorigenesis has been seriously hampered by the lack of specific markers that identify them and permit in-depth analysis. Recently we have shown that rat renal cell tumors exhibited several new antigenic components which were either lacking or undetectable in the control kidney.[6] In the present study, we have purified one of the antigenic components and prepared a monoclonal antibody against this antigen. The present studies deal with the purification, partial characterization, preparation of monoclonal antibody and application of monoclonal antibody for immunohistochemical analyses of the histogenesis of renal tumors in rats treated with EHEN and NTA. Since there is no direct shift from vacuolated epithelium to the preneoplastic lesions, our results indicate that the hyperplastic lesions may derive from epithelium of proximal tubules and not directly from vacuolated epithelium.

The abbreviations used are: NTA, trisodium nitrilotriacetate monohydrate; BCS, bovine calf serum; FCS, fetal calf serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; NR-2, Nara renal cell tumor; MAb, monoclonal antibody; EHEN, N-ethyl-N-hydroxyethylnitrosamine; ELISA, enzyme-linked immunosorbent assay.

MATERIALS AND METHODS

Induction of primary tumors, transplantation and cell culture The regimen of induction of primary renal cell tumors was described previously.[6] Briefly, inbred male Wistar rats were fed on a basal diet containing 1,000 ppm of EHEN (Nakarai Chem., Japan) for two weeks followed by an sc injection of β-cyclodextrin (Nakarai Chem.), at a dose of 45 mg/100 g body weight once a day for 7 days. A small pale tumor in the renal cortex developed after 32 weeks.

Twelve newborn rats of inbred Wistar strain were given sc implants of the minced tumor under cold anesthesia. The tumors thus developed in the animals were passed by serial transplantations in newborn rats. At the same time, we established a cell line from those tumors which was maintained in Eagle's MEM medium (Nissui Pharama, Japan) with 10% BCS (Flow Lab., Australia).

For study of the histogenesis of renal tumors, we treated the rats with EHEN and NTA according to the regimen described previously.[8] The paraffin-embedded tissues were prepared for immunohistochemical studies.[8]

Preparation of tumor extracts Cultured cells of renal cell tumor were pooled, minced and homogenized with 2.5 volumes of cell lysis buffer containing 50 m*M* Tris-hydrochloride (pH 7.4), 0.15 *M* NaCl, 0.5% Triton X-100, 0.5% deoxycholate and 1 m*M* phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 10,000 rpm for 20 min in a Kubota KR/180B rotor.

Protein was estimated by the method of Lowry *et al.*[11] using bovine serum albumin as the standard.

Antigen purification Renal cell tumor extracts were sequentially chromatographed through DEAE-Sephadex A-50, Sephadex G-100, and Con A-Sepharose affinity columns. The buffer systems and elution conditions were as detailed previously.[12] Three ml fractions were collected and protein profiles were monitored at 280 nm with a Perkin-Elmer 55B spectrophotometer. The presence of tumor-associated antigens in each purification step was monitored by analyzing an aliquot of the samples by SDS-polyacrylamide gel electrophoresis.[13]

Production of monoclonal antibodies Six-week-old male BALB/c mice were immunized by intrasplenic injection with approximately 20 μg of partially purified tumor antigens in 50 μl of PBS.[14] The mice were killed three days after injection and fusion was done with myeloma cell P3-X63-Ag 8.653 by the method of Köhler and Milstein[15] with a slight modification as reported previously.[8] Two weeks after the fusion, the supernatant from the wells was analyzed for antibody production by the avidin-biotin-complex-immunoperoxidase technique (ABC method) described below. After cloning by limiting dilution and expanding hybridoma cells, ascites fluid was produced. The ascites fluid was purified using a Sephacryl S-300 column equilibrated in PBS. The purity of the antibody was checked by Ouchterlony immunodiffusion assay.

Immunohistochemical staining Immunohistochemical staining was performed by the ABC method[16] according to the manufacturer's protocol (Vectastain ABC Kit, Vector Lab. Co., Ltd.). Briefly, monoclonal antibodies diluted at 1: 200 to 1: 500 were first incubated with the specimens for 1 h at room temperature, followed by biotinylated secondary antibody and then a preformed avidin-biotinylated horseradish peroxidase complex. The antigen was visualized by incubation in a peroxidase substrate solution.

Two-dimensional electrophoresis Two-dimensional polyacrylamide gel electrophoresis, using isoelectric focusing electrophoresis as the first dimension and SDS in the second dimension, was performed by the method of O'Farrell.[17] Samples of 100 μl containing 300 μg of protein were placed on the top of a rod gel. 2-D Pharmalite (Pharmacia Fine Chem., Sweden), with a pH range of 3 to 10, was used. The gels were run at 2 mA for 4 h, then removed from the rod tubes. A 1% agarose gel was used to keep the cylindrical gel on the 7.5% slab gel.

Western blot of protein After electrophoresis, the separated proteins were transferred to a nitrocellulose sheet and the specific antigen was localized by enzyme-linked immunosorbent assay as described.[18]

RESULTS

Partial purification of tumor-specific antigens and production of monoclonal antibody Since there was no associated reagent that allowed identification of the tumor-associated antigens, at each step of column chromatography we employed polyacrylamide gel electrophoresis to monitor the presence of antigens of interest. The results, which demonstrated several striking differences in the composition of total cellular proteins between control kidney cells (lane A) and renal cell tumors (lane B) before purification, are shown in Fig. 1. The conspicuous differences were the presence in renal cell tumors of at least 13 polypeptides with molecular weights ranging from 20K to 220K (indicated by circles) which were either lacking or undetectable in control kidney cells. The total tumor cell extracts were first

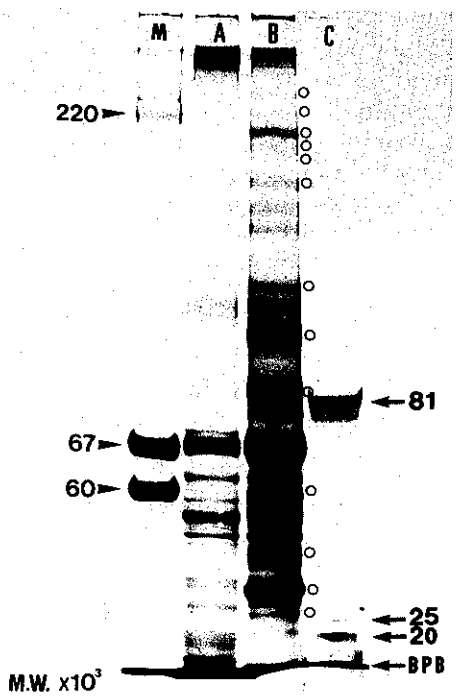

Fig. 1. Electrophoretic analysis of cellular proteins on polyacrylamide gel. After electrophoretic separation, the protein bands were visualized by staining with Coomassie blue. M, molecular weight, standard; A, control kidney; B, renal cell tumors; C, partially purified proteins from renal cell tumors.

chromatographed through DEAE-Sephadex A-50. Three different polypeptides with molecular weights of 81K, 25K and 21K were clearly eluted with some other proteins at a salt concentration of 0.15 *M*. Fractions containing 81K, 25K and 21K polypeptides were pooled and further chromatographed through a Con A-Sepharose affinity column. These three polypeptides (81K, 25K and 21K) were eluted from nonabsorbed fractions (Fig. 1, lane C), indicating a nonglycoprotein nature. It should be noted that these three polypeptides were slightly shifted in mobility due to distortion of the gel in this lane (C) as evidenced by the slight retardation of the bromophenol blue dye front (BPB).

The partially purified tumor-associated antigens were used as the immunogen for production of monoclonal antibodies according to the methods that we reported previously.[8] Several hybridoma lines producing MAb against renal tumor antigens were obtained. All MAbs reacted with anti-mouse IgM but not with other anti-mouse immunoglobulins and formed a precipitin line in Ouchterlony immunodiffusion assay. One of the lines producing MAb, designated NR-2 MAb, was selected for further characterization because of its strong reactivity with renal tumor cells.

Biochemical characterization of NR-2 MAb To identify and characterize the polypeptides that react with NR-2 MAb, we employed the combination of two-dimensional polyacrylamide gel electrophoresis and Western blotting-ELISA techniques. NR-2 MAb recognized a polypeptide of 81K molecular weight with an isoelectric point at pH 4.6 (pI=4.6), as is clearly shown in Fig. 2. This polypeptide is a nonglycoprotein as judged by its inability to bind to a Con A-Sepharose column and to be stained by periodic acid-Schiff reagent.

Microscopic observation Histological changes of renal cortex in the development of renal cell tumor were divided the following four categories as described by Alden and Kanerva[2] and Merski.[9] Vacuolated epithelium consisted of epithelium with cytoplasmic vacuolation.

Simple hyperplasia was composed of proliferative epithelium with clear cells or basophilic cells. Proliferation of the epithelial cells was not marked and therefore tubular structure was retained. Adenomatous hyperplasia, in contrast, was often delineated from the surrounding parenchyma. Cells in these foci in comparison with the cells in the simple and adenomatous hyperplasia were hyperchromatic.

The lesions diagnosed as renal cell tumors were composed of irregularly arranged cells forming either solid or papillary structures with occasional mitotic figures and central necrosis.

Reactivity of NR-2 MAb with renal tissues and tissues in other organs NR-2 MAb was used as a probe to analyze the histogenesis of renal cell tumors with regard to the expression of this antigen. Immunohistochemically, no staining could be detected in any of the vacuolated epithelia presenting from the most to the least severe cytoplasmic vacuolation (Fig. 3). In contrast, strong cytoplasmic staining was observed in both clear cells (Fig. 4) and in basophilic cells (Fig. 5) of the simple hyperplasia. Furthermore, adenomatous hyperplasia and renal cell tumors reacted strongly with NR-2 MAb (Fig. 6). Interestingly, NR-2 MAb also showed a positive reaction with proximal epithelial cells in nontumorous areas. Such epithelial cells were either unchanged or slightly degenerative (Fig. 7a and 7b).

To determine whether NR-2 MAb cross-reacts with cellular components of different origins, we screened the normal tissues and carcinomas in various organs of rats. Approximately 58% of the hyperplastic foci of the liver sections were stained positively by NR-2 MAb, as illustrated in Fig. 8. In normal tissues, the ducts of the salivary glands (30%) and sebaceous glands of the skin (90%) also showed positive reaction with NR-2 MAb (Table I).

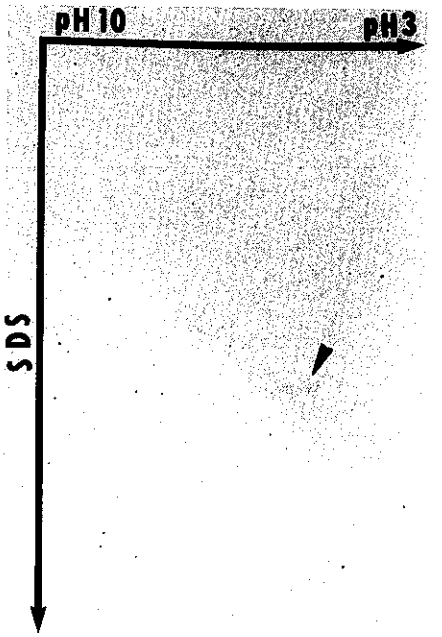

Fig. 2. Identification and biochemical characterization of renal cell tumor-associated antigen by monoclonal antibody NR-2 MAb. Renal cell tumor extracts were subjected to two-dimensional polyacrylamide gel electrophoresis followed by transfer onto a nitrocellulose sheet as detailed in the text. The renal cell tumor-specific antigen recognized by NR-2 MAb was localized by enzyme-linked immunosorbent assay as described.[19] The antigen identified is a polypeptide of molecular weight 81,000 with a pI of 4.6.

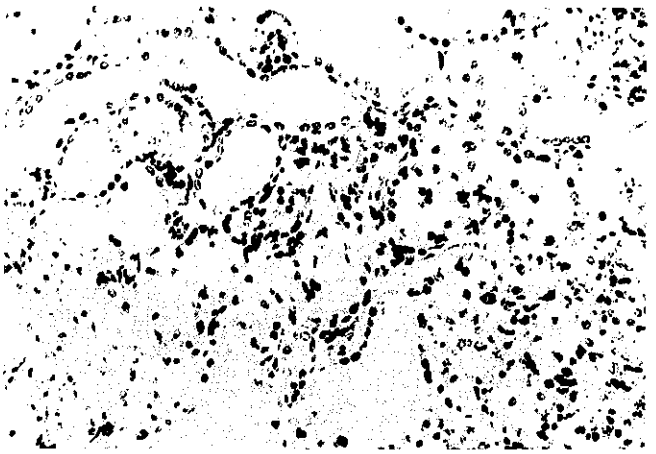

Fig. 3. Immunohistochemical staining of the vacuolated epithelium with NR-2 MAb according to the ABC method.[11] The specimens were first incubated with NR-2 MAb, followed by a biotinylated secondary antibody and then a preformed avidin-biotinylated horseradish peroxidase complex. The presence of antigen was visualized by incubation in a peroxidase substrate (0.1% diaminobenzidine tetrahydrochloride) solution.

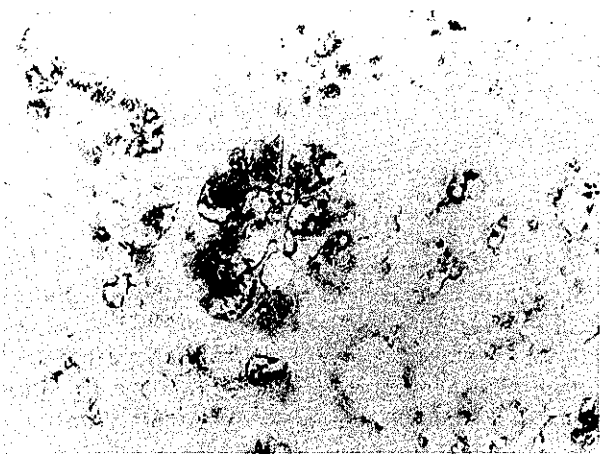

Fig. 4. Immunohistochemical staining of simple hyperplasia of clear cells with NR-2 MAb. A strong cytoplasmic staining was observed. The specimens were counterstained with methyl green. ×200.

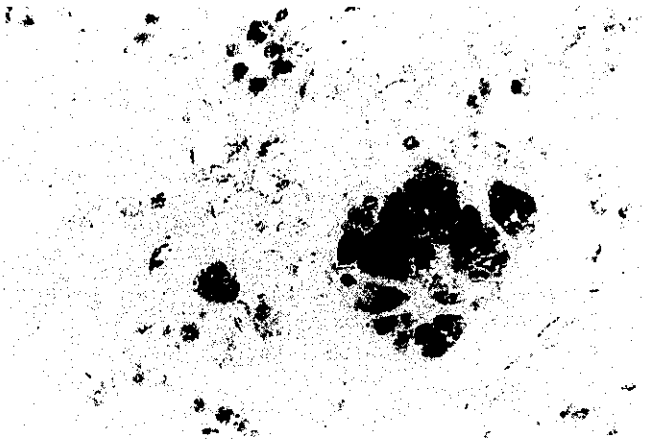

Fig. 5. Immunohistochemical staining of simple hyperplasia of basophilic cells with NR-2 MAb. Note the strong staining in the cytoplasm. Counterstained with methyl green. ×200.

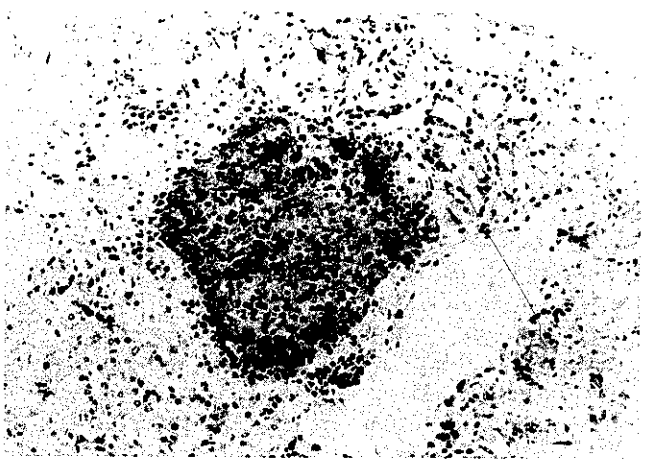

Fig. 6. Immunohistochemical staining of adenomatous hyperplasia with NR-2 MAb. A strong positive staining was observed. Counterstained with hematoxylin. ×100.

DISCUSSION

The development of monoclonal antibodies against renal cell tumors has allowed us to dissect sequentially the pathological and cellular events leading to the formation of renal tumors induced by chemical carcinogens. One of the monoclonal antibodies, NR-2 MAb, that reacts with a renal tumor-associated antigen (polypeptide of 81,000 deltons) was used to probe the histogenesis of renal adenocarcinoma in chemically induced rats. With immunohistochemical staining techniques we have found that NR-2 MAb reacted equally well with simple hyperplasia, adenomatous hyperplasia, and renal cell tumors, despite the different lesions induced by different chemicals. Both clear cells and basophilic cells of the simple hyperplasia showed positive reactions, whereas the vacuolated epithelium was negative. In addition, the proximal tubules in nontumorous areas also expressed NR-2 antigen. These data not only demonstrate a close

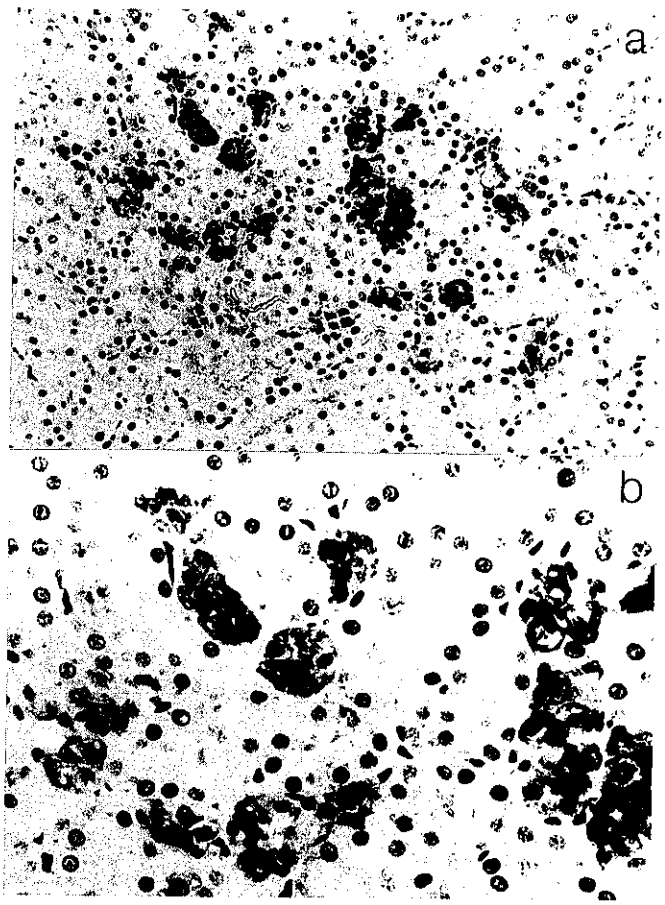

Fig. 7. Immunohistochemical staining of proximal epithelial cells in nontumorous areas with NR-2 MAb. A positive reaction was observed in both unchanged or slightly degenerative proximal epithelium. Counterstained with hematoxylin. a, ×200; b, ×400.

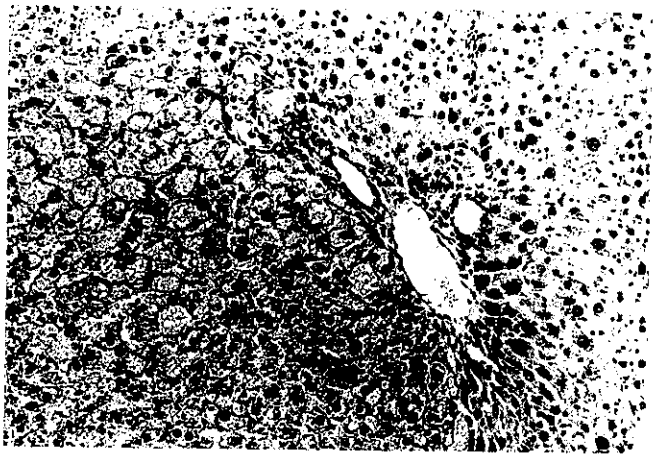

Fig. 8. Immunohistochemical staining of hyperplastic liver foci with NR-2 MAb. A weak but clearly positive staining was observed. Counterstained with hematoxylin. ×200.

Table I. Reactivity of NR-2 MAb with the Normal Tissue and Carcinomas in Various Organs of Rats

Normal tissues	Reactive no. examined	Carcinomas	Reactive no. examined
Thyroid gland	0/10	Esophagus	
Salivary gland	3/10	squamous cell carcinoma	0/13
Esophagus	0/10	Thyroid gland	
Stomach	0/10	papillary carcinoma	0/2
Colon	0/10	follicular carcinoma	0/9
Liver	0/10	Lung	
Pancreas	0/10	adenocarcinoma	0/12
Spleen	0/10	Liver	
Lung	0/10	hepatoma &	
Heart	0/10	hyperplastic foci	7/12
Adrenal gland	0/10	Mammary gland	
Urinary bladder	0/10	adenocarcinoma	0/3
Testis	0/10	papillary carcinoma	0/3
Skin (sebaceous gland)	9/10	Urinary bladder	
Cerebrum	0/10	transitional cell	
Fetal kidney	0/8	carcinoma	0/7

Individual tissues were designated as positive when NR-2 MAb reacted in at least 10% of cells.

relationship among simple hyperplasia, adenomatous hyperplasia, and renal cell tumor, but also suggest that there is no direct shift from vacuolated epithelium to the preneoplastic lesion. Although vacuolation may produce cell death by rupturing the luminal membrane[19] and cause cell damage which, in turn, may develop into hyperplastic lesions,[10] our data have clearly shown that these vacuolated epithelia did not express NR-2 antigen. Thus, our results do not support the postulated causal relationship between NTA-associated tubular toxicity and tumorigenicity.[2]

The tentatively assigned term "clear cell type," which we have defined here, corresponds to "hyperplasia of vacuolated type."[2, 9] Our data revealed that the clear cell type and the basophilic cell type of simple hyperplasia gave similar positive reactions with the NR-2 MAb. Accordingly, these findings suggest that the clear cell is not cytoplasmic vacuolation since the vacuolated epithelium does not react with NR-2 MAb. These data, taken together, indicate that hyperplastic lesions which eventually achieve autonomous growth may arise from apparently initiated epithelia of proximal tubules which express NR-2 antigen, and not directly from vacuolated epithelium.

It is interesting to note that the NR-2 MAb reacted with preneoplastic lesions of the liver (58%), whereas no other neoplasia could be recognized by this monoclonal antibody. In the present study, it is not known whether the renal tumor-associated antigen (81K polypeptide with a pI=4.6) recognized by NR-2 MAb shares some common antigenic determinant sites with the preneoplastic antigen (74K polypeptide with a pI=4.75) expressed in hyperplastic liver nodules and hepatoma.[12]

This is the first report of an immunohistochemical demonstration of the histogenesis of experimental renal cell tumors in rats using monoclonal antibody. Such a study in an experimental animal model should provide information which may eventually make it possible to understand tumorigenesis of renal cell tumors in humans.

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