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H-*ras* p21 AND PEANUT LECTIN IMMUNOREACTIVITY OF HYPERPLASTIC, PRENEOPLASTIC AND NEOPLASTIC URINARY BLADDER LESIONS IN RATS

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Hyperplastic, preneoplastic and neoplastic urinary bladder lesions induced by bladder carcinogens and toxins in the rat were evaluated for immunoreactivity with polyclonal or monoclonal antibodies to H-*ras* p21 or binding to peanut lectin with avidin-biotin immunocytochemistry. A low proportion (<20%) of hyperplastic and neoplastic bladder lesions induced by N-butyl-N-(4-hydroxybutyl)-nitrosamine and fixed in Bouin's fixative only were immunoreactive on the cell membrane with the antibodies to H-*ras* p21. Lectin binding was found for these lesions, as well, even in formalin-fixed tissue and for lesions induced by other carcinogens, but not in regenerative bladder hyperplasias after cyclophosphamide exposure or in bladder exposed to bladder tumor promoters. The latter lesions were also not immunoreactive with antibodies to p21. Our results suggest that this relatively simple technique might be used for identification and screening of tumors for involvement of *ras* oncogenes and carcinogen initiation.

Key words: H-*ras* p21 — Peanut lectin — Rat bladder — Bladder cancer

Oncogene activation or involvement has been demonstrated for a variety of human and rodent tumors.[1,2] An original human bladder cell line, T24, contained H-*ras* activated by point mutation[3] and a *ras*-related protein was found in urine of bladder cancer patients.[4] Activated oncogenes have been found in malignant and benign tumors of rodents but not usually in hyperplastic or preneoplastic tissue, although modulation of oncogene expression can be found in these lesions and tumors.[1,2,5,6] Immunocytochemical demonstration of oncogene protein products in tumors has been described[7,8] and their increased expression may contribute to carcinogenesis.[1] The protein product of H-*ras*, p21, can be readily demonstrated in tissue sections on the cell surface of mouse Harvey virus-induced sarcoma cells with an activated H-*ras* gene.[7] Lectin agglutinability, especially to concanavalin A, has been shown to be a characteristic of rat bladder cells after exposure to carcinogens.[8–13] Also, blood group precursor T-antigen detection by immunoreactivity with peanut lectin was shown in human bladder carcinoma.[14] We have utilized the avidin-biotin-peroxidase complex (ABC) immunohistochemical technique[15] to demonstrate immunoreactivity of antibodies to oncogene proteins and peanut lectin binding in preneoplastic and neoplastic urinary bladder lesions after exposure to carcinogens but not in regenerative hyperplastic lesions after exposure to bladder toxins or tumor promoters.

Six F344 rats (Charles River Japan, Inc., Kanagawa) were exposed to N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) at 500 ppm in the drinking water for 12 weeks.[16,17] At 50 weeks, the bladder was fixed in Bouin's or formalin fixative and embedded in paraffin. Five Harvey sarcoma virus-induced sarcomas in BALB/c mice, fixed in Bouin's fixative, were also studied.[7] Tissue sections were stained for H-*ras* p21 using a 1:25, 1:50 or 1:100 dilution of rabbit polyclonal anti-*ras* p21 pan-reactive antibody to a peptide comprising amino acid positions 29–44 of H-*ras* p21 or a pan-reactive H-*ras* p21 mouse monoclonal antibody, prepared by immunization of mice against the same peptide (Cetus Corp.,

Abbreviations used are: ABC, avidin-biotin-peroxidase complex; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; FANFT, N-[4-(5-nitro-2-furyl)-2-thiazolyl]formamide; BBS, barbital sodium; PB, phenobarbital sodium; CP, cyclophosphamide.

Emeryville, CA).[18] Controls included normal rabbit serum to background staining and omission of the primary antibody. The rabbit Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA) was used with 3,3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen.[7,15] Additional bladder lesions were obtained at various intervals up to 68 weeks from 4–7 F344/NCr rats each fed the bladder carcinogen, N - [4 - (5 - nitro - 2-furyl)-2-thiazolyl] formamide (FANFT) at 2000 ppm for 6 weeks or the bladder promoter barbital sodium (BBS) at 1000 ppm or phenobarbital sodium (PB) at 500 ppm. All these tissues were fixed in formalin. Eight 6-week-old F344/NCr rats were injected ip with cyclophosphamide (CP) at a dose of 100 mg/kg and sacrificed at day 7, 14, or 21. Appropriate untreated controls were used. Their bladders were fixed in Bouin's fixative or formalin. For demonstration of peanut lectin binding *in vivo,* we used a biotinylated peanut lectin (agglutinin) (Vector Laboratories) at a 1:100 dilution and a Vectastain kit without the biotinylated secondary antibody. Controls included omission of the biotinylated lectin.

Immunoreactivity with antibodies to H-*ras* p21 was readily demonstrated focally in less than 20% of bladder lesions in 4 of 6 rats with multiple BBN-induced bladder hyperplasias (simple and papillary-nodular), papillomas and carcinomas fixed in Bouin's fixative but not formalin. In multiple cell foci within the hyperplastic or neoplastic lesions, the p21 immunoreactivity was seen on the cell surface and in the cytoplasm (Fig. 1), especially in better-differentiated tumors. Less than 1% of the tumor cells were reactive in any specific lesion, however. Positive staining was defined as intense immunoreactivity (brown color with DAB chromogen) compared with no nonspecific background staining. All Harvey virus-induced sarcomas were also highly reactive on the cell membrane and cytoplasm (Fig. 2).[7,8] Antibodies were used at dilutions as noted which produced no background (non-

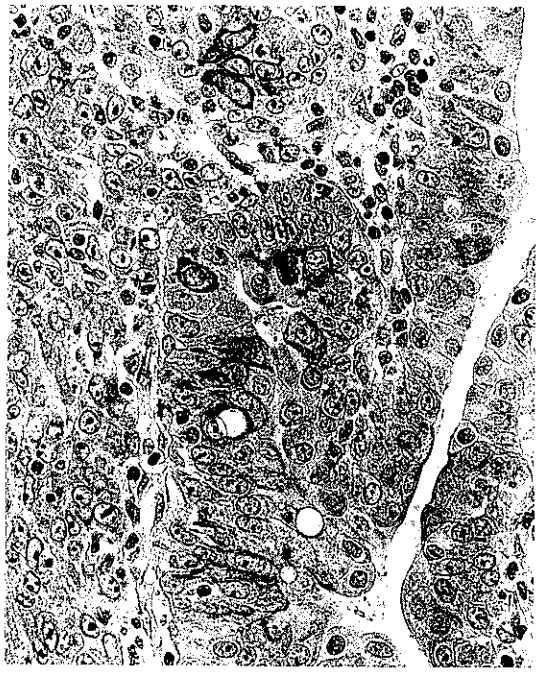

Fig. 1. H-*ras* p21 immunoreactivity on the cell surface of some BBN-induced bladder carcinoma cells in a tumor mass (top) and overlying neoplastic urothelium (middle and bottom). ABC immunocytochemistry, hematoxylin. ×400.

Fig. 2. H-*ras* p21 immunoreactivity with cell membranes and cytoplasm of Harvey virus-induced sarcoma in a mouse. ABC immunocytochemistry, hematoxylin. ×250.

specific) staining. Regenerative bladder hyperplasias induced by cyclophosphamide from 7 to 21 days and normal urothelium fixed in Bouin's fixative were not immunoreactive. The hyperplasias were diffuse and associated with bladder hemorrhage or ulceration and appeared generally different morphologically from those induced by BBN. Formalin-fixed bladder tumors or hyperplasias induced by BBN, FANFT, BBS, or PB were not reactive either. Bouin's fixed lesions were not available for these studies.

Peanut lectin binding was found in the majority of papillary-nodular hyperplasias and papillomas or carcinomas induced by either FANFT (6/6 rats) or BBN (6/6 rats), even in formalin-fixed tissue. Focally, large and small areas of the lesions were immunoreactive and staining was seen on the cell surface and in the cytoplasm (Fig. 3), especially in squamous areas. Cell surface staining was not as sharp as p21 immunoreactivity. Regenerative hyperplasias induced by CP did not show lectin binding in Bouin's or formalin-fixed tissues. Urothelium of rats treated with the promoter BBS or PB only or of control rats also was not reactive.

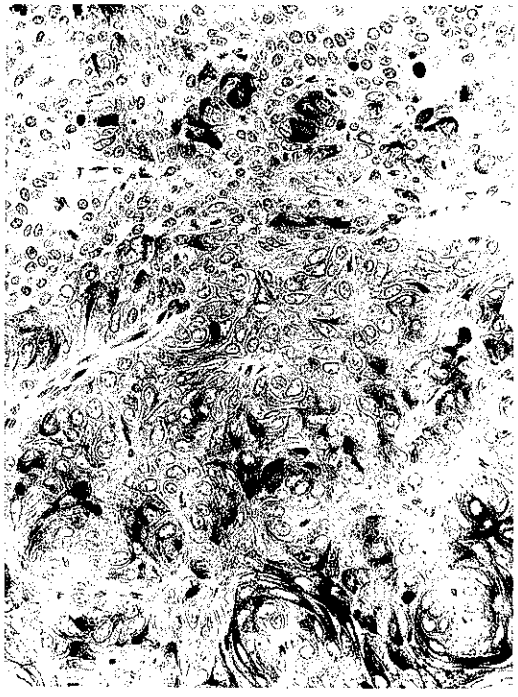

Fig. 3. Peanut lectin binding to metaplastic squamous area (bottom) and transitional cell area (top) of bladder carcinoma induced by BBN. ABC immunocytochemistry, hematoxylin. ×250.

Thus, we have shown that H-*ras* p21 and peanut lectin binding were found in preneoplastic and neoplastic bladder lesions but not in regenerative hyperplasias. This technique may be useful to differentiate these processes and also to provide evidence of oncogene involvement in a tumor, if the proper fixative is employed.

We have previously demonstrated that the fixative is important for immunoreactivity of cell surface antigens in fixed tissue sections.[7,15] The localization of H-*ras* p21 in preneoplastic bladder lesions suggests that enhanced p21 expression occurred as an early stage in carcinogenesis, as suggested in mouse liver adenomas[19] and skin papillomas.[20] Activation, increased expression, or amplification of *ras* genes may have occurred in our bladder tumor model since we used anti-*ras* p21 pan-reactive antibodies to a region of H-*ras* p21 which is not a common site of mutation. We cannot determine the cause of the enhanced p21 immunoreactivity, i.e., involvement of K-*ras*, H-*ras*, or N-*ras*. Characterization of these potential causes of p21 immunoreactivity is in progress. The lectin binding activity of the same preneoplastic and neoplastic lesions could be due to altered cell surface glycoproteins in these carcinogen-exposed cells,[13] but only in those rats exposed to agents that are carcinogens, not solely tumor promoters or toxins. Similar lectin binding findings have been reported in human tumors.[21–23]

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