ras Gene alterations in invasive and non-invasive rat bladder carcinomas
induced by N-methyl-N-nitrosoare

Y. Yura, M. Azuma, K. Uchida, H. Momose & R. Oyasu

Department of Pathology, Northwestern University Medical School, Chicago, Illinois 60611, USA.

Summary We have established a reliable method to induce invasive and non-invasive carcinomas in the heterotopically transplanted urinary bladder of rats by repeated injection of N-methyl-N-nitrosoare (MNU), and examined the alterations of the ras oncogene and ras oncogene product (p21) in the induced tumours. The incidence of muscle-invasive carcinomas was proportional to the total dose of MNU. When 5, 6 or 12 doses of MNU were used, muscle invasive carcinomas developed in 22, 58 or 45% of animals, respectively, after a mean observation period, respectively, of 54 ± 9, 45 ± 13 and 38 ± 3 weeks. Whereas activated H-ras gene was detected in only one non-invasive carcinoma by DNA transfection assay, seven of 18 non-invasive and invasive carcinomas showed activated ras p21 when examined by immunoblot analysis. Amplification or rearrangement of myc or epidermal growth factor (EGF) receptor gene was not observed. The results indicate that alterations of ras gene may be involved in the development of rat bladder carcinomas but not of invasiveness.

Human bladder cancer can be divided into two types; a majority of them are low-grade papillary carcinomas only superficially invasive, whereas as many as 20% of tumours are deeply invasive potentially lethal carcinomas (Kaye & Lange, 1982; Brawn, 1982). When human bladder cancers were tested for transforming activity using NIH3T3 cells, activated H-ras was demonstrated in approximately 10% of randomly selected tumours (Fujita et al., 1984; Fujita et al., 1985), and there was no correlation between H-ras activation and the degree of invasiveness of tumours. As in human carcinomas, the rate of ras oncogene activation is infrequent in carcinogen-induced rat bladder carcinomas (Fujita et al., 1988; Debiec-Rychter et al., 1989). Thus the significance of ras gene alterations in the development of urinary bladder cancer in general and of deeply invasive carcinoma in particular remains unclear. Though several models are available for the induction of rat urinary bladder cancer, the frequency of deeply invasive carcinoma is low and requires an extended period of observation (Kunze, 1979). In this study, the heterotopically transplanted rat urinary bladder system (HTB) was used for the induction of urinary bladder carcinomas. It was developed in our laboratory to study the role of urine on bladder carcinogenesis (Oyasu et al., 1976; Oyasu et al., 1978). The system is also suited to test the effect of topically applied carcinogen because urinary tract infection and subsequent calculus formation, the two common complications which frequently occur after repeated intravesical administration of test substances can be avoided with the HTB system. Though useful for testing direct effects of test compound on the bladder mucosa, disadvantages include meticulous care of the HTB to avoid infection and the fact that periodic spontaneous emptying (micturition) cannot be expected to occur. We had two objectives, to establish a reliable method to induce non-invasive and deeply invasive carcinomas after MNU administration and to examine the alterations of the ras genes and ras gene product (p21) in these tumours.

Materials and methods

Induction of carcinomas in heterotopically transplanted bladder (HTB)

The HTB system was established in male Fischer 344 rats by the published method (Oyasu et al., 1976; Oyasu et al., 1978; Babay et al., 1982). In brief, a bladder taken aseptically from donor rat was connected to a reservoir (Babay et al., 1982) through an intervening silastic tubing. The bladder-reservoir unit was then transplanted into a syngeneic recipient in such a way that the bladder portion was placed within the gluteal muscle and the reservoir portion was in the dorsal subcutaneous tissue. The skin incision was closed with metal clips. Four weeks after transplantation of urinary bladder, recipients in the first two groups received instillation into HTBs 0.5 mg of N-methyl-N-nitrosoare (MNU) (ICN Pharmaceutical, NJ) dissolved in 0.5 ml of physiologic saline once a week for 2 weeks (Figure 1). Since the HTB system is a blind pouch, the injected material will not be lost by micturition, but will be absorbed through the mucosa. Therefore MNU will be taken up by the urothelial cells exerting genotoxic effects. The compound is alkali labile, and carcinogenic effects on other organs are not expected to take place. After 20 weeks, group 2 rats received 0.5 mg of MNU for three consecutive weeks. Group 3 and 4 rats received 0.5 mg of MNU once a week for 6 weeks. After 16 weeks, group 4 rats received additional 6-weekly doses of 0.5 mg MNU. Ten weeks after bladder transplantation, all HTBs received 0.5 ml of normal sterilised rat urine once a week until termination of the experiment. The injected urine is expected to be completely absorbed in 48 h (Hirao et al., 1980). Rats were allowed to live until a majority of the HTBs in the group became markedly distended and the bladder aspirate haemorrhagic. These changes were indicative of tumour development (Oyasu et al., 1976; Oyasu et al., 1978). When large, tumours were divided into four parts, one part used for transplantation in nude mice, a second for explant culture, a third for storage at ~80°C for DNA extraction and the fourth for light microscopic examination. Small tumours were submitted for histologic examination only. Tumours were classified by grade, stage, and histologic type (Oyasu et al., 1987) (see also Table 1 for definition).

Explant culture

Tumour tissue was minced into 1 mm3 pieces and placed in Petri dishes coated with rat tail collagen gel and with a small amount of Ham's F-12 (GIBCO, Grand Island, NY) supplemented with 10% foetal bovine serum, penicillin (100 u ml-1) and streptomycin (100 µg ml-1), and incubated in a humidified atmosphere of 5% CO2 and 95% air. Outgrowths of carcinoma cells were placed on new collagen gel-coated dishes to establish secondary growth. This procedure was repeated twice to remove fibroblast contamination. When a monolayer of carcinoma cells was obtained, the culture was passaged to new dishes. NIH3T3 cells were grown in Dulbecco's
modified essential medium supplemented with 10% calf serum (Flow Laboratories, McLean, VA).

**DNA transfection assay**

DNA extracted (Andersson et al., 1979) from tumour cells was transfected to NIH3T3 cells by the published method (Andersson et al., 1979). Control DNA was obtained from five normal rat urinary bladders.

**Southern blot hybridisation**

DNA (10–20 μg) isolated from tumours on cell lines was digested with BamHI, HindIII or EcoRI under the conditions recommended by the manufacturer (Boehringer Manheim, Indianapolis, IN). The resulting DNA fragments were separated by gel electrophoresis and immobilised on a nitrocellulose membrane. Southern blot hybridisation was performed under stringent conditions (50% formamide, 5 × SSC and 42°C) with 32P-labelled probes (3.0 × 10^6 c.p.m.) obtained by nick translation (Rigby et al., 1977). The probes used were v-H-ras, v-K-ras, v-myc (Oncor, Gaithersburg, MD), p51C- and pHER A64-1 (ATCC, Rockville, MD).

**Immunoblot**

Protein was extracted from tumour cells (Meyers et al., 1989) and ras p21 was concentrated by immunoprecipitation (Finkel et al., 1984) with rat monoclonal antibody Y13-259 (Oncogene Science, Inc., Manhasset, NY), which recognises the products of normal or activated H-ras, K-ras and c-N-ras (Furth et al., 1982). Immunoprecipitates were collected by centrifugation, washed, boiled in sample buffer, and resolved by SDS-polyacrylamide electrophoresis (Laemmli, 1970). Following transfer to nitrocellulose membranes (Towbin et al., 1979) and subsequent blocking with bovine serum albumin in PBS, membranes were incubated with the following antibodies; Y13-259, mouse monoclonal antibodies recognising the twelfth position substitutions with valine (DWP), arginine (R256), glutamic acid (E184) (DuPont, Boston, MA) or classmatched myeloma control proteins MOPC-141 (IgG2b) (Litton Bionetics, Charleston, SC) and MOPC-21 (IgG1) (Cappel, West Chester, PA). As positive controls, T24 human bladder cancer cells (glycine to valine at codon 12), S2-721 cells (NIH3T3 cells transformed by a rat H-ras oncogene activated by a GGA→GA mutation in codon 12, encoding glutamic acid) and 118–413 cells (NIH3T3 cells transformed by a human K-ras oncogene activated by a GGT→CGT mutation in codon 12, encoding arginine) were used. Membranes were then incubated with either rabbit anti-rat horseradish peroxidase or biotinylated anti-mouse horseradish peroxidase, and then incubated with diaminobenzidine substrate to complete the reaction.

**Results**

**Incidence of carcinomas in HTBs**

The treatment with 12 doses (group 4) yielded the highest tumour incidence (100%) despite shorter study period (38 ± 3
weeks) (Table I). The MNU dose of more than 3 mg was effective in inducing deeply invasive carcinomas (P2 and 3) and their incidences in groups 3 (0.5 mg × 6) and 4 (0.5 mg × 12) were, respectively, seven of 12 and nine of 20. The tumours observed in group 1 (0.5 mg × 2) were all small and non-invasive. These results together with our previous findings (Oyasu et al., 1987) indicate that deeply invasive carcinomas can be induced with 3 mg of MNU, that the induction period can be shortened by repeating the same treatment schedule, and that most of the invasive carcinomas show squamous differentiation. No metastasis to regional lymph nodes or distant organs was observed.

**Growth potential and histological features of rat bladder carcinomas transplanted into nude mice**

Since tumours observed in group 1 were small, only tumours grown in groups 2, 3 and 4 were used as transplants. There were 21 non-invasive and eight deeply invasive (P2 and 3) carcinomas. The growth of non-invasive (NI) carcinomas was slow; ten of 21 transplants which had attained more than 1 cm in diameter could be serially transplanted at an interval of 10 to 15 weeks (designated as NI-1 to 10). They were sharply demarcated cystic masses containing clear serous fluid and one or two papillary nodules. Microscopic examination generally confirmed transitional cell character and papillary growth pattern. Eight invasive carcinomas (designated as I-1 to 8) grew rapidly without exception and could be passed at an interval of 4 to 11 weeks. Despite the sharp circumscription they were well differentiated squamous carcinomas invasive to adipose and skeletal muscle tissues. Transplants in general maintained their original morphologic and grade. Focal glandular differentiation (adenocarcinoma) occurred in two tumours.

**Figure 2** Detection of H-ras sequences in NIH3T3 cells transformed by genomic DNA from MNU-induced rat bladder carcinoma. Hybridisation was carried out with a v-H-ras probe. Lane a, NIH3T3 cells, lanes b and c, NIH3T3 transformants derived from non-invasive carcinomas (NI-10 and NI-13, respectively). Molecular weight markers are shown on the left margin.

Two low-grade tumours adapted to grow in vitro, NI-11 and NI-12 (D-44), were not tumorigenic, whereas cultured cells derived from an invasive squamous cell carcinoma (I-8) developed into a squamous cell carcinoma.

**Detection of activated ras oncogenes**

High-molecular weight DNA was prepared from 13 primary tumours and four nude mouse transplants. These included seven invasive and ten non-invasive carcinomas. Seven
samples developed foci. DNA derived from these foci were subjected to Southern blot analysis using ras gene probes. Neither K-ras nor N-ras probe detected DNA fragments other than the endogenous mouse fragments (data not shown), but one of the seven transformants (NI-10) contained additional DNA bands that hybridised with the H-ras probe (Figure 2, lane b). NI-10 was derived from a grade I PO transitional cell carcinoma.

Amplification of H-ras, K-ras, myc and EGF receptor gene was examined in ten primary tumours (five invasive and five non-invasive) and five transplants (two invasive and three non-invasive) and two cell lines (NI-11 and NI-12). Of these samples, non-invasive tumours and three transplants were also used in DNA transfection assay. No amplification was demonstrated in any sample including NI-10, which contained activated H-ras gene.

Detection of activated ras p21 by immunoblot

All of the 18 tumour samples tested demonstrated p21 at various densities (Figure 3). Seven of 18 carcinomas contained activated ras p21 in reaction with monoclonal antibody E184 which specifically recognises the mutation from glycine to glutamic acid at codon 12. The protein extract of NI-10 which was shown to contain activated H-ras gene by Southern blot analysis also expressed activated ras p21. No reactivity was demonstrated with monoclonal antibodies DWP and R256 or negative controls MOPC-21 and 141 (data not shown).

Discussion

In the present study, we not only confirmed the previous observation that the frequency of deeply invasive carcinomas was proportional to MNU dose, but that the highest dose schedule (0.5 mg x 12) was able to shorten the induction period considerably. The tumour implants in nude mice remained considerably stable in their phenotypic expression after repeated passages.

One mechanism of activation of ras genes is induction of mutation at positions 12, 13 or 61 (Tabin et al., 1982; Reddy et al., 1982; Bos et al., 1985; Yusa et al., 1983). In MNU-induced rat mammary carcinomas, the H-ras-1 became activated by single amino acid substitution at the twelfth codon, encoding glutamic acid instead of glycine (Sukumar et al., 1983). To clarify the efficiency of MNU on the ras p21 in MNU-induced rat bladder carcinomas, we examined the reactivity of ras p21 with antibodies which were raised against synthetic peptides showing substitution at codon 12 of ras p21 from glycine to valine, glutamic acid, or arginine. Specificity of these monoclonal antibodies has been adequately demonstrated (Carney et al., 1986; Pullano et al., 1989; Azuma et al., 1990). Immunoblot analysis demonstrated that seven of 18 carcinomas tested contained activated ras p21 with substitution with glutamic acid. Of the nine tumours which were subjected also to DNA transfection assay, three showed twelfth codon mutation and yet in only one of these (NI-10) ras gene activation was demonstrable by Southern blot hybridisation perhaps due to low sensitivity of the assay. It has been observed that although human H-ras-1 genes mutated at codon 12, encoding glutamic acid in place of glycine, generated transformants by transfection assay, the cells displayed a less striking change in morphology as compared to those generated by mutated ras genes which encoded valine (Seeburg et al., 1984). Since rare 'spontaneous' carcinoma occurred in urine-treated HTB without carcinogen treatment (Ozono et al., 1983) there is a possibility that ras mutation seen in some tumours is unrelated to MNU treatment.

In conclusion, our data indicate that approximately one half of non-invasive and invasive carcinomas induced by MNU contain activated ras oncogenes or oncogene product p21, but that their expression cannot be correlated to the aggressiveness of tumours. Our data are consistent with the previous observation that H-ras oncogenes are activated by MNU during the initiation of rat mammary carcinogenesis (Sukumar et al., 1983).

We are grateful to Dr Mariano Barbacid, Squibb Institute For Medical Research, Princeton, NJ, for providing us with S2-721 and 118-413 cells.

This investigation was supported by NIH grant CA 14649.

References

ANDERSSON, P., GOLDFARB, M.M. & WEINBERG, R.A. (1979). A defined subgenomic fragment of in vitro synthesized Moloney sarcoma virus DNA can induce cell transformation upon transfection. Cell, 16, 63.
AZUMA, M., MOMOSE, H. & OYASU, R. (1990). In vitro malignant conversion of low-grade rat urinary bladder carcinoma cells by exposure to N-methyl-N-nitrosourea. Cancer Res., 50, 7062.
BABAYA, K., MIYATA, Y., CHIMIEL, J.S. & OYASU, R. (1982). Effects of rat urine fractionated by molecular weight on bladder carcinosogenesis. Cancer Res., 42, 15.
BOS, J.L., TOKOZI, D., MARSHALL, C.J. & others (1985). Amino-acid substitutions at codon 13 of the N-ras oncogene in human acute myeloid leukemia. Nature, 315, 726.
BRAWN, P.N. (1982). The origin of invasive carcinoma of the bladder. Cancer, 50, 37.
CARNEY, W.P., PEIT, D., HAMER, P. & others (1986). Monoclonal antibody specific for an activated ras protein. Proc. Natl Acad. Sci. USA, 83, 7485.
DEBIEC-RYCHTER, M., ZUKOWSKI, K. & WANG, C.Y. (1989). Chromosomal characteristics and malignancy of urothelial cells from carcinogen-treated rats. J. Natl Cancer Inst., 81, 361.
FINKEL, T., DER, C.J. & COOPER, G.M. (1984). Activation of ras genes in human tumors does not affect localization, modification, or nucleotide binding properties of p21. Cell, 37, 151.
FUJITA, J., OHUCHI, N., IT0, N. & others (1988). Activation of H-ras oncogenes in rat bladder tumors induced by N-butyl-N-(4-hydroxybutyl)nitrosamine. J. Natl Cancer Inst., 80, 37.
FUJITA, J., SRIVASTAVA, S.K., KRAUS, M.H., RHIM, J.S., TRONICK, S.R. & AARONSON, S.A. (1985). Frequency of molecular alteration affecting ras proto-oncogenes in human urinary tract tumors. Proc. Natl Acad. Sci. USA, 82, 3849.
FUJITA, J., YOSHIDA, O., YUASA, Y., RHIM, J.S., HATANAKA, M. & AARONSON, S.A. (1984). H-ras oncogenes are activated by somatic alterations in human urinary tract tumors. Nature, 309, 464.
FURTH, M.E., DAVIS, L.J., FLEURDELYS, B. & SCOLNICK, E.M. (1982). Monoclonal antibodies to the p21 products of the transforming gene of Harvey murine sarcoma virus and of the cellular ras gene family. J. Virol., 43, 294.
HIRAO, Y., IZUMI, K. & OYASU, R. (1980). The effect of normal rat urine on mucosal regeneration in heterotopic urinary bladder. Lab. Invest., 42, 76.
KATZ, K.W. & LANGE, P.H. (1982). Mode of presentation of invasive bladder cancer: reassessment of the problem. J. Urol., 128, 31.
KUNZE, E. (1979). Development of urinary bladder cancer in the rat. In: Carcinogenesis: Current Topics in Pathology, Grundmann, E. (ed.) p. 151. Springer-Verlag, Berlin.
LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680.
MEYERS, F.J., GUMERLOCK, P.H., KOKORIS, S.P., DE VERE WHITEX, R.W. & MCCORMICK, F. (1989). Human bladder and colon carcinomas contain activated ras p21. Cancer, 63, 2177.
OYASU, R., ISAWASHI, T., MATSUMOTO, M., HIRAO, Y. & TABUCHI, Y. (1978). Induction of tumors in heterotopic bladder by topical application of N-methyl-N-nitrosourea and N-butyl-N-(3-carboxypropyl)nitrosamine. Cancer Res., 38, 3019.
OYASU, R., MANNING, D.J., MATSUMOTO, M. & HOPP, M.L. (1976). Heterotopic urinary bladder with a communicating reservoir. Cancer Res., 36, 2261.
OYASU, R., SAMMA, S., OZONO, S., BAUER, K., WALLEMARK, C.-B. & HOMMA, Y. (1987). Induction of high-grade, high-stage carcinomas in the rat urinary bladder. Cancer, 59, 451.

ras ALTERATIONS IN RAT BLADDER CANCER 13
OZONO, S., BABAYA, K., MORIKAWA, A. & OYASU, R. (1983). A minimal dose of N-methyl-N-nitrosourea carcinogenic to heterotopically transplanted rat urinary bladder. Carcinogenesis, 4, 547.

PULLANO, T.G., SINN, E. & CARNEY, W.P. (1989). Characterization of monoclonal antibody R256, specific for activated ras p21 with arginine at 12, and analysis of breast carcinoma of v-Harvey-ras transgenic mouse. Oncogene, 4, 1003.

REDDY, E.P., REYNOLDS, R.K. & SANTOS, E. (1982). A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene. Nature, 300, 149.

RIGBY, P., DICKMAN, R., RHODES, C. & BERG, P. (1977). Labelling of deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase. J. Mol. Biol., 98, 503.

SEEBURG, P.H., COLBY, W.W., CAPON, D.J., GOEDDEL, D.V. & LEVINSON, A.D. (1984). Biological properties of human c-Ha-ras 1 genes mutated at codon 12. Nature, 312, 71.

SUUKMAR, S., NOTARIO, V., MARTIN-ZANCA, D. & BARBACID, M. (1983). Induction of mammary carcinomas in rats by nitrogen mustard involves malignant activation of H-ras-1 locus by single point mutations. Nature, 306, 658.

TABIN, C.J., BRADLEY, S.M., BARGMANN, C.I. & 6 others (1982). Mechanism of activation of a human oncogene. Nature, 300, 143.

TOWBIN, H., STAEHLIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitro-cellulose sheets: procedure and some applications. Proc. Natl Acad. Sci. USA, 76, 4350.

YUASA, Y., SRIVASTAVA, S.K., DUNN, C.Y., RHIM, J.S., REDDY, E.P. & AARONSON, S.A. (1983). Acquisition of transforming properties by alternative point mutations within C-bas/has proto-oncogene. Nature, 303, 775.