Reduced expression of the CDK inhibitor p27KIP1 in rat two-stage bladder carcinogenesis and its association with expression profiles of p21WAF1/Cip1 and p53

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The cyclin-dependent kinase (CDK) inhibitor p27KIP1 exerts its growth suppressive effects by targeting the cyclin–CDK complexes. Reduced protein levels of p27KIP1 have been reported in numerous human cancers and this has been attributed to increased degradation. However, few reports have addressed the significance of p27KIP1 expression in chemical carcinogenesis of rodents. In a rat two-stage urinary bladder carcinogenesis model, with N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) initiation followed by promotion with sodium L-ascorbate (Na-AsA), we evaluated the expression of p27KIP1 protein using immunohistochemistry during various stages of urinary bladder carcinogenesis. In addition, we evaluated the mRNA expression profiles for p27KIP1, p21WAF1/Cip1 and p53 in tumors. Fisher 344 rats were initiated with 0.05% BBN in the drinking water for 4 weeks and then administered 5% Na-AsA in the diet. Immunohistochemical examination revealed p27KIP1 protein to be constitutively expressed in normal urothelium, simple hyperplasia and in most papillary and nodular (PN) hyperplasias and small papillomas, but diminished or absent in large papillomas and in transitional cell carcinomas. An inverse correlation between expression of p27KIP1 and cell proliferation was generally observed. Quantitation of mRNA by multiplex reverse transcription-PCR showed a significant downregulation of p27KIP1, p21WAF1/Cip1 and p53 mRNA in tumors. More than 50% reduction in p27KIP1 mRNA expression was observed in 42 and 47% of tumors at weeks 18 and 24, respectively; similar reduction in p21WAF1/Cip1 mRNA expression was observed in 58 and 73% of tumors at weeks 18 and 24, and in p53 mRNA expression in 50 and 73% of tumors at weeks 18 and 24, respectively. None of the 25 tumors we examined by PCR-single-strand conformational polymorphism analysis had p53 mutations. These data imply that abnormal down-regulation of p27KIP1, p21WAF1/Cip1 and/or p53 in tumor cells may contribute to the malignant progression of tumors during rat two-stage bladder carcinogenesis.

Introduction

Knowledge of cell cycle regulation has advanced rapidly over the past several years due to the discovery of an increasing number of cell cycle regulatory proteins, namely the cyclins, cyclin-dependent kinases (CDKs) and CDK inhibitors. Recently, a number of cell cycle related elements was found to be either inactivated or overexpressed in transformed cell lines or tumors. Although the regulation of cell cycle regulatory proteins has thus been extensively studied in vitro using tumor cell lines, there have been relatively few studies of this kind in vivo using animal tumor models. Recently, cell cycle regulatory events in tumors have been analyzed in vivo using several animal models of carcinogenesis (1–3), including neoplasia in the urinary bladder (4). In contrast to genetic alterations found in human cancers, which are potentially due to a multitude of environmental carcinogens and genetic factors, those in tumors induced by chemical carcinogens in inbred animals are more likely to be attributable to the effects of the carcinogens administered. Usually only a single carcinogen is administered in simple protocols. Induction of neoplasms with carcinogens in rodents followed by analysis of cell cycle related elements in the preneoplastic lesions and tumors at various stages of their development should contribute to understanding the relationship between cell cycle deregulation and the development of malignancies in vivo.

Numerous urinary bladder carcinogenesis models has been developed using rats and mice, typically by administration of chemical carcinogens in the food or the drinking water (5–8). Animal models of urinary bladder carcinogenesis are useful for studies of tumor biology for the following reasons: firstly, progression from preneoplastic lesions to carcinomas can be readily observed, thus allowing comparison of genetic events in precursor lesions with those in tumors. Secondly, stage-specific molecular events in urinary bladder carcinogenesis can be analyzed readily in experimental systems using the multistage carcinogenesis protocol of initiation and promotion. Thus, multistage chemical carcinogenesis of rat urinary bladder provides an excellent model for the study of multistep progression from preneoplastic changes to carcinoma, and studies in this model have led to significant advances in our understanding of urinary bladder carcinogenesis. Chemical carcinogens are generally divided into two categories, genotoxic and non-genotoxic (including tumor promoters). Genotoxic carcinogens are capable of directly interacting with and disrupting the integrity of genomic DNA, and are believed to play a role in the carcinogenic process by causing mutations in proto-oncogenes or tumor suppressor genes (9). In contrast, the mechanisms of action of non-genotoxic carcinogens are much less well understood. One of the most widely employed and
well characterized experiment protocols for urinary bladder carcinogenesis in rats is the two-stage bladder carcinogenesis protocol, which consists of initiation with a genotoxic carcinogen such as N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN) or N-[4-(5-nitro-2-furyl)-2-thiazole]formamide (FANFT) for a period of 4–6 weeks, followed by promotion with a non-genotoxic carcinogen such as sodium L-ascorbate (Na-AsA) or sodium saccharin for the duration of the experiment (6,7). Currently, little is known about the mechanism of promotion by non-genotoxic carcinogens in rat urinary bladder carcinogenesis. A recent study indicated that cyclin D1 overexpression, and subsequently accelerated cell cycle entry, is involved in rat two-stage bladder carcinogenesis (4); consistent with the view that increased cell proliferation might account for the carcinogenicity of non-genotoxic chemicals (10,11). Since cancer cells typically undergo a series of genetic alterations during the course of promotion and progression, additional genetic events are likely to be associated with two-stage bladder carcinogenesis. We postulated that downregulation of CDK inhibitors, negative effectors of the cell cycle, is one possibility.

The CDK inhibitors can be grouped into two categories which differ in sequence homology and their targets of inhibition; the INK4 family containing ankyrin repeat motifs (p16INK4a, p15INK4b, p18INK4c, and p19INK4d (12–15)) and the CIP/KIP family (p21WAF1/Cip1, p27Kip1, and p57Kip2) (16–20). The INK4 family of CDK inhibitors are specific inhibitors of CDK4/6 complexes while p27KIP1 and p21WAF1/Cip1 are able to suppress cell cycle progression via inhibition of a diverse family of preactivated cyclin–CDK complexes (18,19,21–23). In the present study, we focused our attention on altered expression profiles for p27KIP1, p21WAF1/Cip1 and p53 were followed by promotion with Na-AsA (7). In addition, mRNA expression was stretched and fixed in formalin for histological examination. A total of 10 age-matched control rats were killed and their urinary bladder mucosa were removed for molecular assessment.

**Pathological diagnosis**

Serial sections from each block were stained with H&E for histopathological assessment. Lesions of the urinary bladder were classified into simple hyperplasia, PN hyperplasia, papilloma, TCC, squamous cell papilloma and SCC, as described previously (47).

**Immunohistochemistry**

Serial sections of 3 µm thickness were made and spread on poly-L-lysine-coated slides (48). Paraffin sections were immersed in three changes of xylene and hydrated using a graded series of ethanol. Antigen retrieval was performed routinely by microwave heating. Sections were immersed in distilled water or buffer inside a plastic container and were repeatedly heated to boiling temperature and then allowed to cool to room temperature gradually for eight to 10 times (4,49,50); for p27KIP1, immunohistochemistry, distilled water was used, and for PCNA, cyclin D1 or p53 immunohistochemistry, an antigen retrieval fluid (AR-10; BioGenex, San Ramon, CA) was employed. Sections were blocked with serum at 37°C for 30 min and then incubated with either mouse monoclonal anti-p27KIP1 antibody (IgG1; Transduction Laboratories, Lexington, KY) at 1:50 dilution, mouse monoclonal anti-PCNA antibody (PC-10, IgG2a; Dako, Tokyo, Japan) at 1:750 dilution, rabbit polyclonal anti-cyclin D1 antibody (IgG; UBI, Lake Placid, NY) at 1:200 dilution, or rabbit polyclonal antihuman p53 antibody (CM1; Medac Diagnostica, Germany) at 1:200 dilution, overnight at 4°C. Staining was achieved with a Dako LSAB2 kit/AP (Dako, Tokyo, Japan) and developed with either 3-aminobenzidine tetrahydrochloride (Zymed). Sections were counterstained for 5 s with Meyer’s hematoxylin and mounted in non-aqueous mounting media (DIATEX; AB Wilh, Becker, Sweden) for microscopy. A negative control staining was included with each staining procedure by omitting the primary antibody. Sections of human TCCs with intense staining for either p27KIP1 or PCNA were included with each staining procedure and served as positive controls (49,50). The epitope for the anti-p27KIP1 mouse monoclonal antibody corresponds to the C-terminus of mouse p27KIP1. The antibody detects a specific band of 27 kDa on western blot analysis of HeLa Cell extracts, as indicated by its information data sheet. The antibody also reacts with p27KIP1 of human, dog, rat and chicken origin by immunohistochemistry and is immunoreactive in formalin-fixed, paraffin-embedded tissues (28,51). The epitope for anti-PCNA mouse monoclonal antibody is the mouse PCNA antigen and the antibody has species cross-reactivity to human and rat. The anti-cyclin D1 antibody recognizes the 11 C-terminal amino acids (residues 285–295) of human cyclin D1 and has species cross-reactivity to mouse and rat. The anti-p53 antibody (CM1) recognizes both wild and mutant forms of p53 on formalin-fixed, paraffin-embedded specimens. For double staining of p27KIP1 and PCNA or p27KIP1 and cyclin D1, sections were first probed with anti-p27KIP1 antibody overnight and developed with the alkaline phosphatase method (BCIP/NBP chromogen) to produce a dark purple to black color. The epitope for anti-PCNA mouse monoclonal antibody is the mouse PCNA antigen and the antibody has species cross-reactivity to human and rat. The anti-cyclin D1 antibody recognizes the 11 C-terminal amino acids (residues 285–295) of human cyclin D1 and has species cross-reactivity to mouse and rat. The anti-p53 antibody (CM1) recognizes both wild and mutant forms of p53 on formalin-fixed, paraffin-embedded specimens. For double staining of p27KIP1 and PCNA or p27KIP1 and cyclin D1, sections were first probed with anti-p27KIP1 antibody overnight and developed with the alkaline phosphatase method (BCIP/NBP chromogen) to produce a dark purple to black color. The epitope for anti-PCNA mouse monoclonal antibody is the mouse PCNA antigen and the antibody has species cross-reactivity to human and rat. The anti-cyclin D1 antibody recognizes the 11 C-terminal amino acids (residues 285–295) of human cyclin D1 and has species cross-reactivity to mouse and rat. The anti-p53 antibody (CM1) recognizes both wild and mutant forms of p53 on formalin-fixed, paraffin-embedded specimens. For double staining of p27KIP1 and PCNA or p27KIP1 and cyclin D1, sections were first probed with anti-p27KIP1 antibody overnight and developed with the alkaline phosphatase method (BCIP/NBP chromogen) to produce a dark purple to black color. The epitope for anti-PCNA mouse monoclonal antibody is the mouse PCNA antigen and the antibody has species cross-reactivity to human and rat. The anti-cyclin D1 antibody recognizes the 11 C-terminal amino acids (residues 285–295) of human cyclin D1 and has species cross-reactivity to mouse and rat. The anti-p53 antibody (CM1) recognizes both wild and mutant forms of p53 on formalin-fixed, paraffin-embedded specimens. For double staining of p27KIP1 and PCNA or p27KIP1 and cyclin D1, sections were first probed with anti-p27KIP1 antibody overnight and developed with the alkaline phosphatase method (BCIP/NBP chromogen) to produce a dark purple to black color. The epitope for anti-PCNA mouse monoclonal antibody is the mouse PCNA antigen and the antibody has species cross-reactivity to human and rat.
Fig. 1. Immunohistochemical assessment of p27KIP1 in normal epithelium, preneoplastic, and neoplastic lesions of the rat urinary bladder. (A) Normal bladder epithelium demonstrating positive nuclear staining of p27KIP1. Note the reaction of superficial ‘umbrella’ cells of the transitional cell epithelium (×400). (B) Simple hyperplasia strongly positive for p27KIP1 (×400). (C) Nodular type PN hyperplasia with intense and extensive p27KIP1 staining (×100). (D) Positive p27KIP1 nuclear staining (brown) in a PN hyperplasia (×100). (E) Serial section to D stained for PCNA (red) showed negligible staining in the nuclei (×100). (F) Papillary type PN hyperplasia with intense and extensive staining of p27KIP1 (×100). (G) Small papilloma with extensive staining of p27KIP1; the intensity of staining was moderate (×100). (H) Large papilloma with weak staining for p27KIP1 (×100). (I) TCC with abundant stroma; note the intense staining of p27KIP1 present in the fibroblasts but not in the epithelial elements (×200). (J) TCC with negligible p27KIP1 (brown) nuclear staining (×100). (K) Serial section to J stained for PCNA (red) showed intense nuclear staining for the majority of tumor nuclei (×100). (L) TCC with little stroma showing very weak staining of p27KIP1 in the epithelial elements (×200).

Quantitation of p27KIP1 immunohistochemistry

Cells were considered positive for p27KIP1 when definite nuclear staining could be identified. Intense staining of p27KIP1 was consistently observed in simple hyperplasia and in fibroblasts, which served as internal controls for the quality of staining (Figure 1B and I). The intensity of p27KIP1 staining in PN hyperplasias, papillomas and TCCs was compared with regions of simple hyperplasia with strong intensity of staining within the same specimen, and was judged on a semi-quantitative scale of negative staining, weak staining, moderate staining or strong staining by the consensus of two pathologists (C.C.R.Lee and T.Ichihara). All visible PN hyperplasias, papillomas and TCCs, at different stages of development, were evaluated (Figure 1). Lesions with a markedly heterogeneous staining pattern for p27KIP1 were not classified. Few squamous cell carcinomas (SCCs) and squamous cell papillomas were encountered and they are not analyzed in the present study.

Extraction of DNA and RNA

Bladder tumor specimens for RNA extraction were snap frozen in liquid nitrogen and stored at –80°C until they were analyzed. Tumors <5 mm were homogenized whole, and larger lesions were divided into smaller pieces. Total
RNA was isolated from tumors using ISOGEN (Nippon Gene, Toyama, Japan) (52,53), a reagent for simultaneous isolation of RNA and DNA from tissue samples. Tumor tissues were placed in ISOGEN and lysed by repeated passage through a syringe fitted with a 23 gauge needle. After removal of the RNA fraction, DNA was ethanol precipitated and then extracted with SepaGene (Sanko Junyaku, Tokyo, Japan). DNA concentrations were determined with a spectrophotometer (Ultraspec-2000i; Pharmacia LKB, Uppsala, Sweden). Multiplex PCR was used to amplify DNA from single cell conformation polymorphism (SSCP) analysis was carried out on the extracted DNA solution.

**Table I. Nucleotide sequence information for PCR primers and conditions for multiplex RT–PCR**

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Nucleotide sequence</th>
<th>Orientation</th>
<th>GC content (%)</th>
<th>Length of primer (bp)</th>
<th>Length of PCR product (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Target to GAPDH ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>p27kip1</td>
<td>5'-AGG AGA GCT GCT AGT TCA G3'</td>
<td>sense</td>
<td>53</td>
<td>19</td>
<td>207</td>
<td>50</td>
<td>5:1</td>
</tr>
<tr>
<td></td>
<td>5'-TTT CTT CTC TGT TGG TCC C3'</td>
<td>sense</td>
<td>45</td>
<td>20</td>
<td>207</td>
<td>50</td>
<td>5:1</td>
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<tr>
<td></td>
<td>5'-AGC TGG CCT CTC CGC TGT CTT C3'</td>
<td>sense</td>
<td>57</td>
<td>21</td>
<td>213</td>
<td>55</td>
<td>2.5:1</td>
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<tr>
<td></td>
<td>5'-TAA GGC AGA AGA TGG GGA AGA G3'</td>
<td>sense</td>
<td>50</td>
<td>22</td>
<td>225</td>
<td>50</td>
<td>5:1</td>
</tr>
<tr>
<td>p53</td>
<td>5'-CCT CAA TAA GCT GTT CTC CC C3'</td>
<td>sense</td>
<td>50</td>
<td>20</td>
<td>250</td>
<td>50</td>
<td>5:1</td>
</tr>
<tr>
<td></td>
<td>5'-ATG AAG ACC AGA AGC TTC CTG C3'</td>
<td>sense</td>
<td>50</td>
<td>22</td>
<td>560</td>
<td>50 or 55</td>
<td>–</td>
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<tr>
<td></td>
<td>5'-TTC GGG GAA AAT TGG TTA GGG G3'</td>
<td>sense</td>
<td>55</td>
<td>22</td>
<td>560</td>
<td>50 or 55</td>
<td>–</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-ACG TGG CCT TGT CGC TGT CTT-3'</td>
<td>sense</td>
<td>55</td>
<td>22</td>
<td>560</td>
<td>50 or 55</td>
<td>–</td>
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**Results**

**Expression of p27kip1 during various stages of rat bladder carcinogenesis**

Normal rat bladder epithelium constitutively expresses the p27kip1 protein (Figure 1A). Simple hyperplasia, firstly appearing after carcinogen treatment, stained strongly positive for p27kip1 (Figure 1B). PN hyperplasia appeared after week 8 and the majority also stained intensely (Figure 1C, D and F) for p27kip1. Papillomas, which began to appear at week 12, frequently showed diminished staining intensity for p27kip1 relative to simple hyperplasia; generally, smaller papillomas stained more intensely than their larger counterparts (Figure 1G and H). TCCs began to appear at week 18, and the majority of them showed diminished staining. Occasionally no staining was observed (Figure 1I, J and L). Two different histological

**PCR–SSCP analysis**

Exons 5, 6 and 7, and 8 and 9 of p53 were analyzed by the PCR–SSCP method (4,54,55). Fifty nanograms of genomic DNA were amplified in a reaction volume of 5 μl containing 50 nM (6)32PdCTP (>3000 Ci/mmol; NEN/Dupont, Boston, MA). The PCR conditions for exons 5 were 38 cycles of 95°C for 60 s, 58°C for 60 s and 72°C for 60 s, and for exons 6–8 and 8–10 were 38 cycles of 95°C for 60 s, 56°C for 60 s and 72°C for 60 s. PCR products were mixed with 20 μl of stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol, denatured for 5 min at 90°C and loaded (3 μl/lane) to mutation detection enhancement gels (modified acrylamide gels; AT Biochem, Malvern, PA). A polycrylamide-like matrix that has a high sensitivity to DNA conformational differences, and electrophoresed for 16 h at 6 W. Gels were dried and exposed to X-ray film at ~80°C with Kodak X-Omat AR film (Kodak, Rochester, NY) and an intensifying screen. The sequences of primers for the PCR–SSCP analysis were as follows: p53 exon 5, 5'-GATCCCCCTTTCCCTGTCACC-3' (sense) and 5'-CAATGAGCTGCTCCTGTCC-3' (antisense); p53 exon 6 and 7, 5'-CCTCCTGATATTTGTCGCTC-3' (sense) and 5'-CCTCCTGACTTTCTGTCACC-3' (antisense); p53 exon 8 and 9, 5'-CGGTGTGCTAGCTGTCACC-3' (sense) and 5'-GGAATATAATGCCTGGACCC-3' (antisense).

PCR was repeated at least twice and electrophoresis was performed under two different conditions (acylamide gel alone and acrylamide gel with 10% glycerol) to confirm the results. If shifted bands were observed by PCR–SSCP analysis, they were cut out from the acrylamide gel to be sequenced.

**DNA sequencing**

DNA sequences were determined with the ABI PRISM dye terminator cycle sequencing ready reaction kit with AmpliTaq DNA polymerase (Perkin Elmer, Applied Biosystems, Foster City, CA) on the ABI PRISM 310 genetic analyzer (Perkin Elmer, Applied Biosystems).

**Statistical analysis**

Statistical comparisons between the different groups (Figure 4A–C) were completed with StatView software (v.4.02; Abacus Concepts, Berkeley, CA) for the Macintosh microcomputer, and significant differences between groups were determined by the Student's paired r-test. A result was only considered statistically significant if P < 0.05.
Fig. 2. Immunohistochemical assessment of p27KIP1 in rat urinary bladder lesions induced by BBN and promoted by Na-AsA. p27KIP1 staining in urinary bladder lesions was scored semi-quantitatively based on the intensity of staining. Only PN hyperplasias were present at week 8. Both PN hyperplasia and papillomas could be observed at week 12. TCCs are present at weeks 18 and 24. The majority of PN hyperplasias had strong or moderate p27KIP1 staining while the majority of papillomas and TCCs demonstrated moderate or weak staining.

patterns of TCCs could be identified, associated with different patterns of staining; TCCs with abundant stroma generally had negligible staining in the epithelial elements but intense staining of the fibroblasts (Figure 1I), while TCCs with rare stroma generally had detectable levels of staining in the epithelial elements (Figure 1L).

Semi-quantitative evaluation of p27KIP1 protein expression
Sample size requirements for obtaining sufficient amounts of RNA for multiplex RT–PCR analysis did not allow detection of p27KIP1 mRNA levels in any specific type of preneoplastic lesion. However, using immunohistochemical techniques, we were able to examine p27KIP1 protein expression in paraffin-embedded tissue sections. Overall, the majority of PN hyperplasias demonstrated moderate to strong p27KIP1 staining while the majority of papillomas and TCCs exhibited only weak to moderate staining (Figure 2).

Quantitation of p27KIP1, p21WAF1/Cip1 and p53 mRNA expression in tumors
The mRNA levels for cell cycle related elements p27KIP1, p21WAF1/Cip1 and p53 in tumors and normal urothelium were determined by multiplex RT–PCR, a semi-quantitative but highly sensitive method for determining the expression levels of target genes (4). The results shown in Figure 3 are representative of five independent PCR reactions. The great majority of tumors evaluated had reduced p27KIP1 mRNA levels, as compared with normal bladder mucosa (Figure 3). A relatively high proportion of tumors analyzed, 42% (5 of 12) of those at week 18 and 47% (7 of 15) at week 24, demonstrated >50% reduction in their p27KIP1 mRNA levels (Table II). A cut-off value of >50% reduction or incrementation was chosen due to the fact that small individual variation in mRNA levels exists within the control group; however, such variations are always within the cut-off value we selected.

Average p27KIP1 mRNA expression was significantly reduced in tumors at both 18 and 24 weeks (Figure 4A).

Reduced mRNA levels of another CDK inhibitor, p21WAF1/Cip1 were observed in 58% (7 of 12) and 73% (11 of 15) of tumors at weeks 18 and 24, respectively. The respective figures for reduced mRNA levels of p53, an upstream regulator of p21WAF1/Cip1, were 50% (6 of 12) and 73% (11 of 15).

Average p21WAF1/Cip1 mRNA expression in tumors was also significantly reduced at both time points (Figure 4B). Average p53 mRNA expression was significantly reduced at week 24 but not 18 (Figure 4C).

Comparison of the mRNA expression profiles of p27KIP1, p21WAF1/Cip1 and p53 in tumors (Figure 3) revealed a simultaneous reduction in all three in 33% (4 of 12) of the 18 week tumors and 40% (6 of 15) of the 24 week tumors. Comparison between the mRNA expression profiles of p21WAF1/Cip1 and p53 revealed a consensus of reduced mRNA levels in 42% (5 of 12) of the 18 week tumors and 67% (10 of 15) of the 24 week tumors.

Absence of p53 mutations and lack of p53 nuclear accumulation in tumor
Twelve tumors taken at week 18 and 15 taken at week 24 were examined for the presence of p53 mutations by PCR–SSCP analysis. None of the tumors we examined harbored mutations within exons 5–9 of the p53 gene (Table II). Immunohistochemistry for p53 was performed on all TCCs and only two tumors, one from week 18 and another from week 24, showed positive nuclear staining in >20% of their nuclei.

Topographic distribution of p27KIP1 relative to PCNA and cyclin D1
To evaluate the topographic distribution of p27KIP1 relative to PCNA, a marker of cell proliferation in TCCs of human...
Fig. 3. Quantitation of p27<sup>KIP1</sup>, p21<sup>WAF1/Cip1</sup> and p53 mRNA expression by multiplex RT–PCR. Peak intensity of the PCR product derived from the target gene was divided by that for GAPDH, the internal control for multiplex PCR. The results obtained from five separate PCR reactions were averaged to represent the relative gene expression for any particular sample. The average relative gene expressions of control rats (normal bladder mucosa) were assigned an arbitrary value of 1, with which the tumor samples were compared. *, Tumor samples with <50% relative expression of mRNA, in comparison with normal bladder epithelium. Dashed lines indicate the average mRNA level of normal bladder epithelium.

Table II. Molecular assessment of p53 mutations and mRNA expression profiles of p27<sup>KIP1</sup>, p21<sup>WAF1/Cip1</sup> and p53 in rat urinary bladder tumors induced by BBN and promoted by sodium L-ascorbate

<table>
<thead>
<tr>
<th>Duration (weeks)</th>
<th>Effective no. of rats</th>
<th>Effective no. of tumors&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Average size of tumors (mm)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>p53 mutation&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Reduced mRNA expression (5)&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Relative mRNA expression&lt;sup&gt;e&lt;/sup&gt;</th>
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<tr>
<td>18</td>
<td>4</td>
<td>12</td>
<td>3.67 ± 1.61</td>
<td>0</td>
<td>42 (n = 5)</td>
<td>0.62 ± 0.39</td>
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<td>58 (n = 7)</td>
<td>0.50 ± 0.18</td>
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<td></td>
<td>50 (n = 6)</td>
<td>0.69 ± 0.50</td>
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<tr>
<td>24</td>
<td>5</td>
<td>15</td>
<td>5.80 ± 1.70</td>
<td>0</td>
<td>47 (n = 7)</td>
<td>0.55 ± 0.27</td>
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<td></td>
<td>73 (n = 11)</td>
<td>0.45 ± 0.22</td>
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<td></td>
<td></td>
<td>73 (n = 11)</td>
<td>0.33 ± 0.17</td>
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<sup>a</sup>The three largest tumors from each bladder were evaluated.

<sup>b</sup>Tumor size was measured as the maximum cut surface diameter (mm) of the tumor.

<sup>c</sup>Mutations of the p53 gene were determined by PCR–SSCP, followed by DNA sequencing.

<sup>d</sup>Reduced expression of p27<sup>KIP1</sup>, p21<sup>WAF1/Cip1</sup> and p53 tumors was defined as >50% reduction.

<sup>e</sup>The mRNA levels were assessed by multiplex RT–PCR and the values are for expression of mRNA in tumors relative to the average of normal bladder mucosa.
p27KIP1 in urinary bladder carcinogenesis

Fig. 4. Quantitation of (A) p27KIP1, (B) p21WAF1/Cip1 and (C) p53 mRNA expression by multiplex RT–PCR. The values are expressed relative to the control (normal bladder mucosa), given an arbitrary value of 1. N, normal bladder mucosa; T1, 18 week tumors; T2, 24 week tumors; NS, not significant. *P < 0.05; **P < 0.01; ***P < 0.001.

urinary bladder (50,56), immunohistochemistry for each of these antigens was performed separately in serial sections. Double staining was also performed on selected specimens to allow precise localization of p27KIP1 relative to PCNA. A reciprocal expression pattern between p27KIP1 and PCNA was observed. The majority of lesions stained homogeneously positive for either p27KIP1 or PCNA (Figures 1D, E, J and K, and 5B and D). Intratumor heterogeneity was observed in a subset of bladder lesions; regions were positively stained for p27KIP1 but not for PCNA, or positively stained for PCNA but not for p27KIP1 (Figure 5A, C, E, F and G). In well-differentiated TCCs, positive p27KIP1 nuclear staining was found diffusely throughout the epithelial elements, whereas PCNA nuclear staining was confined to the basal cell layers (Figure 5H and I). Double immunohistochemistry for cyclin D1 and p27KIP1 was performed on a selected number of TCCs (Figure 6). A mutually exclusive pattern for cyclin D1 and p27KIP1 was not observed.

Discussion

Despite clear evidence that p27KIP1 can function as a negative regulator of cell cycle progression, its precise role in tumorogenesis is still the subject of active investigation. Since p27KIP1 functions biochemically as a cell cycle inhibitor (21,24,25), a reduction in the protein level of p27KIP1 is likely to provide a cell with a selective growth advantage. We have presented evidence that reduced expression of p27KIP1 is a frequent molecular event in two-stage bladder carcinogenesis of rats. Thus, immunohistochemical examination revealed a dramatic decrease of p27KIP1 protein expression in advanced as compared with early preneoplastic lesions, confirmed by multiplex RT–PCR to be at least partially due to reduction in mRNA levels. Increased degradation of p27KIP1 in tumors is another possibility, since cell cycle regulation of p27KIP1 cellular abundance occurs at the post-transcriptional level via proteasome-mediated degradation (57,58). The regulatory mechanism of p27KIP1 expression in rat bladder tumors is the subject of ongoing investigation. Reduced expression of p27KIP1 protein has not been implicated in human urinary bladder carcinogenesis, but recent studies suggest that patients with breast and colon tumors that display low expression of p27KIP1 have a poor prognosis (27,28,30). The finding of decreased expression of p27KIP1 at both the mRNA and protein levels in rat bladder tumors implies a tumor suppressive role for this CDK inhibitor and it is consistent with a normal role of this protein in growth suppression in non-malignant tissues. A recent publication reported that p27KIP1 knockout mice are predisposed to tumors in multiple tissues when challenged with γ irradiation or the chemical carcinogen N-ethyl-N-nitrosourea (ENU) (59).

Interestingly, the intensity of p27KIP1 staining was somewhat increased in simple hyperplasia as compared with normal bladder epithelium. A possible explanation is that increased p27KIP1 in this setting is a physiological response to counteract proliferative signals generated by exposure to carcinogens. In contrast, loss of p27KIP1 protein expression in the majority of advanced lesions is likely a result of either increased degradation or transcriptional repression of this protein during the course of tumor progression. In a small population of papillomas and TCCs, strong expression of p27KIP1 protein was seen, possibly indicating the existence of a mechanism by which some growing tumor cells may tolerate this inhibitor of cell cycle progression.

Reduced mRNA expression of p21WAF1/Cip1, a CDK inhibitor with structural similarities to p27KIP1, was also observed in the present study. This could play a role in rat two-stage bladder carcinogenesis for the following reasons: firstly, p21WAF1/Cip1 is one of the CDK inhibitors that inhibit kinase activities of preactivated cyclin–CDK complexes (18,19) and a downstream effector of p53-mediated growth suppression (20,60). Secondly, p21WAF1/Cip1 has been shown to induce a cell cycle arrest by direct binding to PCNA (61) and ectopic overexpression of p21WAF1/Cip1 in human brain tumor cells results in G1-arrested cells (62). Thirdly, p21WAF1/Cip1 can function as a tumor suppressor through its ability to control cell cycle progression, and transfection of p21WAF1/Cip1 into malignant cells has been reported to suppress tumorigenicity in vivo (63). Finally, p21WAF1/Cip1 mutations have been reported in TCCs of the human urinary bladder (64,65) and in human bladder cancer cell lines (66).
Fig. 5. Reciprocal expression of p27Kip1 and PCNA in rat urinary bladder lesions and intra-tumor heterogeneity in TCCs. (A) Papilloma with heterogeneous staining of p27Kip1 (dark purple) and PCNA (red), as revealed by double immunohistochemical staining; the lower-right half of the papilloma and the adjacent simple hyperplasia are positively stained for p27Kip1 (dark purple), while the superficial portion of the papilloma has many nuclei positive for PCNA (red) (×50). (B) Two adjacent tumors stained for p27Kip1 (brown); the tumor on the left stained negative while the tumor on the right stained positive (×50). (C) Serial section to A stained for PCNA (red) confirming intense nuclear staining in the superficial portion of the papilloma (×50). (D) Serial section to B stained for PCNA (red); the tumor on the left has many positive nuclei while the tumor on the right has very few (×50). (E) Immunohistochemical staining of p27Kip1 (brown) in a TCC; intense nuclear staining is apparent in the lower portion while negligible staining is present in the upper portion (×200). (F) Serial section to E stained for PCNA (red); intense nuclear staining is observed predominantly in the upper portion (×200). (G) Double immunohistochemical staining of a serial section to E for p27Kip1 (black) and PCNA (red) confirms the mutually exclusive staining patterns for these two antigens within the same lesion (×200). (H) TCC with positive nuclear staining for p27Kip1 (brown) in the epithelial elements; nuclear staining is appreciably stronger in the upper, well-differentiated regions (×100). (I) Serial section to D showing PCNA (red) staining, predominantly in the nuclei of the basal cell layers (×100).

The p53 protein has been described as the cellular gatekeeper for growth and division and a guardian of the genome because of its importance in the mediation of cell cycle arrest and induction of apoptosis in response to DNA damage (67). Inactivation of p53, primarily due to mutations or deletions, is found in 50–55% of all human cancers (68,69). Inactivation of p53 gene appears to have an important role in the tumorigenesis of a subset of urinary bladder carcinomas (70). Thus, alterations are common in high grade, invasive and non-papillary tumors of the urinary bladder (71) and are associated with tumor progression (72). Functional inactivation of the p53 gene in human urinary bladder carcinomas commonly involves an allelic loss of chromosome 17p and mutation of p53 gene on the remaining allele (73). However, mutations of
p53 gene have been reported to be very uncommon in rat bladder tumors induced by the two-stage bladder carcinogenesis protocol; in one experiment in which F344 rats were initiated with FANFT for 6 weeks followed by promotion with 5% Na-AsA until week 78, p53 mutations were observed in only two of 17 bladder tumors (74). In the present study, nuclear accumulation of p53 was detected in only two cases and p53 mutations were not detected in rat bladder tumors induced by the two-stage bladder carcinogenesis protocol of BBN initiation followed by Na-AsA promotion (Table II); reduced mRNA levels were noted in tumors (Table II; Figures 3 and 4C) and may indicate a loss of function for this important tumor suppressor gene. It is possible that a loss or reduction of p53 function may be linked to the reduced expression of p21WAF1/Cip1 mRNA in rat bladder tumors, since p21WAF1/Cip1 has been shown to be a downstream mediator of p53-mediated growth suppression in vitro (20). The observation that functional inactivation of p53 gene in rat bladder tumors induced by the two-stage bladder carcinogenesis protocol may result from reduction in mRNA levels rather than mutations is a novel possibility which deserves further investigation. A relationship between loss of p53 function due to reduction in mRNA levels and the acquisition of a malignant phenotype has been established in in vitro experiments. When an immortalized non-tumorigenic rat urothelial cell line strongly expressing wild-type p53 was transfected with an antisense rat p53 RNA, a significant reduction in p53 mRNA expression was observed; the transfected cell lines acquired a malignant phenotype and formed muscle-invasive, high-grade TCCs when injected s.c. into athymic nude mice (75). Analysis of the transformed cells revealed a decrease in the expression of p21WAF1/Cip1.

Concomitant decrease in p21WAF1/Cip1 and p53 mRNA levels was observed in 42 and 67% of tumors at weeks 18 and 24, respectively (Figure 3). The high concordance rates are consistent with the finding that p21WAF1/Cip1 is a downstream effector of p53-mediated growth suppression (20); a complete concordance between p21WAF1/Cip1 and p53 mRNA expression, however, should not be expected because p21WAF1/Cip1 expression can also be regulated by p53-independent pathways (76). An unexpected finding was the observed high concordance rate between p27KIP1, p21WAF1/Cip1 and p53 mRNA levels, reduction of all three being observed in 33 and 40% of the tumors at weeks 18 and 24, respectively. The significance of this is the subject of ongoing investigations.

It has been reported that in normal human tissues, an inverse correlation between expression of p27KIP1 and cell proliferation is generally observed (34,77). In addition, it has been shown that p27KIP1 gene knock-out mice have increased body weight and multiple organ hyperplasia, suggesting that p27KIP1 protein inhibits cell proliferation in vivo (78–80). Furthermore, p27KIP1 expression is inversely proportional to the proliferative index in some human malignancies (81). To a certain extent, a mutually exclusive staining pattern was also observed in our examination of the topographic distribution of p27KIP1 and PCNA in urinary bladder lesions; lesions with a low proliferative index were mostly positive for p27KIP1, whereas tumors characterized by a higher growth fraction had low p27KIP1 expression. The mutually exclusive pattern of p27KIP1 and
PCNA staining in these lesions indicates that p27\(^{\text{KIP1}}\) plays an important role in negatively controlling cellular proliferation. Overexpression of cyclin D1, a positive regulator of cell cycle progression, has been reported in the two-stage bladder carcinogenesis model (4). An increase in cyclin D1 and a decrease in either p21\(^{\text{WAF1/Cip1}}\) and/or p27\(^{\text{KIP1}}\) are likely to have similar effects on the cell cycle. Double staining for cyclin D1 and p27\(^{\text{KIP1}}\) was therefore performed to determine whether there is a linkage—negative or positive—between the two types of changes. Among the TCCs we evaluated, a heterogeneous staining pattern was generally observed for both cyclin D1 and p27\(^{\text{KIP1}}\) rather than a mutually exclusive pattern. This result is consistent with our previous observation that there does not exist a simple correlation between cyclin D1 overexpression and cell proliferation in TCCs of the urinary bladder (4,50).

The CDK inhibitors p27\(^{\text{KIP1}}\), p21\(^{\text{WAF1/Cip1}}\) and the tumor suppressor gene p53 are negative regulators of cell cycle progression; inactivation of these cell cycle regulatory elements is likely to result in uncontrolled cell proliferation. The observation that reduced expression of p27\(^{\text{KIP1}}\), p21\(^{\text{WAF1/Cip1}}\) and p53 is present in rat bladder tumors induced by two-stage bladder carcinogenesis implies that accelerated cell cycle progression is the mechanism behind the carcinogenicity of non-genotoxic chemicals. The reciprocal expression of p27\(^{\text{KIP1}}\) and PCNA in the great majority of lesions further supports this view.

In conclusion, our results suggest that inappropriate down-regulator of p27\(^{\text{KIP1}}\), p21\(^{\text{WAF1/Cip1}}\) and p53 are associated with malignant tumor development in the rat bladder and may be important in determining the biologic potential of urinary bladder carcinomas. Reduced expression of these cell cycle related elements might account for the increased cell proliferation and the autonomous growth of urinary bladder carcinomas. This study demonstrates that components of the cell cycle machinery have the potential of being targeted and altered during chemical carcinogenesis of the urinary bladder, which partially explains the mechanism of tumor promotion by non-genotoxic carcinogens. Identifying the cellular and molecular targets involved in non-genotoxic chemical carcinogenesis is an important step toward providing a biological basis for the phenotypic changes observed during rat bladder carcinogenesis. It is of interest that the mechanisms involved in down-regulation of these important cell cycle related elements now be determined, because such findings could potentially lead to novel therapeutic strategies for urinary bladder carcinomas.

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