Cyclin D1 Overexpression in Rat Two-Stage Bladder Carcinogenesis and Its Relationship with Oncogenes, Tumor Suppressor Genes, and Cell Proliferation

Chyi Chia R. Lee, Shinji Yamamoto, Hideki Wanibuchi, Seiji Wada, Kazunobu Sugimura, Taketoshi Kishimoto, and Shoji Fukushima

First Department of Pathology [C. C. R. L., S. Y. H. W., S. F.] and Department of Urology [S. W., K. S., T. K.], Osaka City University Medical School, Osaka 545, Japan

ABSTRACT

Overexpression of cyclin D1 has been implicated in the malignant transformation of a variety of human cancers, including urinary bladder carcinomas. However, few reports have addressed the significance of cyclin D1 overexpression in chemical carcinogenesis in rodents. In the present study, we evaluated the oncogenic potential of cyclin D1 in experimental rat urinary bladder carcinogenesis and its relationships to the oncogenes cyclin E, K-ras, and H-ras as well as tumor suppressor genes p53 and p16WAF1/CIP1. In addition, proliferation status of preneoplastic lesions and tumors was assessed by proliferating cell nuclear antigen immunohistochemistry, and the results were comparable to perhaps the most important checkpoint in the mammalian cell cycle, the G1 phase of two-stage bladder carcinogenesis, overexpression of cyclin D1 protein was observed in 17% of papillary or nodular hyperplasias, 66% of papillomas, and 69% of transitional cell carcinomas, whereas nuclear accumulation of p53 was observed in none of the preneoplastic lesions and in fewer than 2% of transitional cell carcinomas. Overexpression of cyclin D1 in preneoplastic lesions and tumors was not dependent on the size of the tumors or their proliferation status. Quantitation of mRNA in tumors by multiplex reverse transcription-PCR showed that average mRNA expression of cyclin D1 and cyclin E was increased, whereas average p21WAF1/CIP1 mRNA expression was decreased. More than 2-fold overexpression of cyclin D1 mRNA was observed in 50 and 60% of tumors at weeks 18 and 24, respectively. Localization of cyclin D1 mRNA expression was demonstrated by in situ hybridization, and the results were comparable to immunohistochemical findings. None of the 25 tumors we examined by PCR-single-strand conformational polymorphism analysis harbored p53 mutations, H-ras mutations, or K-ras mutations. Thus, during the promotion phase of two-stage bladder carcinogenesis, overexpression of cyclin D1 in tumor cells may provide yet another mechanism by which tumors can gain a growth advantage. In contrast, tumors with mutated p53 may not have a growth advantage. Our results suggest that overexpression of cyclin D1 plays a critical role during urinary bladder carcinogenesis.

INTRODUCTION

Cyclin D1 is a member of the G1 cyclin family involved in regulating the transition through the G1 phase of the cell cycle (1, 2), perhaps the most important checkpoint in the mammalian cell cycle (3). Overexpression of cyclin D1 has been reported in various human malignant tumors, such as cancers of the esophagus (4), breast (5, 6), liver (7), lung (8), and urinary bladder (9). Cyclin D1 was initially implicated as an oncogene in parathyroid adenomas (10) and B-cell lymphomas (11), and experiments in transgenic mice have provided support for a role of cyclin D1 in the neoplastic transformation of mammary cells (12). In addition, a limited number of reports have addressed the significance of cyclin D1 overexpression in chemical carcinogenesis in rodents (13–18).

We reported recently that overexpression of cyclin D1 in human TCC of the urinary bladder (9) occurs exclusively in the low-grade, superficial, papillary tumors and is inversely correlated to nuclear accumulation of p53. In the present study, we evaluated the oncogenic potential of cyclin D1 in a well-established two-stage urinary bladder carcinogenesis model in rats, namely, initiation with BBN followed by promotion with Na-AsA (19, 20). Our aim was to assess the role of cyclin D1 overexpression and to analyze the stage-specific events that occur during multistage carcinogenesis of rat urinary bladder. We used immunohistochemical studies to analyze the expression and distribution of cyclin D1 in normal, preneoplastic, and neoplastic lesions of rat urinary bladder. In addition, quantitation and in situ localization of cyclin D1 mRNA expression in tumors were performed. Finally, we concluded the relationships between overexpression of cyclin D1 in relation to alterations of oncogenes cyclin E, K-ras, and H-ras as well as tumor suppressor genes p53 and p21WAF1/CIP1.

MATERIALS AND METHODS

Chemicals

BBN and Na-AsA were purchased from Tokyo Kasei Co. (Tokyo, Japan) and Wako Pure Chemical Industries, (Osaka, Japan), respectively.

Animals

A total of 116 6-week-old, male F344/DuCrj rats (Charles River Japan, Inc., Hino, Japan) were housed five per cage in an animal facility with a 12-h light-dark cycle at a temperature of 22 ± 2°C and 44 ± 5% relative humidity and were given free access to tap water and food (Oriental MF; Oriental Yeast Co., Tokyo, Japan). The animals were observed daily, and body weights were measured weekly throughout the duration of the experiment.

Experimental Design

Rats were divided into two groups, the experimental group and the control group. Rats of the experimental group were administered 0.05% BBN in their drinking water for 4 weeks followed by 3 days of nontreatment and then 5% Na-AsA in a powdered basal diet (Oriental MF) until sacrifice. Rats of the control group were maintained on the basal diet without any treatment. The total period of observation was 24 weeks, and subgroups were sacrificed at weeks 4, 8, 12, 18, and 24 in the experimental group and weeks 4 and 24 in the control group. At the time of sacrifice, 10 urinary bladders from the experimental group and 5 urinary bladders from the control group were fixed in 10% formaldehyde.

Received 5/19/97; accepted 9/5/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported in part by grants-in-aid for cancer research from the Ministry of Education, Science, Sports and Culture of Japan and by a grant from the Japan Science and Technology Corporation, included in the Project of Core Research for Evolutional Science, Sports and Culture of Japan and by a grant from the Japan Science and Technology (CREST) in Japan.

2 To whom requests for reprints should be addressed, at the First Department of Pathology, Osaka City University Medical School, 1-4-54 Asahi-machi, Abeno-ku, Osaka 545, Japan. Phone: 81-6-645-2041; Fax: 81-6-646-3093.

The abbreviations used are: TCC, transitional cell carcinoma; BBN, N-butyl-N-(4-hydroxybutyl)nitrosamine; Na-AsA, sodium L-ascorbate; PN, papillary or nodular; SCC, squamous cell carcinoma; PCNA, proliferating cell nuclear antigen; IPAP, image processor for analytical pathology; SSCP, single-strand conformational polymorphism; RT, reverse transcription; HPRT, hypoxanthine-guanine phosphoribosyltransferase.
neutral buffered formalin; 5 urinary bladders from both the experimental group and the control group were fixed in 4% buffered paraformaldehyde. The urinary bladders were distended with the fixative and then stored overnight at 4°C. After fixation, they were divided sagittally, and the number of tumors in each bladder was recorded. For histological analysis, the bladders were cut into 12 strips, routinely processed for embedding in paraffin, sectioned, and stained with H&E. For molecular assessment, four animals at week 18 and five animals at week 24 were sacrificed, and the three largest tumors from each urinary bladder were removed and stored at —80°C until they were analyzed. After the removal of tumors, the remaining bladder tissues were stretched and fixed in formalin for histological examinations. In addition, 12 control rats were sacrificed at week 24, and their urinary bladder mucosa was removed for molecular assessment.

Pathological Diagnosis
The histopathological lesions of the urinary bladder were classified into simple hyperplasia, PN hyperplasia, papilloma, TCC, squamous cell papilloma, and SCC, as described previously (21).

Immunohistochemistry
Serial sections of 3-μm thickness were made and spread on poly-L-lysine-coated slides. Paraffin sections were immersed in three changes of xylene and hydrated using a graded series of alcohols. Antigen retrieval was performed routinely by immersion of sections in distilled water inside a plastic container and by microwave heating for 20 min at low power. For PCNA immunohistochemistry, sections were blocked with goat serum at 37°C for 30 min and then incubated with mouse monoclonal anti-PCNA antibody (PC-10, IgG2a; DAKO Japan Co., Ltd.) at 1:250 dilution overnight at 4°C. For cyclin D1, p21WAF1/CIP1, or p53 immunohistochemistry, sections were blocked with horse serum and then incubated with either rabbit polyclonal antihuman cyclin D1 antibody (IgG; Upstate Biotechnology, Inc., Lake Placid, NY) at 1:200 dilution, rabbit polyclonal antihuman p21WAF1/CIP1 antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 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 Japan Co., Ltd.) at 42°C for 50 min, followed by heating at 70°C for 15 min. Washes were made to a final stringency of 0.1 X SSC at 50°C. A digoxigenin detection kit (Boehringer Mannheim) was used for detection of hybridized probes. Staining was developed with 5-bromo-4-chloro-3-indoxyl phosphate/nitroblue tetrazolium chloride substrate, and then the sections were counterstained for 5 min with nuclear fast red (Zymed Laboratories, Inc.).

Probe Templates and Probes for in Situ Hybridization
Five μg of total RNA, extracted from the liver of a control rat, served as the template for cDNA synthesis. A cyclin D1 fragment was generated by PCR amplification of cDNA for 45 cycles with the following pair of primers: 5'-ACAGCCTCTGTGCTCGCAAA-3' (upstream) and 5'-CAG-GACCTCTTCTGACAC-3' (downstream). Unincorporated nucleotides were removed by spin-column chromatography. Using a pCR-Script SK(+) Cloning Kit, the PCR products were inserted into a plasmid (pCR-Script SK(+): plasmid; Stratagene, La Jolla, CA) flanked by T7 and T3 promoters. The direction of inserted cyclin D1 sequence was determined by DNA sequencing. cDNA clones containing the template sequence for sense and antisense RNA probe synthesis were propagated, and the plasmid DNA was recovered with a plasmid extraction kit (Plasmid Midi; Qiagen, Inc., Chatsworth, CA). Antisense probes were generated by digestion with Not I (Wako, Osaka, Japan) followed by RNA synthesis with T7 RNA polymerase (Promega, Madison, WI); sense probes were generated by digestion with Hin-dIII (Takara Biomedicals, Otsu, Japan) followed by RNA synthesis with T3 polymerase (Promega).

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 smaller than 5 mm were homogenized whole, and larger lesions were divided into smaller pieces. After homogenization with a polytron homogenizer, total RNA was isolated using Isogen (Nippon Gene, Toyama, Japan). After removal of the RNA fraction, DNA was ethanol precipitated and then extracted with Sepagene (Sanko Junyaku Co., Tokyo, Japan). DNA concentrations were determined with a spectrophotometer and adjusted to a final concentration of 50 ng/μl with InstaGene Purification Matrix (Bio-Rad Laboratories, Hercules, CA). RNA was stored at —80°C and DNA at —30°C until it was analyzed. For the extraction of total RNA from normal rat urinary bladder epithelium, the urinary bladder was divided in half and preincubated in PBS (pH 7.0) containing 30 mm EDTA for 10 min at 37°C. Urothelium was removed by scraping with a clean glass slide, and the cells were precipitated by centrifugation. The urothelial cells were resuspended in Isogen and lysed by repeated aspiration through a syringe fitted with a 23-gauge needle. Control DNA for PCR-SSCP analyses was extracted from the liver and brain of control animals with SepaGene.

Multiplex RT-PCR
Bladder tumors obtained from the 18th- and 24th-week sacrifices were available for RNA extraction, and the expression of cyclin D1, p21WAF1/CIP1 and cyclin E mRNA was examined by multiplex RT-PCR. In addition, total RNA was extracted from the bladder mucosa of 12 control rats, age 24 weeks. RNA concentrations were determined with a spectrophotometer. For cDNA synthesis, 3 μg of total RNA were mixed with 1 μl of oligodeoxynucleotide primer (Biotech International Ltd., Bentley, Australia) and 1 μl of RNase inhibitor (Boehringer Mannheim) in a total volume of 9 μl. The samples were heated to 70°C for 10 min and then placed immediately on ice for 10 min. To each sample, 4 μl of 5 X reverse transcriptase buffer, 2 μl of 0.1 M DTT, 4 μl of deoxynucleotide triphosphate (2.5 mm) and 1 μl of Superscript II reverse transcriptase (Life Technologies, Inc.) were added. Reverse transcriptions were carried out at 42°C for 50 min, followed by heating at 70°C for 15 min. Template RNA was degraded by the addition of RNase (Boehringer Mann-
heim), and the concentration of cDNA was adjusted to 100 ng/μl. Multiplex PCR was carried out with two sets of primers (30 cycles of 95°C for 60 s, 55°C for 60 s, and 72°C for 72 s) with sequences as follows: Cyclin D1, 5'-TGAACACCTATCGGGCGCA-3' (sense) and 5'-GGACGCAGCAACAGACATF-3' (antisense); Cyclin E, 5'-CTGGCTGAATGACTGTCC-3' (sense) and 5'-CTTCTTGGTTGCTGCC-3' (antisense); p21WAF1, 5'-TCTTGFCTGTCGCCCTC-3' (sense) and 5'-GGACGCAGCAACAGACATF-3' (antisense); H-ras exon 1, 5'-GCCTGCTGAAAATGACTGAG-3' (sense) and 5'-CTCTATCGTAGCCCTAC-3' (antisense); K-ras exon 1, 5'-GACTCCTACAGGAACTCGT-3' (sense) and 5'-CCAATAATAACCTTGGTACC-3' (antisense); K-ras exon 2, 5'-AGGACCCTGATCATATTC-3' (sense) and 5'-GCCTGCTGAAAATGACTGAG-3' (antisense); p53 exon 8 and 9, 5'-CTGGCTGTGCCTCCfCU-3' (sense) and 5'-TFAA000QAATfGGAGGCAG-3' (antisense); p21WAF1, 5'-TTAAGGGAATTGGAGCGCA-3' (sense) and 5'-GTCTCAGTGGCAGAAGTC-3' (antisense); Cyclin E, 5'-CTGGCTGAATGACTGTCC-3' (sense) and 5'-CTTCTTGGTTGCTGCC-3' (antisense); and H-ras exon 2, 5'-GACCTCCTAGGAACTCGT-3' (sense) and 5'-CTTCTTGGTTGCTGCC-3' (antisense).

PCR products were electrophoresed through 2% agarose gels containing ethidium bromide and visualized under UV illumination. The images were digitized, recorded onto a diskette, and quantitated with image analysis software (AT Biochem Inc., Malvern, PA) on a personal computer (Power Macintosh 8500, Apple Computer, Inc., Cupertino, CA). Peak intensity of the PCR product derived from the target gene was divided over HPRT, the internal control for multiplex PCR.

Northern Blot Analysis

To confirm the validity of multiplex RT-PCR, a preliminary experiment was performed to quantitate mRNA expression of cyclin D1 relative to HPRT in normal rat tissues using multiplex RT-PCR, and the results were compared to those of Northern blot analysis. The results obtained from the two different methods were in good agreement (data not shown). For Northern blot analysis, 10 μg samples of total RNA per lane were electrophoresed through 1% agarose, denatured for 5 min at 65°C in 1 M NaOH, neutralized in 1 M HCl, mixed with 20 μl of stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol; denatured for 5 min at 80°C; electrophoresed for 4 h at 100 V and transferred to a nylon membrane (Amersham, Little Chalfont, United Kingdom; Ref. 22). Hybridization was carried out overnight at 42°C in a hybridization oven. Washes were performed to a final stringency of 0.1 × SSC and 0.1% SDS at 65°C, and autoradiography was performed with Kodak XAR film (Eastman Kodak, Rochester, NY) exposed for 48 to 72 h at ~80°C between two intensifying screens. After prehybridization at 42°C for 5 h, 32P-labeled cyclin D1-specific cDNA probes were added, and hybridization was carried out overnight at 42°C in a hybridization oven. Washes were performed to a final stringency of 0.1 × SSC and 0.1% SDS at 65°C, and autoradiography was performed with Kodak XAR film (Eastman Kodak, Rochester, NY) exposed for 48 to 72 h at ~80°C between two intensifying screens. After obtaining the results of cyclin D1 hybridization, membranes were stripped and rehybridized to a HPRT probe to confirm equivalent loading of RNA in all lanes.

Probes for Northern Blot Analysis. Five μg of total RNA, extracted from the liver of a control rat, was the template for cDNA synthesis. The probes were generated by PCR amplification of cDNA for 45 cycles. The PCR products were electrophoresed through a 1% agarose gel to be sequenced. If shifted bands were observed by PCR-SSCP analysis, they were cut out from the acrylamide gel to be sequenced.

SSCP Analysis

Exons 5, 6, and 7, and 8 and 9 of p33 and exons 1 and 2 of K-ras and H-ras were analyzed by the PCR-SSCP method (23, 24). Fifty ng of genomic DNA were amplified in a reaction volume of 5 μl containing 50 nM [α-32P]dCTP (38 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s). PCR products were mixed with 20 μl of stop solution containing 95% formamide, 20 mM EDTA, 0.05% bromphenol 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 Inc., Malvern, PA) and electrophoresed for 16 h at 6 W. The sequences of primers for the PCR-SSCP analysis were as follows: p53 exon 5, 5'-GATTCTTTTCTCTCTCTAC-3' (sense) and 5'-ACAGCAAGTGGCAGTCTCA-3' (antisense); p53 exon 6 and 7, 5'- CTTCTGACCTTATTCTTGCTC-3' (sense) and 5'-CCTTCTGTACTTCTTGCTC-3' (antisense); and 5'-CCTTCTGACCTTATTCTTGCTC-3' (sense) and 5'-CCTTCTGTACTTCTTGCTC-3' (antisense); p53 exon 8 and 9, 5'-CCTTCTGTACTTCTTGCTC-3' (sense) and 5'-CCTTCTGTACTTCTTGCTC-3' (antisense); and 5'-CCTTCTGTACTTCTTGCTC-3' (sense) and 5'-CCTTCTGTACTTCTTGCTC-3' (antisense); and 5'-CCTTCTGTACTTCTTGCTC-3' (sense) and 5'-CCTTCTGTACTTCTTGCTC-3' (antisense); and 5'-CCTTCTGTACTTCTTGCTC-3' (sense) and 5'-CCTTCTGTACTTCTTGCTC-3' (antisense).

PCR-SSCP was repeated at least twice 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 a Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using the Applied Biosystems Model 373A DNA Sequencing System.

Statistical Analysis

Unless otherwise indicated, statistical analyses were completed with StatView software (Version 4.02, Abacus Concepts, Inc., Berkeley, CA) for the Macintosh microcomputer, and significant differences were determined by the Student's t test. A result was only considered significant if P was <0.05.

RESULTS

Overexpression of Cyclin D1 during Various Stages of Rat Bladder Carcinogenesis. Seventy-five rats from the experimental group and 20 rats from the control group were examined for overexpression of cyclin D1 by immunohistochemistry. Normal bladder mucosa was negative for cyclin D1 staining. Overexpression of cyclin D1 in preneoplastic lesions and tumors was defined as positive when nuclear staining of >5% of nuclei (9) was evident. The results are summarized in Table 1. At week 4, PN hyperplasia appeared, and positive nuclear staining of cyclin D1 was noted in 28% of these lesions (Figs. 1, C and D, and 2A; Table 1). At week 12, PN hyperplasia became more numerous, and papillomas appeared. Overexpression of cyclin D1 during various stages of rat bladder carcinogenesis was defined as more than 5% positive nuclear staining.

Table 1 Immunohistochemical assessment of the incidence of cyclin D1 overexpression in rat urinary bladder lesions induced by BBN and promoted by sodium l-ascorbate

<table>
<thead>
<tr>
<th>Duration (weeks)</th>
<th>Effective no. of rats</th>
<th>PN hyperplasia</th>
<th>Papilloma</th>
<th>TCC</th>
<th>Others</th>
<th>Total no. of lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>15</td>
<td>52 of 183 (28%)</td>
<td>38 of 50 (76%)</td>
<td>73 of 95 (77%)</td>
<td>52 of 183 (28%)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>200 of 1185 (17%)</td>
<td>48 of 73 (66%)</td>
<td>125 of 181 (69%)</td>
<td>239 of 1236 (19%)</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>20</td>
<td>89 of 654 (14%)</td>
<td>11 of 25 (44%)</td>
<td>52 of 86 (69%)</td>
<td>214 of 826 (26%)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>15</td>
<td>38 of 234 (16%)</td>
<td>97 of 148 (66%)</td>
<td>101 of 345 (29%)</td>
<td>606 of 2590 (23%)</td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>15</td>
<td>379 of 2256 (17%)</td>
<td>125 of 181 (69%)</td>
<td>5 of 5 (100%)</td>
<td>214 of 826 (26%)</td>
<td></td>
</tr>
</tbody>
</table>

* Overexpression of cyclin D1 in urinary bladder lesion is defined as more than 5% positive nuclear staining.

* Papilloma, transitional cell papilloma.

* Others, includes squamous cell papilloma and SCC.

* A squamous cell papilloma.

* Includes one squamous cell papilloma and three SCCs.
staining of cyclin D1 was detected in 16% of PN hyperplasia lesions, in 44% of papillomas, and in 60% of TCCs (Table I). Two squamous cell papillomas (Fig. 4, A and B), one at week 12 and another at week 18, and three SCCs (Fig. 4, C and D) at week 18 were observed; these were also cyclin D1 positive. The intensity of cyclin D1 staining was highly variable among the various lesions of the urinary bladder, and generally weak in simple hyperplasias and PN hyperplasias, but intense in papillomas and TCCs.

**Quantitation of Cyclin D1 and PCNA Immunohistochemistry by Image Analysis.** The results of cyclin D1 and PCNA immunohistochemistry were quantitated on an IPAP image analyzer. Positively stained lesions were selected for quantitation, and the results are presented in Fig. 5. Among the cyclin D1-positive lesions, in lesions with more than 5% positive nuclear staining, cyclin D1-positive indices were not significantly different between PN hyperplasia, papilloma, or TCC. However, progressive increases in PCNA-positive indices from PN hyperplasia to papilloma to TCC were observed.

**Topological Distribution of Cyclin D1 Relative to PCNA.** To determine whether the increased expression of cyclin D1 in preneoplastic lesions and tumors was simply due to increased cell proliferation, serial sections were stained for both cyclin D1 and PCNA (25). In simple hyperplasias (Fig. 1, A and B), the distribution of cyclin D1-positive nuclei was heterogeneous and did not correspond to PCNA-positive nuclei. Similar findings were obtained for PN hyperplasias; the distribution of cyclin D1-positive nuclei was generally heterogeneous (Fig. 1C and D). In the larger PN hyperplasias, nuclear staining of cyclin D1 was sometimes regional (Fig. 2A). In papillomas, cyclin D1-positive nuclei were located superficially (Fig. 2, B–D), whereas PCNA was diffusely positive. Two patterns of cyclin D1 staining were observed in TCCs, either regional (Fig. 3A) or diffuse (Fig. 3B). PCNA staining in TCCs was generally diffuse, irrespective of the cyclin D1 status (Fig. 3C); in fact, many of the TCCs that were negative for cyclin D1 also exhibited extensive PCNA staining. Mitotic cells were frequent in TCCs, but they were always devoid of cyclin D1 staining (Fig. 3D). The squamous metaplasias and the SCC had distinct patterns of cyclin D1 staining; basal cells were positive for both cyclin D1 and PCNA (Fig. 4, A, B, and D). Double staining of cyclin D1 and PCNA in the SCC (Fig. 4C) clearly demonstrated the expression of both of these proteins in individual basal cells (Fig. 4D).

**Quantitation of Cyclin D1, p21WAF1/Cip1, and Cyclin E mRNA Expression in Tumors.** PCR products of cyclin D1, cyclin E, and p21WAF1/Cip1 in the multiplex RT-PCR reaction were quantitated against HPRT as a standard. To establish the baseline expression of various genes, mRNAs were extracted individually from 12 control animals, and RT-PCR reactions were carried out for each. The average mRNA levels in the control animals were assigned an arbitrary value of 1. Expression of cyclin D1 (Fig. 6A) and cyclin E (Fig. 6B) was increased in tumors, whereas average p21WAF1/Cip1 mRNA expression was decreased (Fig. 6C). The average cyclin D1 mRNA expression in tumors at week 18 was more than two times higher than in normal bladder mucosa and was increased further in tumors at week...
The average cyclin E mRNA expression peaked at week 18 and decreased slightly at week 24. The average p21WAF1/Cip1 mRNA expression was decreased at week 18 and decreased further at week 24. More than 2-fold overexpression of cyclin D1 mRNA was observed in 50 and 60% of tumors at weeks 18 and 24, respectively (Table 2). The expression of cyclin D1 mRNA varied widely among the tumors and did not appear to be correlated to tumor size (Fig. 7). These findings further validated the results of cyclin D1 immunohistochemistry.

Localisation of Cyclin D1 mRNA Expression. Serial sections were made from the paraformaldehyde-embedded specimens and were available for immunohistochemistry and in situ hybridization of cyclin D1. Following immunohistochemistry of cyclin D1, positively stained specimens were included for in situ hybridization. The pattern of cyclin D1 mRNA expression generally corresponded to that for the protein. Hybridization with the antisense probe showed a distribution in the cytoplasm of tumor cells as blue granules (Fig. 3E); hybridization with the sense probe did not generate any signals (Fig. 3F).

Nuclear Accumulation of p53 in TCCs. Two of the TCCs evaluated immunohistochemically, one from the 18th week (Fig. 8A) and another from the 24th week (Fig. 8B), were found to have nuclear accumulation of p53 protein in more than 20% of their tumor nuclei, in line with the reported mutations of the p53 gene in human TCCs of the urinary bladder (26). Strong, diffuse staining of p53 was not present in any of the preneoplastic lesions. Some of the larger TCCs were stained very faintly with the anti-p53 antibody, but this probably represented expression of the wild-type p53, because the antibody CM1 recognizes both mutant and wild-type p53 in formalin-fixed, paraffin-embedded specimens. Overall, p53-positive tumors comprised fewer than 2% of all tumors that we evaluated. In addition, both of the p53-positive tumors (Fig. 8E) were devoid of cyclin D1 staining (Fig. 8D).

Elevated Cyclin D1 mRNA Levels Corresponds to Protein Overexpression. Serial sections were made from paraformaldehyde-embedded specimens for cyclin D1 immunohistochemistry and in situ hybridization. Increased mRNA expression in the cytoplasm of tumor cells, detectable by in situ hybridization, corresponded to the results of immunohistochemistry. On the basis of the results of mRNA quantitation by multiplex RT-PCR, 50% of the tumors from week 18 and 60% of the tumors from week 24 had more than 2-fold expression of cyclin D1 mRNA (Table 2; Fig. 7). This result was comparable to the results of immunohistochemistry, because more than 60% of the TCCs from weeks 18 or 24 had more than 5% of their tumor nuclei overexpressing cyclin D1 (Table 1).

Absence of p53, H-ras, and K-ras Mutations in Tumors. Twelve tumors from week 18 and 15 tumors from week 24 were examined for the presence of p53, H-ras, and K-ras mutations by PCR-SSCP analysis. None of the tumors we examined harbored mutations within exons 5–9 of the p53 gene, exons 1 and 2 of the H-ras gene, or exons 1 and 2 of the K-ras gene (Table 2).
Expression of p21WAF1/CIP1 in Tumors and Preneoplastic Lesions. Nuclear staining of p21WAF1/CIP1 was sometimes observed in preneoplastic lesions and in TCCs. Occasionally, PN hyperplasia showed nuclear staining of p21WAF1/CIP1 (Fig. 9A). In TCCs, the pattern of p21WAF1/CIP1 staining was usually superficial and confined to small regions (Fig. 9B).

Topographic Distribution of Cyclin D1 Relative to p21WAF1/CIP1 and PCNA. To evaluate the relationship between cyclin D1 expression relative to p21WAF1/CIP1 and cell proliferation, the expression of p21WAF1/CIP1, cyclin D1, and PCNA was assessed by immunohistochemistry. Consistent with our previous observations, cyclin D1 expression was limited to the superficial cells in a papilloma (Fig. 9C).
Two patterns of p21WAF1/Cip1 expression were observed in this papilloma, exclusively nuclear or predominantly nuclear with extensive cytoplasmic staining. The exclusively nuclear pattern of p21WAF1/Cip1 staining was located superficially, whereas the cytoplasmic staining was confined to deeper regions (Fig. 9, D and F). PCNA positive nuclei were distributed diffusely and were not confined to any particular region (Fig. 9E).

**DISCUSSION**

We have presented evidence that aberrant expression of cyclin D1 is an early and frequent molecular event in two-stage bladder carcinogenesis in rats. Thus, nuclear staining of cyclin D1 was not present in normal bladder mucosa but appeared in simple hyperplasias as early as 4 weeks after the administration of BBN. PN hyperplasias,
which are putative neoplastic lesions of the rat urinary bladder (21, 27–29), appeared at week 8, and a substantial number of these lesions were cyclin D1 positive. On the basis of our observations, we hypothesize that PN lesions with extensive cyclin D1 overexpression are more likely to progress to advanced lesions, such as papillomas or carcinomas in the current bladder carcinogenesis protocol, and that overexpression of cyclin D1 in neoplastic lesions is probably related to the acquisition of autonomous growth properties. This conclusion is supported by the following: (a) the percentage of cyclin D1-positive lesions at weeks 8, 12, 18, and 24 remained relatively constant (ranged from 19 to 29%; Table 1); (b) the total number of cyclin D1-positive PN lesions at weeks 8 and 12 (n = 252) closely approximated the total number of cyclin D1-positive advanced lesions that developed later, namely papillomas, TCCs, squamous cell papillomas and SCCs (n = 227); and (c) among the cyclin D1-positive lesions, lesions with more than 5% cyclin D1-positive nuclei, the percentage of cyclin D1-positive nuclei in PN hyperplasia closely approximated those of papilloma and TCC (Fig. 5).

The D-type cyclins have a very short half-life of about 30 min, and their expression is generally highly growth factor inducible (1). Using sensitive and semiquantitative multiplex RT-PCR, we have demonstrated conclusively here that cyclin D1 is activated transcriptionally in bladder tumors. Substantially increased expression of cyclin D1 mRNA was noted for the majority of bladder tumors, and the results were shown to be comparable with those for immunohistochemistry. In situ hybridization permitted localization of cyclin D1 mRNA expression in the cytoplasm of tumor cells. The increase in cyclin D1 mRNA expression did not appear to be correlated to the size of tumors; therefore, we hypothesize that overexpression of cyclin D1 in tumors may not be directly related to increased cell proliferation.

Nuclear staining of cyclin D1 was usually intense and extensive in papillomas and TCCs after weeks 12 and 18, respectively. The following observations suggest that cyclin D1 overexpression in papillomas and TCCs is unlikely to be the direct consequence of increased cell proliferation: (a) the distribution of cyclin D1-positive nuclei in papillomas was almost exclusively superficial (Fig., 2, B–D) and was in contrast to the PCNA-positive nuclei, which were located predominantly in the basal cell layers or the proliferative compartments; (b) the distribution of cyclin D1-positive nuclei in some TCCs (Fig. 3A) was confined to small regions near the surface, whereas the distribution of PCNA-positive nuclei in TCCs (Fig. 3C) was diffuse and extensive; (c) among the cyclin D1-positive lesions with more than 5% positive nuclear staining, the cyclin D1-positive indices did not differ significantly among PN hyperplasias, papillomas, and TCCs, whereas the percentages of PCNA-positive nuclei increased progressively from PN hyperplasia to papilloma to TCC (Fig. 5); and (d) a wide variation in terms of cyclin D1 expression was observed in tumors; many tumors showed either rare staining for cyclin D1 or minimal increases in cyclin D1 mRNA levels, whereas no such variation was noted for PCNA staining. In fact, several previous reports have noted no direct relationship between cyclin D1 overexpression and increased PCNA staining in tumors of rodents (13–16).

Two findings lead us to hypothesize that overexpression of cyclin D1 may have provided a growth advantage for tumors under the conditions of the current two-stage bladder carcinogenesis protocol: (a) more than 2-fold overexpression of cyclin D1 mRNA was ob-

---

**Table 2 Molecular assessment of cyclin D1 mRNA overexpression and p53, K-ras, and H-ras gene mutations 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</th>
<th>Average size of tumors (mm)</th>
<th>Cyclin D1 mRNA overexpression</th>
<th>Mutations&lt;sup&gt;a&lt;/sup&gt;&lt;br&gt;</th>
<th>p53</th>
<th>K-ras</th>
<th>H-ras</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>4</td>
<td>12</td>
<td>3.67 ± 1.61</td>
<td>6 of 12 (50%)</td>
<td>0 of 12 (0%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>5</td>
<td>15</td>
<td>5.80 ± 1.70</td>
<td>9 of 15 (60%)</td>
<td>0 of 15 (0%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<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> Cyclin D1 mRNA expression was assessed by multiplex RT-PCR, and overexpression of cyclin D1 mRNA in tumors is defined as more than 2-fold mRNA expression in comparison to the average of normal bladder mucosa.  
<sup>d</sup> p53, K-ras, and H-ras gene mutations were assessed by PCR-SSCP analysis.
served in 50 and 60% of the tumors at weeks 18 and 24, respectively (Table 2), and (b) the majority of papillomas and TCCs evaluated showed nuclear staining of cyclin D1 (Table 1). In contrast, fewer than 2% of the TCCs we examined by immunohistochemistry had nuclear accumulation of p53, a feature that closely relates to mutations in the p53 gene in human high-grade bladder cancers (26). None of the TCCs we examined by PCR-SSCP analysis contained mutations of p53 within exons 5-9. Both of these findings suggest that p53 alterations are very rare in bladder tumors induced with BBN and promoted by Na-AsA. In a similar two-stage rat bladder carcinogenesis model, initiation with N-[4-(5-nitro-2-furyl)-2-thiazole]formamide for 6 weeks followed by promotion with 5% sodium ascorbate until week 78, p53 mutations were observed in only 2 of 17 bladder tumors (30).

Rat bladder tumors induced under the current two-stage carcinogenesis protocol were almost exclusively papillary and noninvasive, features that closely correlate to overexpression of cyclin D1 in TCCs of human bladder (9). Papillary TCCs are often multifocal and only occasionally progress, but flat-type tumors, such as carcinomas in situ, frequently progress to invasive disease. Potentially, two molecular pathways may be involved in the progression of TCC, one of which features the inactivation of the p53 gene, which occurs frequently in TCCs; cells involved in active proliferation within the tumor were positive for both cyclin D1 and PCNA (Fig. 4D). The pattern of cyclin D1 staining in these transformed lesions does not resemble that in PN hyperplasias, papillomas, or TCCs, but it is similar to that in mouse skin carcinogenesis (17), also a model involving cyclin D1 overexpression in squamous epithelium.

Cyclins are positive regulators of cell cycle progression. Accumulating evidence suggests that overexpression of cyclin E may also participate in tumorigenesis (32). Although overexpression of cyclin E has not been implicated in urinary bladder carcinogenesis of rodents, it has been found in mouse mammary tumors (14), in N-methyl-N-nitrosourea-induced primary rat mammary tumors (18), and in N-nitrosomethylbenzylamine-induced rat esophageal papillomas (15). The following observation suggests that overexpression of cyclin D1 may have a dominant effect over cyclin E in two-stage bladder carcinogenesis of rats. The results of multiplex RT-PCR analysis showed more than 2-fold average cyclin D1 mRNA expression present in tumors from week 18 when compared to that of normal bladder mucosa, and the expression was increased further in tumors from week 24 (Fig. 6A). In contrast, only moderate increases of cyclin E mRNA expression occurred in the week 18 tumors, but the level of expression decreased in the tumors by week 24 (Fig. 6B). These findings also suggest that cyclin D1 is overexpressed preferentially in the two-stage bladder carcinogenesis protocol. Consistent with our results, preferential expression of cyclin D1, but not cyclin E, has been observed in tumors derived from the two-stage mouse skin carcinogenesis protocol using 7, 12-dimethylbenz(a)anthracen as the initiator and 12-O-tetradecanoylphorbol-13-acetate as the promoter; in contrast, increased expression of cyclin E was seen in most tumors induced by a complete carcinogenesis protocol with benzo(a)pyrene (33). The significance of increased cyclin E mRNA expression in bladder tumors remains to be determined. In addition, it remains to be seen whether increased expression of cyclin D1 and cyclin E has cooperative effects in rat bladder carcinogenesis.

Although the significance of H-ras and K-ras mutations in rat bladder tumors induced in two-stage bladder carcinogenesis has not been elucidated completely, our results indicate that alterations of these candidate oncogenes are not likely to be a common genetic defect. This assumption is based on the results of PCR-SSCP analysis,
In addition, p21WAF1/CIP1 has also been shown to induce a cell-cycle arrest by direct binding to PCNA (37). We evaluated the topographic expression of p21WAF1/CIP1 in preneoplastic lesions and tumors of the rat urinary bladder by immunohistochemistry and determined the average expression of p21WAF1/CIP1 mRNA levels in tumors. When we applied immunohistochemistry of p21WAF1/CIP1 to preneoplastic lesions and tumors, aberrant expression of p21WAF1/CIP1 was observed occasionally in some PN hyperplasias, papillomas, and TCCs (Fig. 9, A, B, D, and F). The expression of p21WAF1/CIP1 was generally confined to small regions within the tumors and did not correspond to

which showed the absence of H-ras and K-ras mutations in the week 18 and week 24 tumors (Table 2). In an analogous two-stage rat bladder carcinogenesis model (initiation with BBN for 4 weeks followed by promotion with 3% uracil until week 24), H-ras and K-ras mutations were not found in any of the 20 urinary bladder tumors examined (34).

The activity of cyclin-CDK complexes is regulated by two families of proteins that inhibit cell cycle progression. p21WAF1/CIP1 is a member of the CDK inhibitors that inhibit kinase activities of preactivated G1 cyclins, such as the cyclin D1-CDK4 complexes (35, 36).

In addition, p21WAF1/CIP1 has also been shown to induce a cell-cycle arrest by direct binding to PCNA (37). We evaluated the topographic expression of p21WAF1/CIP1 in preneoplastic lesions and tumors of the rat urinary bladder by immunohistochemistry and determined the average expression of p21WAF1/CIP1 mRNA levels in tumors. When we applied immunohistochemistry of p21WAF1/CIP1 to preneoplastic lesions and tumors, aberrant expression of p21WAF1/CIP1 was observed occasionally in some PN hyperplasias, papillomas, and TCCs (Fig. 9, A, B, D, and F). The expression of p21WAF1/CIP1 was generally confined to small regions within the tumors and did not correspond to

Fig. 8. Immunohistochemistry of p53 in bladder TCC and its relationship with cyclin D1 and PCNA. A, an 18-week TCC shows nuclear accumulation of p53. B, a 24-week TCC with nuclear accumulation of p53. C, H&E staining of a serial section corresponding to B. D, cyclin D1 staining of another serial section shows only a few positive nuclei. E, low magnification of B showing extensive p53 staining. F, proliferative status of the TCC as demonstrated by PCNA staining. Magnification: A and B, ×50; C–F, ×25.
the expression of cyclin D1 or PCNA; this may have been due to the altered constituents of cyclin complexes in tumors (38, 39). There is some evidence that posttranslational modification, as well as the stoichiometry of p21WAF1/Cip1 and cyclin-CDK complexes, may regulate its activity (40). We observed decreased average p21WAF1/Cip1 mRNA expression in tumors compared to normal bladder mucosa (Fig. 6C). p21WAF1/Cip1 can act 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 (41). The observation that the average p21WAF1/Cip1 mRNA expression in tumors was lower than that of normal bladder mucosa suggests that p21WAF1/Cip1 expression may be transcriptionally repressed. It is possible that changes in the overall level of p21WAF1/Cip1 mRNA expression could play a role in two-stage urinary bladder carcinogenesis of rats.

In conclusion, aberrant expression of cyclin D1, but not p53 inac-
ivation, appears to be associated with oncogenic transformation of the transitional epithelium in this well-characterized model of experimental urinary bladder carcinogenesis. The present study not only demonstrates the importance of cyclin D1 overexpression during molecular carcinogenesis of bladder tumors in rats but also provides an incentive for additional research on the role of cyclin D1 in the oncogenesis and progression of bladder cancers.

REFERENCES

30. Ito, N., and Fukushima, S. Promotion of urinary bladder carcinogenesis in experi-
Cyclin D1 Overexpression in Rat Two-Stage Bladder Carcinogenesis and Its Relationship with Oncogenes, Tumor Suppressor Genes, and Cell Proliferation

Chyi Chia R. Lee, Shinji Yamamoto, Hideki Wanibuchi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/57/21/4765

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.