Cell Cycle Checkpoint Function in Bladder Cancer

Sharon C. Doherty, Stephanie R. McKeown, Valerie McKelvey-Martin, C. Stephen Downes, Anthony Atala, James J. Yoo, Dennis A. Simpson, William K. Kaufmann

Background: Cell cycle checkpoints function to maintain genetic stability by providing additional time for repair of DNA damage and completion of events that are necessary for accurate cell division. Some checkpoints, such as the DNA damage G1 checkpoint, are dependent on p53, whereas other checkpoints, such as the decatenation G2 checkpoint, are not. Because bladder transitional cell carcinomas (TCCs) often contain numerous chromosomal aberrations and appear to have highly unstable genomes, we analyzed cell cycle checkpoint functions in a panel of TCC lines.

Methods: Cell cycle arrest was induced in normal human fibroblasts (NHF1-hTERT) and normal human uroepithelial cells (HUCs), and TCC lines and checkpoint functions were quantified using flow cytometry and fluorescence microscopy. The inducers and checkpoints were ionizing radiation (i.e., DNA damage) (G1 and G2 checkpoints), the mitotic inhibitor colcemid (polyploidy checkpoint), or the topoisomerase II catalytic inhibitor ICRF-193 (decatenation G2 checkpoint). Four of the five TCC lines expressed mutant p53.

Results: HUCs had an effective G1 checkpoint response to ionizing radiation, with 68% of cells inhibited from moving from G1 into S phase. By contrast, G1 checkpoint function was severely attenuated (<15% inhibition) in three of the five TCC lines and moderately attenuated (<50% inhibition) in the other two lines. NHF1-hTERT had an effective polyploidy checkpoint response, but three of five TCC lines were defective in this checkpoint. HUCs had effective ionizing radiation and decatenation G2 checkpoint responses. All TCC lines had a relatively effective G2 checkpoint response to DNA damage, although the responses of two of the TCC lines were moderately attenuated relative to HUCs. All TCC lines had a severe defect in the decatenation G2 checkpoint response.

Conclusion: Bladder TCC lines have defective cell cycle checkpoint functions, suggesting that the p53-independent decatenation G2 checkpoint may cooperate with the p53-dependent G1 checkpoints to preserve chromosomal stability and suppress bladder carcinogenesis. [J Natl Cancer Inst 2003;95:1859–68]

Transitional cell carcinomas (TCCs) account for more than 90% of bladder cancers (1). The molecular, genetic, and cellular changes that occur in TCC of the bladder are numerous and include chromosomal alterations, loss of heterozygosity, and loss of cell growth regulation. Loss of heterozygosity has been reported for many chromosomal regions in TCCs, including sites...

Affiliations of authors: Cancer and Ageing Research Group, School of Biomedical Sciences, University of Ulster, Coleraine, Northern Ireland (SCD, VMM, CSD); Radiation Science Research Group, School of Applied Medical Science and Sports Studies, University of Ulster, Jordanstown, Northern Ireland (SCD, SRM); Laboratory for Tissue Engineering and Cellular Therapeutics, Children’s Hospital and Harvard Medical School, Boston, MA (AA, JJY); Department of Pathology and Laboratory Medicine, Lineberger Comprehensive Cancer Center and Center for Environmental Health and Susceptibility, University of North Carolina at Chapel Hill, Chapel Hill (DAS, WKK).

Correspondence to: William K. Kaufmann, PhD, Department of Pathology and Laboratory Medicine, Lineberger Comprehensive Cancer Center and Center for Environmental Health and Susceptibility, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295 (e-mail: bill.kaufmann@med.unc.edu).

See “Notes” following “References.”

DOI: 10.1093/jnci/djg120

Journal of the National Cancer Institute, Vol. 95, No. 24, © Oxford University Press 2003, all rights reserved.
on 3p, 4p, 8p, 9p, 9q, 11p, 13q, 14q, 17p, and 18q (2–5). Several of these genetic alterations have been linked with inactivation of tumor suppressor genes. In bladder TCC, two of the most frequently inactivated tumor suppressor genes are the retinoblastoma (RB) and p53 genes. Genetic alterations in RB and p53 occur in approximately 35% and 50% of bladder cancers, respectively, and are associated with high-stage and high-grade TCCs (6–9). The RB and p53 genes encode key proteins of the cell cycle checkpoints that arrest cells in G1. Deletion of 9p21 is also a common and early event in the development of TCC (10); this region encodes three distinct proteins (p16, ARF, and p15 in humans) that are negative regulators of RB and p53 function. Thus, bladder cancers display mutations in cell cycle checkpoint genes that are associated with malignant growth and genetic instability.

There are two major classes of cell cycle checkpoints: DNA damage checkpoints and dependency checkpoints. DNA damage checkpoints delay cell cycle transitions from G1 to S and from G2 to M, thereby providing more time for DNA repair. Essential components of the G1 checkpoint include ATM, p53, RB, Chk2, and p21Waf1 (11,12). DNA damage activates ATM kinase, which phosphorylates p53 and Chk2, leading to the induction and activation of p53. In turn, p53 transactivates p21Waf1, which inhibits the G1 cyclin–dependent kinases that normally inactivate RB, and thereby represses the E2F transcription factors that initiate S phase (13,14). Key protein regulators of the G2 checkpoint include ATM, ATR, NBS1, Plk1, BRCA1, Chk1, Cdc25C, Wee1, Myt1, and 14-3-3σ/ε (15–18). G2 arrest or block that occurs after DNA damage is achieved by three mechanisms; two act immediately and simultaneously, and the third acts after a delay of several hours. The first mechanism is an ATM- and BRCA1-dependent phosphorylation of Cdc25C by the Chk1 protein kinase (19). This phosphorylation event promotes an interaction between Cdc25C and 14-3-3 proteins that sequesters Cdc25C into the cytosol and prevents activation of the nuclear pool of Cdk1/cyclin B1, thereby delaying mitosis (20,21). The second mechanism is the exclusion of cyclin B1 from the nucleus (22) by the Crm1 nuclear exporter protein (23). In the absence of DNA damage, Plk1 phosphorylates cyclin B1 within the nuclear export sequence, blocking interaction with Crm1 and promoting nuclear accumulation of cyclin B1 (24). In the presence of DNA damage, ATM- and ATR-dependent inhibition of Plk1 sustains nuclear exclusion of cyclin B1, thereby delaying mitosis. These immediate ATM-dependent G2 checkpoint responses to DNA damage are independent of p53 (25,26). The delayed third mechanism involves the p53-dependent induction of 14-3-3σ and p21Waf1 and may serve to sustain mitotic delay after high doses of ionizing radiation (26,27).

Dependency checkpoints, such as the spindle damage, polyploidy, and decatenation checkpoints, are those that delay cell cycle phase transitions when essential cell cycle events have not been completed. The spindle damage checkpoint delays anaphase and chromosome segregation until metaphase has been completed. p53 enforces a polyploidy checkpoint that prevents initiation of DNA synthesis pending completion of mitosis and cytokinesis (28). The decatenation checkpoint delays entry into mitosis until newly synthesized daughter DNA molecules are sufficiently decatenated by DNA topoisomerase II to permit chromosome condensation and segregation (29,30). ICRF-193 is a topoisomerase II catalytic inhibitor that keeps the enzyme in the form of a closed clamp so that it cannot form a covalent complex with DNA. When cells in G2 phase are incubated briefly with ICRF-193, decatenation is blocked and entry into mitosis is delayed (29,31,32). The mitotic delay in response to ICRF-193 is not a DNA damage checkpoint because it is independent of ATM and p53 activities and does not involve activation of Chk1 or Chk2 (30,33). Genetic evidence for the decatenation checkpoint function was demonstrated by the expression of a kinase-inactive ATR allele that ablated the mitotic delay induced by ICRF-193 (30). A requirement for the breast and ovarian cancer tumor suppressor gene BRCA1 in the decatenation checkpoint was also indicated, suggesting that malignant progression might include inactivation of the decatenation checkpoint function (30). The decatenation checkpoint delays mitosis by sustaining cytosolic sequestration of Cdk1–cyclin B1 complexes through the inhibition of Plk1 activity (16,30).

Ablation or attenuation of cell cycle checkpoint function is associated with increased growth and genetic instability, two hallmarks of cancer. In this study, we hypothesized that the inactivation of checkpoint functions may contribute to the genetic instability in bladder cancer. We therefore evaluated whether there were differences in the function of DNA damage and dependency checkpoints in human uroepithelial cells (HUCs) and a series of bladder cancer cell lines.

**MATERIALS AND METHODS**

**Cell Lines and Cell Culture**

Normal HUCs were obtained from biopsy tissue from pediatric patients at Children’s Hospital in Boston, MA. The primary cultures were established at Harvard Medical School and shipped to the University of North Carolina at Chapel Hill Lineberger Comprehensive Cancer Center at passages 3–5. The cells were confirmed to be of bladder epithelial origin as described (34) and were grown using serum-free keratinocyte medium (Invitrogen Life Technologies, Carlsbad, CA) supplemented with bovine pituitary extract at 50 μg/mL and epidermal growth factor at 10 ng/mL. Checkpoint function was analyzed in HUCs within two passages of their receipt at the University of North Carolina at Chapel Hill.

The following bladder cancer lines were obtained from American Type Culture Collection (Manassas, VA): T24, J82, TCC-SUP, RT4, and UM-UC-3. The T24 and RT4 lines were maintained in McCoy’s 5A medium supplemented with 10% fetal bovine serum (FBS). The TCC-SUP and UM-UC-3 lines were maintained in minimum essential medium (MEM) supplemented with 1 mM sodium pyruvate and 10% FBS. The J82 line was maintained in MEM with nonessential amino acids and Earle’s basic salt solution supplemented with 10% FBS. Diploid human fibroblasts, obtained from neonatal foreskin at the University of North Carolina at Chapel Hill, were engineered to express the catalytic subunit of telomerase (NHFL1-hTERT) and maintained in MEM supplemented with 10% FBS and 2 mM glutamine as checkpoint-proficient controls (16,30). All cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO2. Four of the five TCC lines have mutant p53 (Table 1).

**G1 Checkpoint Function**

G1 checkpoint function was quantified using a flow cytometric assay, as described (35–37). HUC cells, NHFL1-hTERT, and the bladder TCC lines, when growing logarithmically, were...
\*NHF1-hTERT = normal human fibroblasts expressing the human catalytic subunit of telomerase; DC = decatenation; PP = polyploid; RB = retinoblastoma; ND = not determined; wt = wild-type allele; mt = mutant allele; hd = homoyzogous deletion (ARF and p16).

†Cell cycle checkpoint scoring: + = effective or modestly attenuated (although the DNA damage G1 checkpoint response in J82 and T24 lines was significantly altered in comparison to that of HUCs. >90% of G1 cells in these cancer cell lines delayed entry into mitosis); +/- = moderately attenuated (TCC lines with reduced DNA damage G1 and G2 checkpoint function relative to HUCs, but with an intermediate level of response between HUCs and severely attenuated TCC lines; - = severely attenuated (cells with <15% G1 arrest after irradiation, with more than an eightfold increase in the number of polyploid nuclei after incubation with colcemid, or <62% G2 delay during incubation with ICRF-193).

‡Cytogenetic analysis of NHF1-hTERT revealed a diploid 46 XY karyotype with no chromosomal aberrations (56). The ploidy of HUCs was estimated from DNA content as determined by flow cytometry (37,41). The ploidy of cancer cell lines was obtained from information provided by American Type Culture Collection (Manassas, VA).

§Tumor suppressor gene status from Sanchez-Carbayo et al. (53). Because gene status was not determined directly in HUCs and fibroblasts, the inferred status is shown in parentheses.

Retinoblastoma gene status implied from the expression of protein localized to the nucleus. + = presence of nuclear expression; - = absence of nuclear expression.

\gamma-irradiated with 1.5 Gy from a \(^{137}\text{Cs}\) source (Gammacell 40; MDS Nordion, Ottawa, Ontario, Canada) at a dose rate of 86 cGy/min and then returned to the incubator. Control cultures were sham-treated, with the same movements to and from the irradiation source. At 6, 9, or 12 hours after irradiation or sham treatment, 10 \(\mu\text{M}\) bromodeoxyuridine (BrdU) was added to the media for 2 hours to label cells actively synthesizing DNA. Cells were released from dishes with trypsin, washed once with phosphate-buffered saline (PBS), and fixed in 70% ethanol in PBS overnight. Cells were incubated with 0.08% pepsin in 0.1 M sodium borate. The nuclei were washed in 70% ethanol in 80\% NaCl, 0.1% sodium azide, and 0.5% Tween 20 before an incubation for 2 hours on ice in 80 \(\mu\text{L}\) Buffer A and counterstained with propidium iodide at 50 \(\text{mg/mL}\) in Buffer A, and RNase A (5 \(\text{mg/mL}\)) was added. A FACScan flow cytometer (BD Biosciences) was used to quantitate the percentage of cells in the first quarter of S phase (BrdU-labeled nuclei with 2–2.5 \(\text{N}\) DNA content). Analysis of cell cycle distribution was performed using the Summit Program (DakoCytomation, Carpinteria, CA). A total of 3 \(\times 10^4\) nuclei were analyzed per sample. Independent determinations of radiation-induced G1 arrest were conducted three times with bladder cancer and fibroblast cell lines. Three independent HUC preparations were analyzed twice, and a fourth preparation was analyzed once. The percentage of cells in early S 6–9 hours after irradiation was expressed as a fraction of the sham-treated control to provide an index of the proportion of G1 cells evading the DNA damage checkpoint. G1 checkpoint function in bladder cancer lines was arbitrarily judged to be severely attenuated when less than 15% of G1 cells arrested entry to S and moderately attenuated when more than 15% but less than 50% of G1 cells arrested entry to S.

**Polyploidy Checkpoint Function**

Cells growing logarithmically were incubated with colcemid to depolymerize microtubules and arrest cells in metaphase. BrdU was added to a final concentration of 10 \(\mu\text{M}\) 2 hours before harvesting for flow cytometric analysis (described above). Because the initiation of DNA synthesis depends on the completion of mitosis and cytokinesis, polyploidy checkpoint function was evaluated as the colcemid-induced increase in the percentage of nuclei with DNA content 2.2 to 4 times (i.e., polyploid nuclei in S, G2, and M) the DNA content of the control cells in G1. NHF1-hTERT cells displayed a normal karyotype (37) and were used as a control for diploid (2N) DNA content. Because the HUC nuclei were predominately diploid, with a minority of tetraploid nuclei, and the bladder cancer lines all predominately displayed a G1 nuclear DNA content of 3N or greater, appropriate adjustments were carried out during flow cytometry to capture profiles of BrdU labeling against DNA content. Polyploidy checkpoint function in bladder cancer and fibroblast lines was analyzed once. Cell lines were judged to have a severely attenuated polyploidy checkpoint if the fraction of polyploid nuclei was increased by more than eightfold over the control after a 48-hour incubation with colcemid.

**G2 Checkpoint Function**

Mitotic delay to assess the G2 checkpoint function was determined by quantifying mitotic cells as previously described (36,37). Cells growing logarithmically were \gamma-irradiated with 1.5 Gy or were sham-treated. After incubation for 2 hours at
37 °C, cells were fixed on dishes with a solution of methanol: glacial acetic acid (3:1), rinsed with water, and then stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride, 1 μg/mL) in Antifade (Molecular Probes, Eugene, OR). The number of mitotic cells in a minimum of 2000 cells per sample was counted by fluorescence microscopy.

Mitotic cells were also quantified by a second method that used flow cytometry to measure the expression of a mitosis-specific form of phosphohistone H3 (38). Cells were harvested by release from dishes with trypsin 2 hours after irradiation and, after one wash with PBS, fixed with 1% formaldehyde in PBS for 20 minutes at room temperature. The cells were stored overnight in 70% ethanol at 4 °C and then incubated with 0.5 μg of a rabbit anti-phosphohistone H3 antibody (Upstate Biotechnology, Lake Placid, NY) in 100 μL of Buffer A, which was detected with an FITC-labeled goat anti-rabbit antibody (20 μg/mL in Buffer A; Santa Cruz Biotechnology, Santa Cruz, CA). DNA was stained with propidium iodide (50 μg/mL in Buffer A). For each sample, 3 × 10⁶ cells were evaluated by two-parameter (DNA content versus H3 expression) analysis using a BD Biosciences FACSscan flow cytometer (16,38). DNA damage G₂ checkpoint function was calculated as the fraction of mitotic cells in irradiated cultures and expressed as a percentage of the fraction of mitotic cells in sham-treated control cultures (mitotic index treated/mitotic index control × 100%).

To quantify decatenation checkpoint function, cells were incubated with 2 μM ICRF-193 (provided by Dr. Andrew Creighton, St. Bartholomew’s Hospital Medical College, London, U.K.), or control solvent (0.5% dimethyl sulfoxide) in medium. After 2 hours, the mitotic index was determined, as described above.

Each bladder TCC line and the fibroblast line were evaluated for both G₂ checkpoint responses three times by the microscopic method and up to three times using the cytometric method. Each of three independent HUC preparations was evaluated once for each G₂ checkpoint by the microscopic method. Cells were judged to have effective or modestly attenuated G₂ checkpoint functions if less than 10% of irradiated or ICRF-193-treated G₂ cells failed to delay entry to mitosis. Lines with greater than 10% but less than 35% of cells evading the checkpoint were judged to have a moderately attenuated checkpoint response, whereas lines with greater than 35% of cells evading the checkpoint were judged to have a severely attenuated checkpoint response.

Statistical Analysis

Irradiation- and ICRF-193-induced G₁ and G₂ checkpoint functions were expressed as the ratio of the index of DNA synthesis or mitosis, respectively, in irradiation- or ICRF-193-treated cells divided by the same index in sham- or solvent-treated control cells. Mean ratios from multiple independent experiments were compared by Student’s t test on log-transformed data using Statistical Analysis Software, version 8.2 (SAS Institute, Cary, NC). Tukey’s method was used to adjust for multiple comparisons between HUCs and bladder TCC lines.

RESULTS

DNA Damage G₁ Checkpoint Function

DNA damage G₁ checkpoint function can be measured by the selective emptying of the early S-phase compartment 6–12 hours after exposure to irradiation. This selective emptying of early S is a consequence of p53- and ATM-dependent induction of p21waf1, which inhibits G₁ cyclin–dependent kinase complexes and arrests the progression of cells from G₁ phase into S phase (13,39). Because the G₁ checkpoint response of normal HUCs was unknown, we determined the percentage of cells in S phase 6, 9, and 12 hours after radiation or sham treatments. At 6 hours after irradiation, there was a selective 73% reduction in the percentage of cells in the first quarter of S, with little change in the fractions of cells in other quarters of S, compared with sham-treated control cells (Fig. 1, A and B). By 9 hours post-irradiation, there was a reduction in the percentages of cells in the first half of S phase and, by 12 hours post-irradiation, the percentages of cells in each of the first three quarters of the S phase compartment were reduced by 50%–60% relative to the sham-treated control (Fig. 1, B). The normal HUC thus displayed a radiation-induced emptying of S phase that spread with time from the beginning (i.e., front) to the end (i.e., back) of the compartment. This selective emptying of the S phase compartment from front to back is characteristic of a p53-dependent G₁ checkpoint response (40,41). In a total of four experiments using different normal HUC strains that were tested 6–9 hours after irradiation, the percentage of irradiated cells in the first quarter of S was, on average, 32% (95% confidence interval [CI] = 14% to 50%) of the percentage of sham-treated control for a 68% inhibition of progression of G₁ cells into S (Fig. 1, D). In comparable experiments in which NHF1-hTERT cells were tested 6 hours after irradiation (Fig. 1, C and D), the percentage of irradiated cells in early S was 5% (95% CI = 2% to 7%) of the sham control, representing a 95% inhibition of progression from G₁ into S. Compared with the G₁ checkpoint response in normal HUCs, the G₁ checkpoint response to irradiation was statistically significantly reduced in all of the bladder cancer lines (P < 0.001) and, after adjustment for multiple comparisons, four of five TCC lines were statistically significantly different from HUCs (Fig. 1, C and D). Little or no reduction in the percentage of S phase cells was detected among the TCC-SUP (104%, 95% CI = 86% to 129%), UM-UC-3 (86%, 95% CI = 82% to 89%), or J82 (93%, 95% CI = 85% to 100%) lines when tested 6 hours after irradiation, whereas a partial reduction in the percentage of S phase cells was detected among the RT-4 (57%, 95% CI = 51% to 62%) and T24 (51%, 95% CI = 46% to 56%) lines. Thus, in comparison with HUCs, three of five bladder cancer lines displayed a severe attenuation of DNA damage G₁ checkpoint function, whereas two others displayed a moderate attenuation of G₁ checkpoint function.

Polyploidy Checkpoint Function

Flow cytometry was used to assess growth arrest during incubation with colcemid as a measure of polyploidy checkpoint function in the bladder TCC lines. Colcemid depolymerizes microtubules and arrests mitotic cells in metaphase. After a
variable interval of metaphase arrest during incubation with colcemid, mitosis collapses and cells reform interphase nuclei with decondensed chromatin surrounded by nuclear envelope (i.e., restitution nuclei). p53 signals to prevent these restitution nuclei from initiating DNA synthesis (28,37,41). Cells with wild-type p53 do not initiate DNA synthesis or enter S phase from the 4N compartment during incubation with colcemid, whereas cells with mutant or inactive p53 do. The standard

Fig. 1. G₁ checkpoint function in normal human uroepithelial cells (HUCs), five bladder cancer cell lines, and normal human fibroblasts expressing the human catalytic subunit of telomerase (NHF1-hTERT), which serves as a positive control. A) HUCs were γ-irradiated with 1.5 Gy (gamma) or were sham-treated (sham). After 6, 9, or 12 hours, bromodeoxyuridine (BrdU) was added to the culture medium for 2 hours. Cells were harvested and stained for flow cytometric analysis with a fluorescein isothiocyanate–labeled anti-BrdU antibody (to identify S-phase nuclei) and propidium iodide (to label DNA). The three panels show the time-dependent emptying of the early S-phase compartment after irradiation of HUCs. The early S-phase compartment, or first quartile, is identified by the inset box. The percentage near the inset box refers to the fraction of events within the early S-phase compartment. For the irradiated cells, the percentage of cells in the early S-phase compartment is also expressed as a percentage of early S-phase compartment nuclei from sham-treated control cells. Data shown at 6 and 9 hours are representative of results from several preparations of HUCs. B) Percentage of irradiated HUCs in each quartile of S-phase over time expressed as a percentage of the sham-treated control cells. C) Comparison of BrdU incorporation by NHF1-hTERT, T24, and J82 cell lines 6 hours after irradiation (1.5 Gy) or sham treatment. Representative data are shown. D) G₁ checkpoint function in NHF1-hTERT, HUCs, and bladder cancer cell lines was determined as the mean fraction of early S-phase compartment cells in irradiated cells expressed as a percentage of the sham-treated control cells. Error bars enclose the upper 95% confidence interval above the mean of multiple experiments (n = 3–4). Values statistically significantly different from those for HUCs are marked with * (P<.001) and # (P = .004) and are not adjusted for multiple comparisons. When adjusting for all pairwise comparisons using Tukey’s method, all comparisons between bladder transitional cell carcinoma cell lines and HUCs remained statistically significant at the .05 level.
protocol to assay polyploidy checkpoint function involved adding colcemid 24 hours after seeding cells onto culture dishes and monitoring BrdU incorporation into polyploid nuclei 48 hours after the addition of colcemid. Insufficient numbers of HUCs were available in these studies to permit quantitation of polyploidy checkpoint function.

To provide a comparison for analysis of polyploidy checkpoint function in the bladder TCC lines, NHF1-hTERT fibroblasts, which have an effective checkpoint function, were used as the positive control. After a total of 48 hours of incubation in colcemid, which culminated with a 2-hour BrdU pulse, NHF1-hTERT fibroblasts displayed an accumulation of unlabeled nuclei with 4N and less DNA content representing restitution nuclei, i.e., those nuclei that had collected a variable fraction of the nuclear DNA within a single nucleus (Fig. 2) (41,42). The tetraploid nuclei normally present in the culture also arrested with 8N and less DNA content. BrdU-labeled tetraploid S phase nuclei were detected infrequently (<1% of the total nuclei) after 48 hours in colcemid, demonstrating the efficiency of this polyploidy checkpoint.

After incubation with colcemid, the bladder TCC lines also accumulated unlabeled restitution nuclei with DNA content similar to that found in G2 nuclei (4N) or less. Three of the five bladder TCC lines reinitiated DNA synthesis, as detected by BrdU incorporation, from the restitution compartment (Fig. 2). A representative example (Fig. 2) shows that at least 58% of nuclei from T24 cells had polyploid DNA content after incubation with colcemid. Although the T24 line displayed a moderately defective DNA damage G2 checkpoint response, this line displayed severely defective p53-dependent polyploidy checkpoint function. This polyploidization was not a universal feature of all bladder cancer lines, however, because the RT4 (Fig. 2) and TCC-SUP lines with moderately and severely defective DNA damage G1 checkpoint function, respectively, did not display the trait. All of the bladder cancer lines displayed a substantial defect in at least one of the p53-dependent G1 checkpoints (DNA damage and polyploidy), and two lines (J82 and UM-UC-3) were severely defective in both.

DNA Damage G2 Checkpoint Function in Normal HUCs and Bladder Cancer Lines

Irradiation was also used to assess the G2 checkpoint response to DNA damage. Cells were treated with 1.5 Gy and then incubated for 2 hours to allow cells that were in mitosis at the time of treatment to finish mitosis and move into G1. When the G2 checkpoint is intact, few cells progress into M phase. When the G2 checkpoint is not intact, irradiated cells progress into M phase. Cells in M phase were quantified by fluorescence microscopy and by the expression of phosphohistone H3 (Fig. 3, A). The two methods for enumeration of mitotic cells yielded equivalent results (Fig. 3, B). G2 checkpoint function was quantified as the percentage of G2 cells that evaded radiation-induced mitotic delay.

In NHF1-hTERT cells and HUCs, the G2 checkpoint response to irradiation was effective, with not more than 5% (95% CI = 1% to 4% for NHF1-hTERT and 95% CI = 2% to 9% for HUCs) of cells evading the checkpoint. The RT4 cancer line had an effective G2 checkpoint response to DNA damage, with 4% (95% CI = 3% to 6%) of cells evading the checkpoint. Compared with the DNA damage G2 checkpoint response in normal HUCs, the T24 and J82 cell lines had a modest but statistically significant attenuation of response, with 9% (95% CI = 5% to 12%; P = .03) of J82 cells and 9% (95% CI = 7% to 11%; P = .02) of T24 cells evading the G2 checkpoint, whereas the UM-UC-3 and TCC-SUP lines had even greater attenuation of the response, with 15% (95% CI = 11% to 21%; P < .001) of UM-UC-3 cells and 24% (95% CI = 22% to 26%; P < .001) of TCC-SUP cells evading the G2 checkpoint. Thus, although the efficiency of G2 checkpoint response to DNA damage was statistically significantly reduced in four of five bladder cancer lines relative to that in normal HUCs, three of the bladder cancer lines responded to radiation effectively, with greater than 90% inhibition of mitosis.

Decatenation Checkpoint Function in Normal HUCs and Bladder Cancer Lines

The DNA topoisomerase II gate–blocking inhibitor ICRF-193 was used to assess decatenation checkpoint function (43).

![Fig. 2. Polyploidy checkpoint function in normal human fibroblasts expressing the human catalytic subunit of telomerase (NHF1-hTERT), which serves as a normal control, and bladder transitional cell carcinoma cell lines. Cells growing in log-phase were incubated for 48 hours with colcemid, a mitosis inhibitor. Bromodeoxyuridine (BrdU) was added to the culture medium for the last 2 hours of incubation, and cells were then harvested and stained for flow cytometry with a fluorescein isothiocyanate–labeled anti-BrdU antibody (to identify S-phase nuclei) and with propidium iodide (to label DNA). The T24 cell line reinitiated DNA synthesis in polyploid nuclei (>4N, identified by the polygon) during incubation with colcemid, whereas the RT4 and NHF1-hTERT cell lines did not. Representative data are shown.](https://academic.oup.com/jnci/article-abstract/95/24/1859/2520548)
Daughter DNA molecules are intertwined (catenated) following DNA replication, and topoisomerase II is needed to remove DNA catenations before chromatid segregation at anaphase of mitosis. Human cells appear to monitor decatenation during G2 and delay entry to mitosis when incubated with ICRF-193. Similar to the DNA damage G2 checkpoint response, an effective decatenation checkpoint response produces a severe reduction in the mitotic index 2 hours after the addition of ICRF-193. Cells with defective decatenation checkpoint function would be expected to proceed from G2 into M with intertwined daughter chromatids.

NHF1-hTERT fibroblasts and normal HUCs expressed a highly reproducible and strong G2 delay in response to ICRF-193, with mitosis being inhibited by approximately 95% (Fig. 4). By contrast, all bladder TCC lines showed severe and statistically significant (P<.001) attenuation of the decatenation checkpoint response (Fig. 4). In fact, the numbers of cells in mitosis after ICRF-193 treatment in the UM-UC-3 and T24 cell lines exceeded the values seen in vehicle-treated control cells by 20%–30%. This was likely to be a result of ICRF-193 slowing mitotic progression in cells that were unable to delay in G2 (44). The bladder cancer lines displayed reduced G2 delay when incubated with ICRF-193. Consequently, many cells progressed into mitosis, with anaphase bridges often observed as an indicator of blocked decatenation (data not shown).

**DISCUSSION**

In this study, cell cycle checkpoint function was quantified in several independent cultures of normal HUCs and five bladder TCC lines to test whether defects in checkpoint function might be associated with the genetic instability typically observed in bladder cancer. Radiation-induced DNA damage and ICRF-193-induced inhibition of DNA decatenation triggered effective checkpoint responses in normal HUCs. However, although the DNA damage G2 checkpoint function was effective in three bladder cancer lines, with more than 90% of G2 cells arrested by irradiation, the decatenation checkpoint function was uniformly defective in bladder cancer lines, with less than 62% of G2 cells arrested by the topoisomerase II inhibitor ICRF-193. Similarly,
all cancer lines displayed at least one defect in the functions of the p53-dependent DNA damage and polyploidy checkpoints. These functional defects in the bladder TCC lines (Table 1) suggest that cell cycle checkpoints may represent barriers to bladder carcinogenesis.

Accurate segregation of a replicated genome into two daughter cells requires that sister chromatids move to opposite poles of the mitotic spindle during anaphase. It is thought that, after replication, a small amount of sister chromatid DNA remains catenated as a result of sterically excluded topoisomerase I by the replication machinery (45). This catenation may hold the sister chromatids together until anaphase, when topoisomerase II-mediated decatenation allows separation of the sister chromatids. ICRF-193 is a topoisomerase II catalytic inhibitor that does not damage DNA. ICRF-193 binding to topoisomerase II holds the enzyme in the closed-clamp conformation (43). When ICRF-193 is bound to topoisomerase II, ATPase activity is diminished, and the conformation change required to reopen the clamp is prevented. In this study, we found that cell cycle progression from G2 to mitosis was suppressed when HUCs were treated with ICRF-193 but not when bladder TCC lines were treated with ICRF-193. Thus, these bladder TCC lines had severe attenuation or complete ablation of the decatenation checkpoint response. Failure of the decatenation checkpoint may result in cells entering into mitosis with incompletely decatenated chromosomes. These entangled chromosomes cannot be segregated properly, resulting in aneuploidy through nondisjunctional errors or polyploidy after mitotic collapse (29,33). Insufficient decatenation before mitotic entry also can generate chromatid breaks and exchanges, producing nonreciprocal translocations and severe chromosomal destabilization (16,29). Thus, defects in decatenation checkpoint function may produce abnormalities of chromosome number and structure.

DNA damage checkpoints provide cells with additional time to repair damaged DNA before initiating DNA synthesis and mitosis. Repair of DNA lesions before DNA synthesis and mitosis reduces the introduction of mutations and chromosomal aberrations as permanent heritable changes within the genome. We found that, after irradiation, G1 arrest occurred in HUCs, with the early S-phase compartment emptying by an average of
68% relative to that in sham-treated controls 6–9 hours post-radiation. An absence or attenuation of the G1 checkpoint response has been shown in normal human prostate, breast, skin, and bronchial epithelial cells (46–48), suggesting that epithelial cells might generally display defective G1 checkpoint function. However, an effective G1 checkpoint response to DNA damage was shown in human thyroid epithelial cells (39) and rat hepatocytes in vivo and in vitro (41), suggesting that not all epithelial cells are generally defective. Further work will be required to determine whether the G1 checkpoint function is dependent on epithelial cell type or is affected by conditions of cell culture. Results of a recent study with rat mammary epithelial cells (49) suggested that p53 function was developmentally regulated with expression of p53 being induced by estrogen. Thus, cell-type-dependent variation in G1 checkpoint function may reflect differences in expression of critical effector genes such as p53.

DNA damage and polyploidy checkpoint functions depend on p53 and RB proteins (28,50,51). Previous analyses of gene expression and mutations in the bladder cancer lines revealed high expression of mutant p53 in the UM-UC-3, TCC-SUP, and J82 lines, low expression of mutant p53 in the T24 line, and low expression of wild-type p53 in RT4 (Table 1) (52,53). In view of these results (52,53), most of the functional defects we observed in the DNA damage and polyploidy checkpoints were not surprising. The reduced DNA damage G1 checkpoint function in the RT4 line, which expresses wild-type p53, may be explained by its very low expression of RB and the homozygous deletions of ARF and p16 (52,53). The effective polyploidy checkpoint function in the TCC-SUP line was unexpected in view of the mutation in p53, the absence of expression of RB in the nucleus, and the severe attenuation of DNA damage G1 checkpoint function. A larger study with more cancer lines and primary tumors is needed to establish an association between functional defects in the DNA damage and polyploidy checkpoints in bladder cancer.

The combination of defects in p53-dependent checkpoints and the p53-independent decatenation checkpoint may amplify genomic destabilization (33). Premature entry into mitosis by cells with insufficiently decatenated chromatids should produce stress on the mitotic spindle because chromatid separation is impeded. If sufficient numbers of chromatids are affected, mitosis may be sufficiently retarded to induce mitotic collapse and, in cells with intact p53 and RB signaling pathways, the polyploidy checkpoint would block initiation of DNA synthesis. Cells with defective polyploidy checkpoint function may be more likely to undergo polyploidization after catenation-induced mitotic collapse. Insufficient decatenation before mitosis could also cause nondisjunction errors, leading to aneuploidy, or induce chromatid breaks (16), leading to translocations after repair. A recent analysis (54) described 10 translocations among the 74 chromosomes in the T24 line, which is evidence of great instability of chromosome number and structure. Our results indicate that TCC of the bladder may possess a combination of defects in cell cycle checkpoints that could increase genetic instability and contribute to malignant progression.

A recent report (55) demonstrated that the decatenation checkpoint requires WRN, a DNA helicase that is defective in Werner syndrome, a familial cancer syndrome associated with premature aging. Cells from Werner syndrome patients did not arrest entry to mitosis when incubated with the bisdioxopiperazine ICRF-187, and expression of wild-type WRN protein in these cells restored ICRF-187–induced G2 arrest. WRN was required for ATR-dependent phosphorylation of BRCA1 in response to ICRF-187 (55). Decatenation checkpoint function thus appears to require two genes—BRCA1 and WRN—that are mutated in familial cancer syndromes and an essential gene—ATR. Analysis of expression of mRNA and protein for these genes may prove to be helpful for understanding the mechanisms of defective decatenation checkpoint function in bladder cancer.

In this study, we showed that cell cycle checkpoint functions may frequently be altered in bladder cancer lines. Defective checkpoint function in the cancer lines was determined by direct comparison with normal bladder uroepithelial cells. Beyond the mutations in p53 and ARF/p16 and the reduced expression of RB, we know nothing of the levels of expression of the many other key regulators of cell cycle checkpoints in bladder TCC and HUCs. Further studies with more cancer lines and HUCs should try to connect functional defects with changes in the activity or expression of key regulators such as BRCA1 and WRN.

References


