[CANCER RESEARCH 63, 760–765, February 15, 2003]

Advances in Brief

Adenoviral-mediated Retinoblastoma 94 Produces Rapid Telomere Erosion, Chromosomal Crisis, and Caspase-dependent Apoptosis in Bladder Cancer and Immortalized Human Urothelial Cells but not in Normal Urothelial Cells[1]

Xinqiao Zhang, Asha S. Multani, Jain-Hua Zhou, Jerry W. Shay, David McConkey, Li Dong, Chang-Soo Kim, Charles J. Rosser, Sen Pathak, and William F. Benedict[2]

Departments of Genitourinary Medical Oncology [X. Z., J-H. Z., L. D., C-S. K., W. F. B.], Cancer Biology [A. S. M., D. M., S. P.], and Urology [C. J. R.], The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, and Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas Texas 75390 [J. W. S.]

Abstract

Retinoblastoma (RB)94, which lacks the NH_2-terminal 112 amino acid residues of the full-length RB protein (RB110), is a more potent tumor and growth suppressor than RB110. In this study, Ad-RB94, but not Ad-RB110, produced marked growth inhibition, cytotoxicity, caspase-dependent apoptosis, and G_2-M block in the human RB-negative, telomerase-positive bladder cancer cell line UM-UC14. This effect was completely inhibited by pretreatment with caspase inhibitors ($P < 0.0001$). Similar results were seen in RB-positive and other RB-negative bladder cancer cell lines. Ad-RB94 produced rapid telomere length shortening and loss of telomere signal, which was associated with polyploidy and chromosomal aberrations ($P < 0.001$). Ad-RB94, however, showed no cytotoxicity to telomerase-negative human normal urothelium cells but was highly cytotoxic to telomerase-positive human E6 and E7 immortalized urothelial cells ($P < 0.0001$). In addition, telomerase-negative cells, which maintain their telomere length through an alternative lengthening of telomeres DNA recombination pathway, showed no cytotoxicity to RB94. These results suggest that the induction of rapid telomere erosion and chromosomal crisis by RB94 in telomerase-positive cancer and in telomerase-expressing immortalized human cells is a major factor in its selective and potent tumor suppression and cytotoxic activity. The lack of cytotoxicity to normal cells should also provide a high therapeutic index when used in gene therapy protocols for the treatment of bladder and other cancers.

Introduction

RB94[3] is produced by translation of the wild-type RB gene from the second in-frame AUG codon and lacks the NH_2-terminal 112 amino acids present in RB110 (1). RB94 has markedly more tumor suppressor potency than wild-type RB110 and is active against both RB+ and RB− tumors, including bladder cancer (1, 2). Because RB94 retains the same key functional domains (such as A, B, and C pockets, nuclear signaling) as RB110, it becomes critical to understand the mechanism(s) of increased potency for RB94. Differences reported thus far include the observation that RB94 has a longer half-life than RB110, remains in its active hypophosphorylated form for an extended period of time, and causes unusual nuclear morphological changes (1, 2). Although these differences may be important to the unique properties of RB94, they do not address the mechanism(s) of increased tumor suppression and cell kill produced by RB94.

An adenoviral construct containing RB94 under tetracycline control enabled us to examine changes that are RB94 specific because the protein is not produced in the presence of doxycycline, although adenoviral proteins are still made. Therefore, changes seen after Ad-RB94 treatment and that are blocked by doxycycline can be considered to be RB94 specific. Focus was given initially to the effect of RB94 on RB-negative bladder cancer cells and immortalized urothelial cells (telomerase positive) as well as normal urothelial cells (telomerase negative) because gene therapy trials using RB94 are being planned for bladder cancer.

We report that Ad-RB94 is cytotoxic to every bladder cancer cell expressing RB94, and this is associated with rapid telomere erosion and chromosomal crisis. Ad-RB110 treatment does not produce these changes. In addition, Ad-RB94 suppresses the growth of E6 and E7 immortalized human urothelial cells and produces similar telomere and chromosomal changes. In contrast, no cytotoxicity, telomere attrition, or chromosomal abnormalities were seen in normal urothelial cells after Ad-RB94 treatment. On the basis of these results, we present one hypothesis to explain the basis for the specificity of RB94 cytotoxicity to human cancer cells and to genetically altered, immortalized cells but not to normal cells.

Materials and Methods

Cell Lines. The bladder cancer cell lines 5637, UM-UC9, UM-UC11, and UM-UC14, the immortalized urothelial cells α-E6-1, α-E6-2, and ϵ E7, and the normal human urothelial cells were obtained from Dr. Barton Grossman (Department of Urology, University of Texas M. D. Anderson Cancer Center). The α-E6-1, α-E6-2, and ϵ-E7 cells were originally obtained from Catherine Reznikoff (3, 4). The immortalized telomerase-negative SUSM1 fibroblasts have been described previously (5). Except for normal urothelial cells, all of the other cells were grown in T-75 tissue culture flasks in modified minimum essential medium supplemented with 10% FCS and incubated at 37°C in 5% CO_2 and 95% air. The normal urothelial cells were grown in keratinocyte growth medium without serum as described previously (6).

Ad-RB94 Construction. The RB94 gene was inserted into an expression cassette under a tTA-inducible cytomegalovirus promoter. This was, in turn, inserted into plasmid pΔE1sp1A obtaining shuttle plasmid pEW22. We also inserted a tTA protein expression cassette into plasmid pBHG11 through plasmid pABS.4, obtaining master plasmid pEW23. By cotransfection of pEW22 and pEW23 into 293 cells, we obtained the recombinant adenovirus expressing RB94 under the control of the tetracycline system. After initial harvesting, the viral vector underwent plaque to plaque purification. Subse-

Received 11/11/02; accepted 1/7/03.

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] This study was supported by a grant from the Retina Research Foundation and the Tobacco Settlement Funds as Appropriated by the Texas State Legislature. It was also supported by the Bladder SPORE CA091846 and Lung SPORE CA70907. C. J. R. was funded by the American Foundation of Urological Disease.

[2] To whom requests for reprints should be addressed, at Department of Genitourinary Medical Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 794-4013; Fax: (713) 794-4019; E-mail: wbenedic@mdanderson.org.

[3] The abbreviations used are: RB, retinoblastoma; RB110, full-length wild-type RB protein of M_r 110,000; Ad-RB110, adenoviral-mediated RB110; RB94, RB protein of M_r ~94,000; Ad-RB94, adenoviral-mediated RB94; BOC, BOC-Asp(Ome)-fluoro-methyl ketone; zVADfmk, benzyloxycarbonyl-val-ala-Asp(OMe)-fluoro-methyl ketone; FISH, fluorescence *in situ* hybridization; Q-FISH, quantitative-FISH; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly(ADP-ribose) polymerase; TRF2, telomeric-repeat binding factor 2; ALT, alternative lengthening of telomere.

quently, 9 strains among a total of 108 candidate strains were tested for infection frequency and RB94 production. Ad-RB94-13B5 was selected as the final RB94 viral construct for future studies.

Adenovirus Infection. Adenoviral vectors were produced and obtained from the Viral Core Facility at the University of Texas M. D. Anderson Caner Center. Ad-RB110 and AdCMVLacZ were initially obtained from Canji, Inc. (San Diego, CA). Cells were exposed to the various adenovirus-mediated genes at an multiplicity of infection of 100 for 1–3 h in medium without serum. The virus was then removed and complete control medium added. Transfection frequency was ≥50% for 5637, UM-UC9, UM-UC11, and UM-UC14 cells and >75% for normal urothelial cells or E6 and ϵ E7 immortalized cells. AdCMVLacZ was used initially to test for transfection efficiency. Because Ad-RB94 is under control of tetracycline regulation, doxycycline (1 μg/ml) was added to the medium at the time of adenoviral exposure and thereafter in specific experiments to block Ad-RB94 expression.

Pretreatment with Caspase Inhibitors. To examine whether Ad-RB94 produced cytotoxicity was caspase dependent, cells were pretreated overnight with 100 μM BOC or zVADfmk purchased from Enzyme Systems Products (Livermore, CA). The cells were then washed with medium and exposed to Ad-RB94 with or without inhibitor for 4 h. The virus was then removed, and the cells were re-fed with complete medium with or without inhibitor until harvested.

MTT Assay. The various cells were seeded into 24-well plates at 4×10^4 cells/well before infection. The cells were infected for 2.5 h as described above, and at different time points the medium was removed, and 200 μl of medium were added containing 1 mg/ml MTT. After 3 h, the reaction was stopped with 200 μl of *N*,*N*-dimethylformamide lysis buffer, and the resultant solution read at A^{595} with a microreader.

Determination of the Cell Cycle and Subdiploid Population by the Fluorescence-activated Cell Sorting Analysis. Cell cycle analysis and the percentage of subdiploid cells were determined as described previously (7, 8). The UM-UC14 cells were plated into 100-mm dishes at 2×10^6 cells/dish before infection. After infection with Ad-RB94 with or without doxycycline or the caspase inhibitors described above, the control and treated cells were harvested with trypsin-EDTA and washed in 1 ml of cold PBS. Approximately 1×10^6 cells from each treatment were resuspended in 0.5 ml of propidium iodide solution (50 μg/ml propidium iodide, 0.1% Triton X-100, and 0.1% sodium citrate in PBS). Cells were then incubated at 4°C for 2 h in the dark, and then the fluorescence was read on a Coulter Epics (R) XL (Beckman-Coulter, Brea, CA). The percentage of subdiploid cell populations was calculated using the multigraph program. The cells were also processed for their cell cycle status by fluorescence-activated cell sorting analysis as described above.

Cytogenetic Analysis. Chromosome preparations were made from treated and untreated cells by routine air-dried techniques (9). In brief, cells were treated with Colcemid (0.04 μg/ml) for 25 min at 37°C and to hypotonic treatment (0.075 M KCl) for 20 min at room temperature. The cells were then fixed in methanol and acetic acid (3:1 by volume) for 15 min and washed three times in the fixative. The slides were air-dried, coded for blinded analysis, and later decoded for the evaluation of results. Several parameters of chromosomal crisis were evaluated including the frequencies of endoreduplication, polyploidy, and chromosome aberrations (as evidenced by both chromosomal and chromatid-type breaks), as well as telomeric associations.

Q-FISH Analysis of Telomeric DNA. Q-FISH analysis was performed using a commercially available Cy-3-conjugated telomere peptide nucleic acid probe following the manufacturer's protocol (Dako Corporation, Carpinteria, CA) as described previously (10). The slides were counterstained with 4′,6-diamidino-2-phenylindole (0.1 μg/ml) and examined using a Nikon photomicroscope with a UV-2A filter for 4′,6-diamidino-2-phenylindole and Cy-3. Fifty to 100 nuclei/sample were photographed, and quantification of telomeric DNA was performed using the Metaview Imaging System software program (Universal Imaging Co., Westchester, PA). From each sample, at least 25 interphase nuclei were quantified for determining the mean telomeric area as compared with the total nuclear area.

Western Blot Analysis. Cells were washed twice with PBS and lysed at 5×10^4 cell/μl in lysis buffer (1% Triton X-100, 1 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, and protease inhibitor mixture; Complete, Mini; Roche Molecular Biochemical, Indianapolis, IN) on ice for 30 min. Equal amounts of lysate were separated by SDS-PAGE on a 10% acrylamide gel. Proteins were transferred to a nitrocellulose membrane (Pierce Biotechnology IN, Rockford, IL) and incubated with mouse monoclonal antibody PARP or rabbit polyclonal antibody TRF2 (Santa Cruz Biotechnology IN, Santa Cruz, CA) either overnight at 4°C or 3 h at room temperature, respectively. It was then incubated with the appropriate secondary antimouse or antirabbit horseradish peroxidase-conjugated antibody (Bio-Rad, Hercules, CA) at room temperature for 2 h. Detection was performed using the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom). An anti-β-actin blot was made in parallel as a loading control.

Telomerase Assay. Cells were plated at a density of 4×10^5 cells in 60-mm tissue culture dishes and allowed to adhere overnight. The medium was removed, and cells were treated with Ad-RB94 with or without doxycycline, Ad-RB110, or AdCMVLacZ for 3 h. Subsequently, the cells were collected at 24 h and 48 h, washed with PBS, centrifuged, and the cell pellets were stored at −80°C. Telomerase activity was detected using the TRAPeze Telomerase Detection Kit (Intergen, Purchase, NY), and ^{32}P was used for the detection of telomerase activity.

Telomere Length Assay. The UM-UC14 cells were seeded into 100-mm tissue culture dishes at 2×10^6 cells before infection. The cells were infected by Ad-RB94 or medium with or without doxycycline as described above and harvested at 48 h for cell genomic DNA isolation. Cell genomic DNA was purified by standard protocols and was measured by A^{260} for quantitation. Telomere length was measured by the Telo TAGGG Telomere Length Assay Kit (Roche Diagnostics Corporation, Indianapolis, IN) with slight modification. A total of 3 μg of purified genomic DNA was digested with *Hin*fI and *Rsa*I. The DNA fragments were then separated by overnight electrophoresis in a 0.8% agarose gel at 1V/cm and transferred to a nylon membrane for Southern blotting. The blotted DNA fragments were then hybridized to a digoxigenin-labeled probe specific for telomeric repeats, incubated with a digoxigenin-specific antibody coupled to alkaline phosphate, and visualized using an alkaline phosphatase substrate.

Statistical Analysis. For the MTT results, the analysis was done using the General Lineal Models of the Statistica software (StatSoft, Inc., Tulsa, OK). Q-FISH results were analyzed by Student's *t* test using Slidewrite Plus (Advanced Graphics Software, Inc., Encinitas, CA).

Results and Discussion

RB94 has shown significant growth suppression against all human tumor cell lines tested to date no matter what the cancer type while RB110 has not (1, 2). To additionally investigate the distinct properties of RB94 compared with RB110 and to further study the effect of RB94 in urothelial cells, we initially examined the effects of Ad-RB94 and Ad-RB110 expression in the RB-negative bladder cancer cell line UM-UC14. The use of an RB-negative cell line allows the expression of either protein to be readily monitored by immunochemical analysis in each cell after gene transfer. This cell line also was chosen because it produces no p16 as determined by both Western blotting and immunochemical analysis (data not shown), and therefore, RB110 should not be growth suppressive in p16 negative cells. This allowed the unique features of RB94 to be more readily determined. Indeed Ad-RB110 was not growth suppressive in UM-UC14 cells (Fig. 1*A*). Ad-RB94, however, caused dramatic growth suppression ($P < 0.0001$), which was completely prevented by the simultaneous addition of doxycycline (Fig. 1*A*), because RB94 expression is under tetracycline control. In contrast, no cytotoxicity was produced by Ad-RB94 against any normal human cells tested to date, including urothelial cells (Fig. 1*B*) and several fibroblast cell lines. Ad-RB94 also caused similar growth suppression in UM-UC11 and UM-UC 9 cells, which are RB positive and p16 negative, and 5637 cells, which are RB negative and p16 positive (data not shown). These results imply not only that RB94 would be a much more effective agent than RB110 for potential gene therapy use but also that RB94 should have a high therapeutic index.

Moreover, we found that RB94 compared with RB110 can suppress the growth of genetically altered human urothelial cells that have not gained the capacity to produce tumors. For example, the telomerase-positive E6 immortalized cell line, α-E6-1, showed marked cytotox-

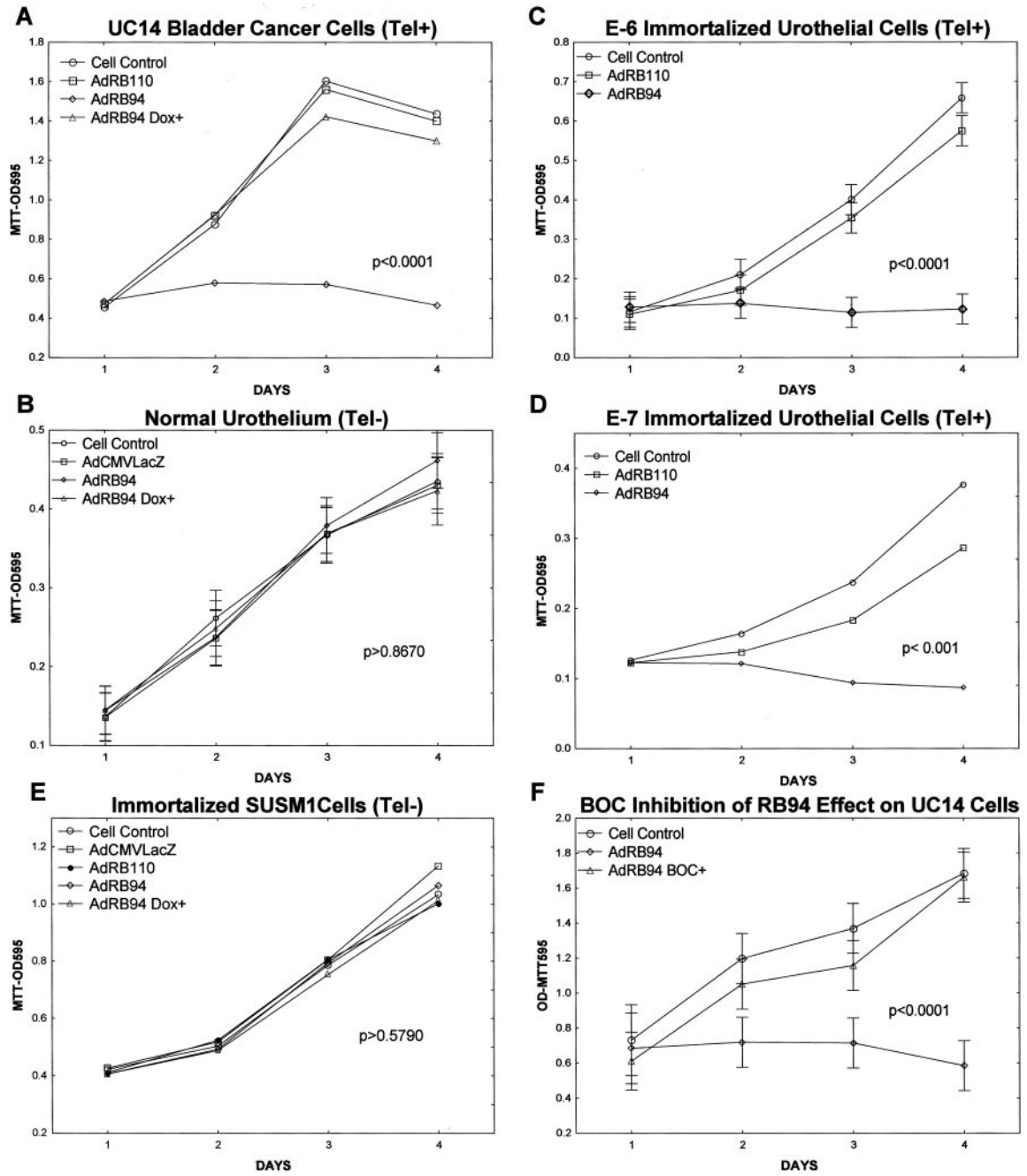

Fig. 1. Cells were exposed to the various adenoviral-mediated genes shown in the insert. *A*, growth suppression by Ad-RB94 but not Ad-RB110 in telomerase-positive (Tel+) UM-UC14 bladder cancer cells. Ad-RB94 growth suppression was inhibited by doxycycline (Dox+). *B*, lack of Ad-RB94 toxicity in telomerase-negative (Tel−) normal human urothelial cells. *C* and *D*, growth suppression by Ad-RB94 but not by Ad-RB110 in telomerase-positive (Tel+) α-E6-1 and ϵ-E7 immortalized human urothelial cells, respectively. *E*, lack of Ad-RB94-induced cytotoxicity in the telomerase-negative SUSM1 fibroblast cell line, which is immortalized by an ALT pathway mechanism. *F*, inhibition of Ad-RB94 induced growth suppression of UM-UC14 cells by the caspase inhibitor BOC.

icity in response to RB94. whereas wild-type RB110 did not (Fig. 1*C*; $P < 0.0001$), as did a similarly E6 immortalized telomerase-positive cell clone, α-E-6-2 (data not shown). In addition, Ad-RB94 produced the same growth suppression in telomerase-positive ϵ-E7 immortalized urothelial cells (Fig. 1*D*; $P < 0.001$). The growth inhibition produced by Ad-RB94 in both the E6 and E7 immortalized cells strongly suggests that the p53 or RB pathways are not involved in the cytotoxicity caused by RB94. These results are also consistent with our previous studies that also indicated that RB94 is active in RB or p53 mutant as well as RB or p53 wild-type tumor cells (1, 2). The latter results also imply that RB94 may be cytotoxic and suppress the growth of genetically altered premalignant urothelium as well as recognizable bladder cancer, whereas it will not be cytotoxic to normal urothelium.

There is a fraction of immortalized cells and a few cancer cell lines that have been found to be telomerase negative, maintaining their telomere length through an ALTs' pathway, rather than by an increase in telomerase activity (11). When either immortalized or cancer cell lines containing an ALT pathway were tested for their cytotoxicity to Ad-RB94, none showed any cytotoxicity to Ad-RB94. This included immortalized fibroblast cell line SUSMI (Fig. 1*E*) and the osteosarcoma cell line U2OS (data not shown). Therefore, ALT pathway cells

represent the only immortal or cancer cell lines examined that have not been sensitive to the effect of Ad-RB94 treatment and suggests that the cytotoxic effects of Ad-RB94 may be mediated through a telomerase related mechanism.

Because ALT pathway cells are by definition telomerase negative and also contain chromosomes having particularly large telomeres, telomere status was subsequently examined after Ad-RB94 treatment. Ad-RB94 caused rapid telomere erosion in UM-UC14 and E6 immortalized urothelial cells but not in normal urothelial cells ($P < 0.001$). An example of the marked decrease in telomere signal found after Ad-RB94 treatment is shown for UM-UC14 cells 48 h after Ad-RB94 treatment in Fig. 2*A*, plate 3 (left). Telomere erosion could be blocked by the addition of doxycycline (Fig. 2*A*, plate 4) and was not seen after Ad-RB110 treatment (Fig. 2*A*, plate 2). Consistent with the rapid loss of telomere signal observed by FISH analysis was the marked shortening of telomere length observed in UM-UC14 cells at the same time point after Ad-RB94 treatment (Fig. 2*A*, right). A similar rapid telomere erosion was seen in other bladder cancer cell lines and in the immortalized telomerase-positive urothelial cells after treatment with Ad-RB94 (data not shown). This rapid effect at the telomere level may be one of the key mechanisms by which RB94 produces its cytotoxic effect on cancer cells as well as genetically altered telomerase-positive cells.

Chromosomal changes were also studied concurrently with the evaluation of telomere status. Marked chromosomal aberrations were seen in the metaphases present within 24 h after Ad-RB94 treatment, which increased at 48 h and was blocked by doxycycline (Fig. 2*B*). These changes included chromosomal breaks, gaps, and telomere associations. Such findings imply that Ad-RB94 produces rapid telomere erosion, resulting in massive genomic instability and chromosomal crisis leading to cell death in cancer cells but not in normal

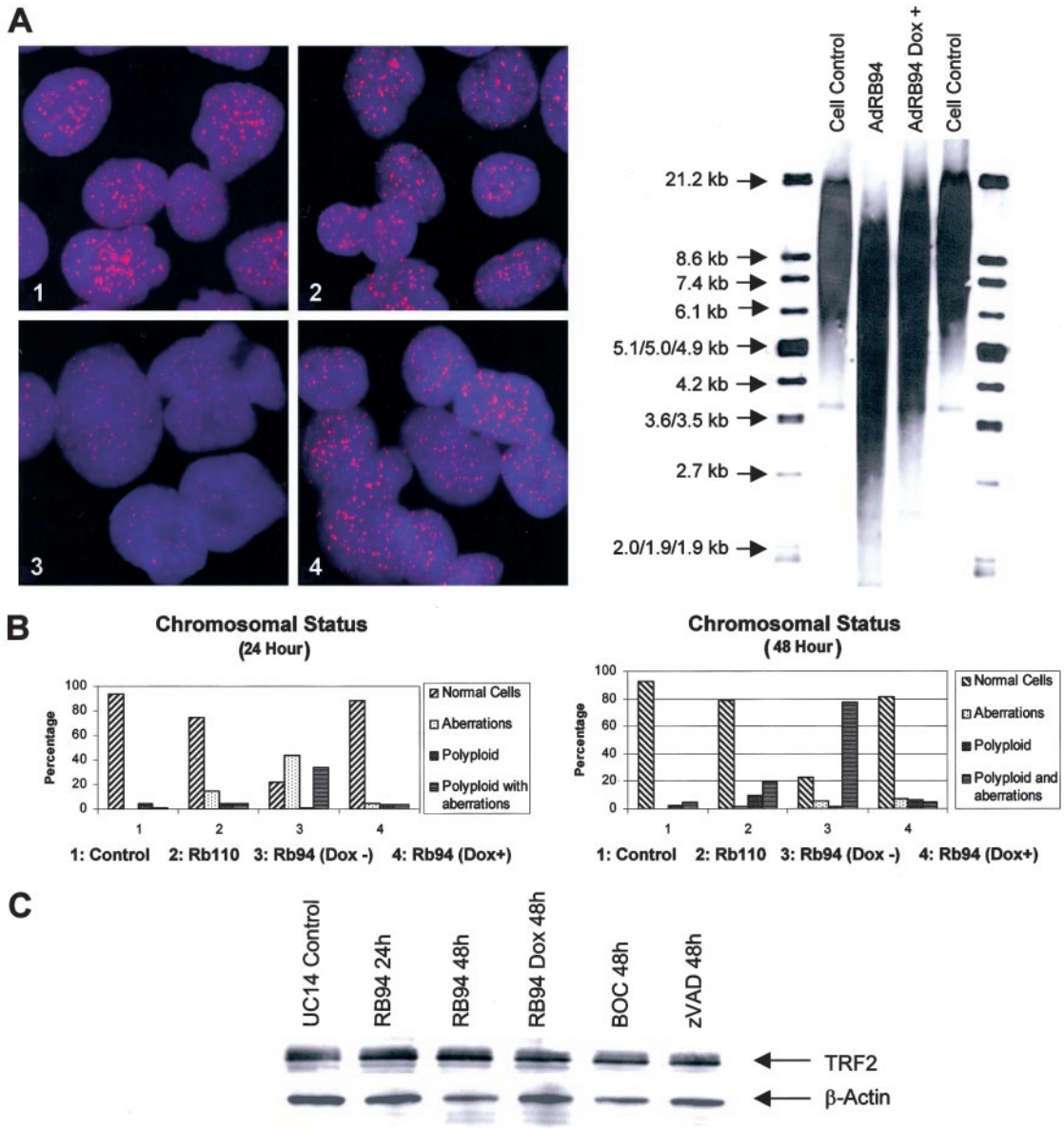

Fig. 2. *A*, *left*, loss of telomere signal is seen 48 h after treatment of UM-UC14 bladder cancer cells with Ad-RB94 (plate 3), which was blocked by doxycycline (plate 4). The specificity of Ad-RB94 to produce rapid telomere erosion was highly significant ($P < 0.001$). Plates 1 and 2 are control and Ad-LacZ treated cells, respectively. *Right*, marked telomere shortening is shown 48 h after Ad-RB94 treatment of UM-UC14 cells consistent with the marked decrease in telomere signal shown in *A* (plate 3) on the *left*. *B*, UM-UC14 cells were exposed to Ad-RB110, Ad-RB94 (DOX−), or Ad-RB94 (DOX+) at a multiplicity of infection of 100 for 1 h. Chromosomal preparations were made 24 and 48 h after exposure and examined for chromosomal aberrations using a blinded protocol. The majority of metaphases examined had chromosomal aberrations in the Ad-RB94 (Dox−)-treated cells with the number of polyploid cells containing chromosomal abnormalities increasing from ~35 to ~80% 24–48 h, respectively, after exposure in those cells that reached metaphase. These chromosomal changes were blocked by the presence of doxycycline (Dox+). *C*, no decrease in TRF2 level was found in Ad-RB94-treated UM-UC14 cells.

cells. In addition, there was a marked increase in the number of polyploid cells present, which increased from 24 to 48 h after Ad-RB94 exposure (Fig. 2*B*). This increase in polyploidy is consistent with the occurrence of abnormal karyokinesis.

Some of the telomere and chromosomal changes produced by Ad-RB94 also have been previously observed after treatment with other agents (12). However, it was not determined whether a similar result could be produced by these same agents in telomerase-positive, immortalized cells or in ALT pathway cells. Moreover, in these latter studies (12), such changes were found to be associated with a decrease in TRF 2. Ad-RB94, however, has not produced a decrease in TRF2 (Fig. 2*C*), illustrating yet another unique feature of Ad-RB94 produced cytotoxicity.

We also wished to study whether or not the cytotoxicity observed after Ad-RB94 treatment was caspase dependent. Therefore, the caspase inhibitor BOC-Asp(Ome)-FMK (BOC) or zVADfmk was added prior and during Ad-RB94 exposure. Both BOC and zVAD blocked the effect of Ad-RB94 treatment in the UM-UC14 cell line ($P < 0.0001$; Fig. 1*F*), indicating that the RB94 produced growth suppression and cell death is caspase dependent. This was additionally supported by the fact that Ad-RB94 produced significant PARP cleavage in UM-UC14 cells, which was blocked by simultaneous doxycycline exposure (Fig. 3*A*). In addition, a marked increase the number of cells in sub-G_1 (22.3%) occurred 48 h after Ad-RB94 treatment in the UM-UC14 cells, which was blocked by both doxycycline and BOC (Fig. 3*B*). The percentage of G_2-M also increased significantly after Ad-RB94 exposure (Fig. 3*B*).

The establishment of immortalized human cells involves escape from cellular senescence. Normal human cells, which are in general telomerase negative, undergo slow telomere attrition over time in culture, finally resulting in marked genomic instability and chromosomal crisis. The vast majority of cells do not survive such chromosomal crisis. Before the establishment of rare immortalized cells, loss of p53 and RB pathway function commonly occurs. The few cells that may finally survive usually have elevated telomerase activity. This increase in telomerase activity is a critical mechanism of immortalizaton, allowing telomeres to remain sufficiently long to prevent senescence (13). Such genetically altered, telomerase-positive cells nevertheless are incapable of producing tumors, and additional mutations must occur before they can become actual cancer cells capable of forming tumors. On the basis of our present findings, we hypothesize that RB94 causes rapid telomere attrition, resulting in massive genomic instability, chromosomal crisis, and subsequent caspase-dependent cell death. We have shown that both telomerase-positive cancer and immortalized cells are sensitive to the effects of Ad-RB94, whereas telomerase-negative normal and ALT pathway cells are not. On the basis of the above considerations, our present hypothesis on the mechanism of RB94-produced cytotoxicity is diagramed in Fig. 4 and outlined in the figure legend.

Using the telomerase repeat amplification protocol assay, we have not found that RB94 directly effects telomerase activity itself in cells and at the time points in which RB94 produces significant cytotoxicity (Fig. 2*C*). This result is similar to that previously found using paclitaxel (14). Others have recently reported that Ad-RB94 decreases telomerase activity in head and neck cancer cells using the same telomerase repeat amplification protocol assay (15). This decrease in telomerase activity was seen only 5–6 days after Ad-RB94 treatment when most of the cells should not have been viable. However, no decrease in telomerase activity was present in the head and neck cancer cells between 1 and 4 days after Ad-RB94 exposure, which were times that marked growth suppression was shown (15). Therefore, these results (15) are consistent with our findings that changes in telomerase activity *per se* is not responsible for Ad-RB94-produced cytotoxicity. Nevertheless, one of the key mechanisms of RB94-induced cell death may involve the telomere complex and some regulatory aspect of telomerase rather than by a decrease in telomerase activity.

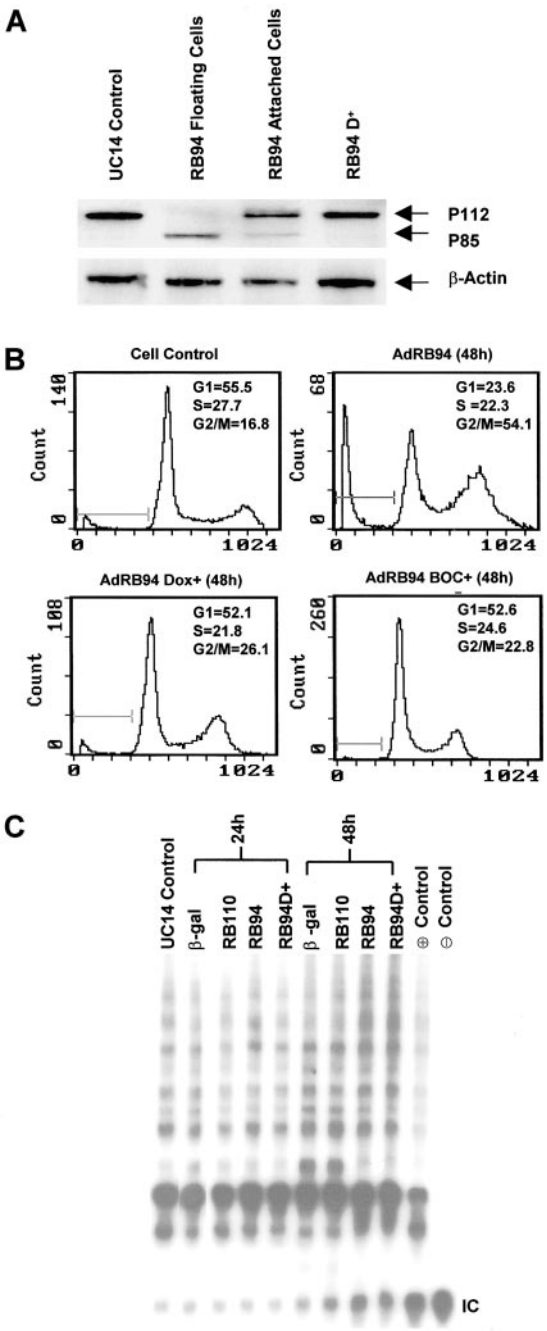

Fig. 3. A. PARP cleavage (P85) in floating and attached cells 48 h after Ad-RB94 treatment of UM-UC14 cells. *B*, increase in G_2-M cells as well as the sub-G_1 population (*bar*) 48 h after Ad-RB94 treatment of UM-UC14 cells. Both floating and attached cells were examined together. *C*, absence of telomerase activity changes in UM-UC14 bladder cancer cells 24 and 48 h after Ad-RB94 treatment.

Whatever the exact unique molecular interactions are by which RB94 produces cytotoxicity and tumor suppression, the results presented here already strongly suggests that Ad-RB94 gene therapy may be highly effective for the treatment of bladder and other cancers. In addition, the finding that Ad-RB94 also kills genetically altered, immortalized urothelial cells, but not normal urothelial cells, may have practical value in treating genetically abnormal, premalignant cells in which telomerase is already active. This suggests the possibility that Ad-RB94 may be cytotoxic not only to cancer cells but also

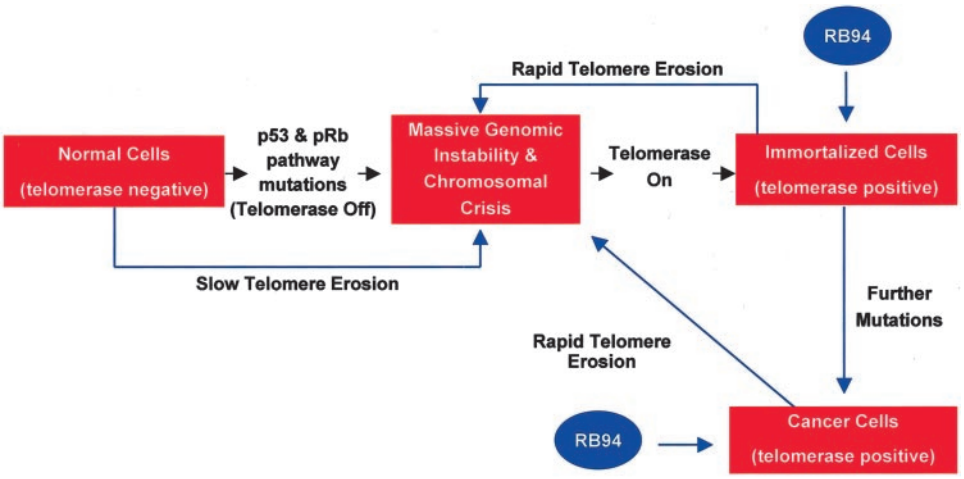

Fig. 4. Diagram of a proposed key pathway of RB94 produced cell death in human immortalized or cancer cells. Slow telomere erosion occurs during passage of telomerase-negative normal cells finally resulting in chromosomal crisis and cell death when p53 and pRB pathways are abrogated. The rare immortalized cells that may be generated are usually telomerase positive, and additional mutations must subsequently occur in order for these cells to actually be able form tumors. RB94, in turn, can produce cell death in these telomerase positive immortalized and cancer cells but not normal telomerase-negative cells by causing rapid telomere erosion and subsequent chromosomal crisis.

to the adjacent normal appearing, genetically altered urothelial cells that are adjacent to the actual malignant lesions (16) while sparing the normal urothelium. Future studies should determine whether this is indeed the case.

Acknowledgments

We thank Drs. Peng Huang and Gordon Mills for their helpful discussions.

References

1. Xu, H-J., Xu, K., Zhou, Y., Li, J., Benedict, W. F., and Hu, S. X. Enhanced tumor cell growth suppression by an internal AUG codon initiated retinoblastoma protein. Proc. Natl. Acad. Sci. USA, *91:* 9837–9841, 1994.
2. Xu, H-J., Zhou, Y., Seigne, J., Perng, G-S., Mixon, M., Zhang, C., Li, J., Benedict, W. F., and Hu, S-X. Enhanced tumor suppressor gene therapy via replication-deficient adenovirus vectors expressing an N-terminal truncated retinoblastoma protein. Cancer Res., *56:* 2245–2249, 1996.
3. Reznikoff, C. A., Yeager, T. R., Belair, C. D., Savelieva, E., Puthenveettil, J. A., and Stadler, W. M. Elevated p16 at senescence and loss of p16 at immortalization in human papillomavirus 16 E6, but not E7, transformed uroepithelial cells. Cancer Res., *56:* 2886–2890, 1996.
4. Belair, C. D., Yeager, T. R., Lopez, P. A., and Reznikoff, C. A. Telomerase activity: a biomarker of cell proliferation, not malignant transformation. Proc. Natl. Acad. Sci. USA, *94:* 13677–13682, 1997.
5. Bryan, T. M., Englezou, A., Gupta, J., Bacchetti, S., and Reddel, R. R. Telomere elongation in immortal human cells without detectable telomerase activity. EMBO J., *14:* 4240–4248, 1995.
6. Liebert, M., Wedemeyer, G., Chang, J. H., Stein, J. A., McKeever, P. E., Carey, T. E., Flint, A., Steplewski, Z., Buchsbaum, D. J., Wahl, R. L., and Grossman, H. B. Comparison of antigen expression on normal urothelial cells in tissue section and tissue culture. J. Urol., *144:* 1288–1292, 1990.
7. Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods, *139:* 271–279, 1991.
8. McConkey, D. J., Goodrich, D., Bucana, C., and Klostergaard, J. The human retinoblastoma gene product suppresses ceramide-induced apoptosis in human bladder tumor cells. Oncogene, *13:* 1693–1700, 1996.
9. Pathak, S. Chromosome banding techniques. J. Reprod. Med., *17:* 25–28, 1976.
10. Multani, A. S., Ozen, M., Furlong, C. L., Zhao, Y. J., Hsu, T. C., and Pathak, S. Heterochromatin and interstitial telomeric DNA homology. Chromosoma (Berl.), *110:* 214–220, 2001.
11. Henson, J. D., Neumann, A. A., Yeager, T. R., and Reddel, R. R. Alternative lengthening of telomeres in mammalian cells. Oncogene, *21:* 598–610, 2002.
12. Multani, A. S., Ozen, M., Narayan, S., Kumar, V., Chandra, J., McConkey, D. J., Newman, R. A., and Pathak, S. Caspase-dependent apoptosis induced by telomere cleavage and TRF2 loss. Neoplasia, *2:* 339–345, 2000.
13. Sherr, C. J., and DePinho, R. A. Cellular senescence: mitotic clock or culture shock? Cell, *102:* 407–410, 2000.
14. Multani, A. S., Li, C., Ozen, M., Imam, A. S., Wallace, S., and Pathak, S. Cell-killing by paclitaxel in a metastatic murine melanoma cell line is mediated by extensive telomere erosion with no decrease in telomerase activity. Oncol. Rep., *6:* 39–44, 1999.
15. Li, D., Day, K. V., Yu, S., Shi, G., Liu, S., Guo, M., Xu, Y., Sreedharan, S., and O'Malley, B. W. The role of adenovirus-mediated retinoblastoma 94 in the treatment of head and neck cancer. Cancer Res., *62:* 4637–4644, 2002.
16. Czerniak, B., Li, L., Chaturvedi, V., Johnston, D. A., and Benedict, W. F. Genetic modeling of urinary bladder carcinogenesis. Genes Chromosomes Cancer, *27:* 392–402, 2000.

Adenoviral-mediated Retinoblastoma 94 Produces Rapid Telomere Erosion, Chromosomal Crisis, and Caspase-dependent Apoptosis in Bladder Cancer and Immortalized Human Urothelial Cells but not in Normal Urothelial Cells

Xinqiao Zhang, Asha S. Multani, Jain-Hua Zhou, et al.

Cancer Res 2003;63:760-765.