Adenovirus-Mediated Ribonucleotide Reductase R1 Gene Therapy of Human Colon Adenocarcinoma

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ABSTRACT

Ribonucleotide reductase is the enzyme responsible for the reduction of ribonucleotides to their corresponding deoxyribonucleotides for DNA synthesis. Ribonucleotide reductase is a multisubunit complex containing two polypeptides, R1 and R2. In addition to catalytic and allosteric regulatory functions, the R1 subunit appears to act as a novel tumor suppressor. Previous studies demonstrated that overexpression of mouse R1 resulted in suppression of tumorigenicity and metastatic potential, whereas expression of antisense RNA, complementary to *R1* mRNA, increased anchorage-independent growth of ras-transformed NIH 3T3 cells. The current study investigated the potential of *R1* gene therapy for human cancer using a recombinant adenovirus encoding the human *R1* gene (rAd5-*R1*).

Recombinant viruses were constructed by FLP-mediated site-specific recombination and demonstrated high infectivity of a human colon carcinoma cell line (Colo320 HRS), as assessed by expression of a viral encoded β-Gal gene (rAd5-*LacZ*). *R1* mRNA and protein were overexpressed in Colo320 HRS cells infected with rAd5-*R1* compared with untreated or rAd5-*LacZ*-infected cells. Infection with rAd5-*R1* inhibited Colo320 HRS cell proliferation, *in vitro*, in a time- and dose-dependent manner. When Colo320 HRS cells were treated with rAd5-*R1*, before injection into CD-1 mice, there was complete inhibition of tumor growth compared with treatment with rAd5-*LacZ*. Furthermore, intratumoral injection of rAd5-*R1* into Colo320 HRS tumor xenografts inhibited tumor growth in CD-1 mice compared with rAd5-*LacZ* treated mice ($P = 0.0001$).

These results demonstrate gene-specific antitumor effects of R1 and suggest that rAd5-*R1* gene therapy has the potential to improve currently available treatments for colon cancer.

INTRODUCTION

Colorectal cancer is the second most common cancer in developed countries. The worldwide prevalence of colorectal cancer exceeds 3.5 million, and ~783,000 new cases of the disease occur each year. This corresponds to 10–15% of all newly diagnosed cancers (1, 2). The annual death rate from colorectal cancer, worldwide, is ~300,000 with 1 in 50 persons dying from the disease each year (1, 2). Current treatment of colorectal cancer involves surgical resection and the use of cytotoxic drugs, treatments that are highly invasive and have significant long-term and short-term side effects. Thus, there is a need for innovative therapies that effectively treat patients, maintain patient quality of life, and exhibit more favorable safety profiles. Over the last decade gene therapy has emerged as a viable alternative to conventional cancer treatment (3). In addition to demonstrating potential for effective treatment of human malignancies, it appears to have a low risk of adverse side effects.

RNR[2] catalyzes the reaction in which 2′-deoxyribonucleotides (dADP, dGDP, dUDP, and dCDP) are generated from the corresponding ribonucleotide 5′-diphosphates (ADP, GDP, UDP, and CDP). This is the rate-limiting step in the production of 2′-deoxyribonucleoside 5′-triphosphates required for DNA replication. RNR consists of two protein subunits, R1 and R2. The R1 subunit is a 160-kDa homodimer that contains the catalytic site, two allosteric effector-binding sites, and redox active disulfides that participate in the reduction of substrates (4, 5). The R2 subunit is a 78-kDa homodimer that contains a nonheme iron that participates in catalysis by forming an unusual free radical on the aromatic ring of a tyrosine residue. Both R1 and R2 subunits are required to form the active site of the enzyme. The genes for R1 and R2 are located on separate chromosomes, and the corresponding mRNAs are differentially expressed throughout the cell cycle. The level of R1 protein remains relatively stable throughout the cell cycle, whereas R2 expression is highly cell cycle-dependent, with highest expression concurrent with DNA replication. The RNR enzymatic activity is also regulated by allosteric control mechanisms involving positive and negative effectors (5, 6).

In addition to being a component of the RNR holoenzyme, the R1 subunit appears to play an important role in determining the malignant potential of tumor cells (7). Using a retroviral vector, stable expression of biologically active mouse R1 in *ras*-transformed mouse fibroblast cells (10T ½) led to significantly reduced colony-forming ability in soft agar. The decrease

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[2] The abbreviations used are: RNR, ribonucleotide reductase; Ad5, adenovirus type 5; LOH, loss of heterozygosity; MOI, multiplicity of infection; β-Gal, β-galactosidase; pfu, plaque-forming unit(s); XTT, sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) bezene sulfonic acid hydrate.

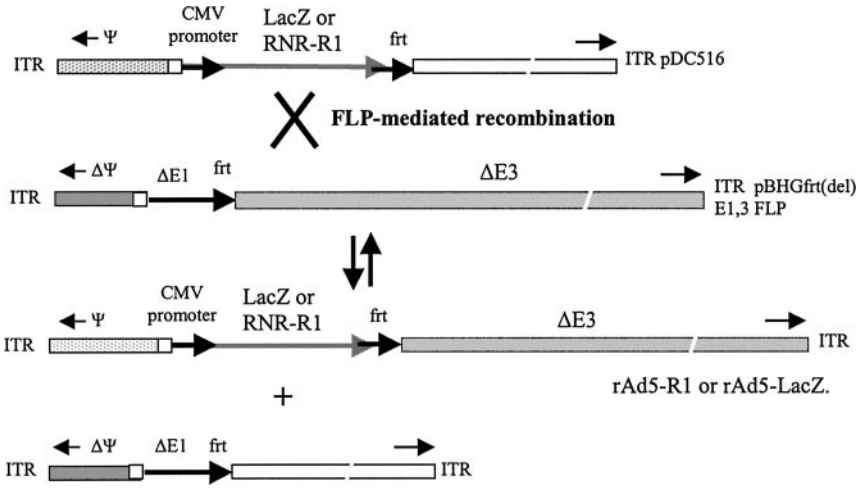

Fig. 1 Construction of rAd5-*R1* and rAd5-*LacZ*. Ad5 genomic plasmid (pBHGfrtdelE1, 3FLP) encoding FLP and carrying an frt site for FLP-mediated recombination with the shuttle plasmid pDC516 is illustrated. Both rAd5-*R1* and rAd5-*LacZ* were generated by FLP-mediated site-specific recombination after cotransfection of 293 cells with the two plasmids. A shuttle plasmid pDC516 contains the left arm with an expression cassette replacing E1 region. ψ,: packaging signal; *ITR,* inverted terminal repeats; *CMV,* cytomegalovirus.

in anchorage-independent growth was accompanied by marked suppression of malignant potential *in vivo*. In three *ras*-transformed cell lines, R1 overexpression resulted in decreased tumorigenicity in syngeneic mouse models. In addition, the ability to form lung metastases by cells overexpressing R1 was reduced by >85%. The characteristics of reduced malignant potential were also demonstrated with R1 overexpressing human colon carcinoma cells. In contrast, *R1* expressed in an antisense orientation significantly increased anchorage-independent growth of ras-transformed NIH 3T3 cells (7). These results suggested that elevated expression of mouse Rl leads to suppression of transformation, tumorigenicity, and metastatic properties of tumor cells.

Although best characterized as the large subunit of the RNR complex, R1 may also be the large subunit component of the complex responsible for generation of dNTPs for DNA repair. p53R2, a recently identified p53-regulated R2 paralogue, is the putative small subunit of the ribonucleotide reductase complex involved in DNA repair (8–10). Studies have demonstrated that R1 can form a functional complex with p53R2 (11). The requirement of R1 for more than one reductase complex would explain the uncoupled nature of R1 and R2 expression.

The *R1* gene has been mapped to chromosome 11p15.5 (12). Interestingly, the centromeric part of 11p15.5 contains a region of frequent LOH in many solid malignancies including lung, breast, ovarian, and stomach cancers (13–19). LOH resulting from an allelic loss of a polymorphic locus is often useful in the identification of tumor suppressor genes. The frequency of LOH within this region is also correlated with metastatic tumor spread (20, 21). This region, termed LOH11A, has been identified with a polymorphic marker, D11S12 (22). Coincidentally, the *R1* gene was found just 50 kb from D11S12. Subsequently, frequent allele loss was determined at a *Sac*I polymorphism within intron IX of *R1*, with 48% of lung cancers being affected (21). Genetic complementation studies using chromosome 11 and fragments containing segment 11p15.5 resulted in reduced tumorigenesis in a syngeneic mouse model and growth inhibition of a number of tumor cell lines *in vitro* (23–27). Taken together, these results suggest that *R1*, encoded within the region of frequent allele loss, may account for at least part of the observed tumor-suppressing activity in the 11p15.5 region.

In this study, the potential therapeutic application of the tumor-suppressing activity of human R1 was explored. A replication-defective recombinant adenoviral construct carrying the human *R1* gene (rAd5-*R1*) was constructed by FLP-mediated site-specific recombination in 293 cells, and was subsequently used to investigate whether it could control proliferation of human colon cancer Colo320 HRS cells *in vitro* and *in vivo*. The results demonstrate that rAd5-*R1* inhibits the growth of Colo320 HRS supporting additional investigation of the potential clinical application of *R1*-based gene therapy in the treatment of colon cancer.

MATERIALS AND METHODS

Cell Lines and Cell Culture. Human colon adenocarcinoma cells (Colo320 HRS) were obtained from the American Type Culture Collection (Manassas, VA). Human embryonic kidney cells (293) were supplied by Microbix Biosystems (Toronto, Ontario, Canada). Cells were maintained in α-MEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin (100 units/ml), and streptomycin (10 units/ml; HyClone, Logan, UT) in a humidified 37°C incubator with 5% CO_2 in air. Cells (293) were used for construction and propagation of recombinant adenoviruses at passage numbers 35–42.

Construction of Recombinant Adenoviruses. A full-length human *R1* cDNA was isolated from a human liver cDNA library (Invitrogen, Burlington, Ontario, Canada) by PCR using a 5′ primer: ATA GAT *GAG CTC* ATG CAT GTG ATC AAG CGA GAT G and a 3′ primer: AGT ACT *CTC GAG* TCA GGA TCC ACA CAT CAG ACA T, which contain *Sal*I and *Xho*I restriction sites indicated in italics, and the start and stop codons are underlined. These restriction sites were used for subcloning into a shuttle plasmid, pDC516 (Microbix Biosystems, Toronto, Ontario, Canada). The *Escherichia coli LacZ* gene (for expression of β-Gal) was amplified from the expression vector pSH18–34 (Invitrogen) by PCR.

The PCR products (*R1* and *LacZ* cDNAs) were cleaved

with the restriction enzymes, purified, and subcloned into the shuttle plasmid, pDC516, containing a mouse cytomegalovirus promoter (see Fig. 1). The sequences of the *R1* and *LacZ* inserts were analyzed by standard sequencing techniques. These constructs were cotransfected with the pBHGfrt(del)E1,3FLP plasmid (Micobix Biosystems) into *E1* complementing 293 cells (28) using standard calcium phosphate transfection methods. The pBHGfrt(del)E1,3FLP plasmid contains the entire *E1*, *E3*-deleted Ad5 genome, a gene encoding FLP recombinase and an frt site to allow for homologous recombination. Twenty-four h after transfection, the medium was aspirated, and cells were overlaid with 5 ml of medium containing 1.0% agarose and 0.1% yeast extract. The culture dishes were incubated at 37°C until the onset of cytopathic effect and plaque formation. A number of individual plaques were collected from plates and stored in 10% glycerol as candidate positive adenoviral stocks. These were repurified by repeating the above plaque formation procedure. Purified recombinant adenoviruses were characterized by restriction mapping and PCR analysis, and rAd5-*R1* or rAd5-*LacZ* clones were subsequently propagated in 293 cells. Recombinant adenovirus stocks were prepared by cell lysis after three freeze-thaw cycles (frozen at −80°C), twice purified by a CsCl gradient, and desalted by dialysis in 10 mM Tris (pH 7.5) and 10% glycerol. Aliquots of purified virus were stored at −80°C in PBS containing 10% glycerol. The viral titer was estimated by absorbance reading at 260 nm and verified by plaque formation in 293 cells. The titer is reported as pfu per ml. The stock solutions were confirmed to be free of replication-competent adenovirus by PCR analysis and infection of Colo320 HRS cells.

Northern Blot Analysis. Northern blot hybridization was carried out to evaluate expression of *R1* mRNA in human colon adenocarcinoma Colo320 HRS cells infected with rAd5-*R1*. Cells were seeded into 100-mm dishes 24 h before infection and then left untreated or infected with rAd5-*R1* or rAd5-*LacZ* at MOIs of 250. Total RNA was extracted from 10^7 cells 24 h after infection using the TRIzol RNA isolation kit (Life Technologies, Inc., Burlington, Ontario, Canada). Twenty μg of RNA was resolved on 1% denaturing formaldehyde agarose gels and transferred to Hybond nylon membrane (Amersham-Pharmacia, Mississauga, Ontario, Canada) by capillary elution. cDNA probes for *R1* mRNA, and 18S and 28S rRNAs were amplified from a human liver cDNA library (Invitrogen, Burlington, Ontario, Canada) by PCR, purified by agarose gel electrophoresis, and recovered using the QIAQUICK gel extraction kit (Qiagen, Missisauga, Ontario, Canada). The probes were radiolabeled with [α-^{32}P]dCTP (Amersham-Pharmacia) using the Oligo labeling kit (Amersham-Pharmacia) and purified with a separation column. After prehybridization, the membranes were hybridized with the labeled probes for 15 h in a solution containing 50% formamide, 0.12 M Na_2HPO_4, 7% SDS, and 1 mM EDTA. The membranes were washed in 2× SSC containing 0.5% SDS at room temperature and then exposed to Kodak X-ray film for autoradiography. The rRNA probes (18S and 28S) were used as internal controls to ensure RNA integrity and equal gel loading.

Detection of R1 Protein by Western Blot. Colo320 HRS cells were treated as described above. Twenty-four h after infection, the cells were harvested and lysed in a SDS sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, and 7.2 mM β-mercaptoethanol. Protein concentration in the lysates was quantified by trichloroacetic acid method or with a Bio-Rad protein assay kit using BSA as the standard. Total protein lysates (40 μg/lane) were resolved on 12% SDS-polyacrylamide gels and proteins transferred to polyvinylidene difluoride membranes. Blots were treated with blocking agent (5% nonfat milk in Tris-buffered saline) for 1 h at room temperature. The blots were subsequently probed with monoclonal antihuman R1 and R2 antibodies (Oncogene Science, Uniondale, NY) at a dilution of 1:500 and monoclonal antihuman glyceraldehyde-3-phosphate dehydrogenase antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:10,000 for 1 h at room temperature. After washing with Tris-buffered saline-Tween 20 three times, a secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) at a dilution of 1:10,000 was added and incubated at room temperature for 1 h. The blot was washed, and secondary antibody detected using an enhanced chemiluminescence detection reagent kit (Amersham-Pharmacia) and exposed to Kodak X-ray film for autoradiography.

XTT Assay for Cell Proliferation. Approximately 1×10^4 Colo320 HRS cells, in 100 μl of culture medium, were plated onto 96-well microtiter plates (Costar) and incubated overnight at 37°C. The medium was removed, replaced with medium containing rAd5-*R1* or rAd5-*LacZ* at different MOIs, and incubated at 37°C for the duration indicated in the text. After incubation, 50 μl of an XTT labeling mixture (XTT at a final concentration of 0.3 mg/ml, XTT labeling reagent and electron-coupling reagent; XTT cell proliferation kit II; Boehringer-Roche Diagnostics, Montreal, Quebec, Canada) was added to each well and incubated for 4 h. The spectrophotometric absorbance was measured at a wavelength of 480 nm using a microtiter plate reader (μQuant; Bio-Tek Instruments, Winooski, VT). The reference wavelength was set at 690 nm. Each condition was performed in triplicate and the results reported as the average.

Tumorigenicity in CD-1 Nude Mice. Six to 8-week-old, female, CD-1 athymic nude mice, (Charles River, Montreal, Quebec, Canada) were acclimatized in a pathogen-free facility at Sunnybrook and Women's Health Science Center for at least 1 week. Animal protocols were in compliance with the Guide for the Care and Use of Laboratory Animals in Canada, and approved by the Animal Care Committees of the Sunnybrook and Women's College Health Science Center and the University of Toronto. Human Colo320 HRS cells were seeded onto 150-mm plates and treated overnight with rAd5-*R1* or rAd5-*LacZ* at an MOI of 300. The cells were harvested and viable cells counted. Approximately 1.5×10^7 cells were s.c. injected into the right flank of CD-1 nude mice. The tumor dimensions (length, width, and height) were measured using calipers twice a week over a 3-week period. Tumor volume was calculated by the formula $L \times W \times H/2$, where L indicates length, W indicates width, and H indicates height. The mice were sacrificed when the tumor burden reached ~15% of total body weight and excised tumors were weighed.

Intratumoral Injections of Recombinant Adenoviruses. Approximately 5×10^6 Colo320 HRS cells were s.c. injected into the right flank of female CD-1 nude mice. Once tumors

reached an approximate volume of 50–100 mm^3, on day 5, mice were randomized by tumor size into two groups (10 mice/group) to receive either rAd5-*LacZ* or rAd5-*R1* treatment. Adenoviral stocks were diluted in 25 μl of sterile PBS (~2.8×10^8 pfu) and injected intratumorally once a day for 5 consecutive days. The tumor volume was measured as above. The mice were sacrificed at day 36 after viral injection.

Statistical Analysis. Student's two-tailed *t* test was used to calculate the statistical significance of differences between experimental groups. Results are presented as mean ± SE (error bars).

RESULTS

Construction of Recombinant Adenoviruses. A schematic outlining the construction of the recombinant adenoviruses, rAd5-*R1* and rAd5-*LacZ*, is illustrated in Fig. 1. They were constructed through FLP-mediated site-specific recombination by cotransfecting 293 cells with the Ad5 genomic plasmid pBHGfrt(del)E1,3FLP and a shuttle plasmid pDC516 bearing the genes of interest (Fig. 1). *R1* and *LacZ* genes were first cloned into a small high copy number shuttle plasmid, pDC516. Recombination in cotransfected 293 cells introduced the *R1* and *LacZ* genes into infectious Ad5 DNA while simultaneously excising the gene encoding for FLP recombinase (29, 30). High efficiency site-specific recombination catalyzed by the FLP recombinase resulted in the "rescue" of the expression cassette to the left end of an *E1*-deleted Ad5 vector, generating rAd5-*R1* or rAd5-*LacZ* (Fig. 1). This *E1* substitution with the expression cassette severely impairs the ability of the recombinant adenoviruses to replicate, restricting their propagation to 293 cells that supply the Ad5 *E1* gene products in *trans* (28).

Identification of recombinant adenoviruses was carried out using newly generated recombinant adenoviruses prepared from the culture of transfected 293 cells. Restriction mapping was performed to analyze the viral genomic structure. DNA from selected clones was subjected to restriction digestion with *Hind*III, which produced DNA fragments of expected size for *R1* and *LacZ* inserted into the adenovirus genome, indicating successful DNA recombination (data not shown). Recombinant adenoviral DNA was prepared from purified rAd5-*R1* and rAd5-*LacZ*, and subjected to PCR analysis to additionally confirm the presence of *R1* and *LacZ* genes in the viral DNA (data not shown). Shuttle plasmid vectors bearing the transgenes were used as controls for the expected size of the *R1* and *LacZ* genes. The infectivity of adenoviruses in different cell lines, even those with the same tissue origin, varies considerably, and as such needs to be determined for each cell line. rAd5-*LacZ*, carrying the β-Gal gene, was used to infect Colo320 HRS cells at an MOI of 200 and the extent of gene transfer was assessed 1 day after infection. The cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside solution to determine β-Gal expression. Hydrolysis of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside by β-Gal resulted in blue staining in ~85% of Colo320 HRS cells (data not shown). The demonstration of LacZ expression in a high percentage of cells suggests that these recombinant adenoviruses are suitable for expression of transgenes in Colo320 HRS cells.

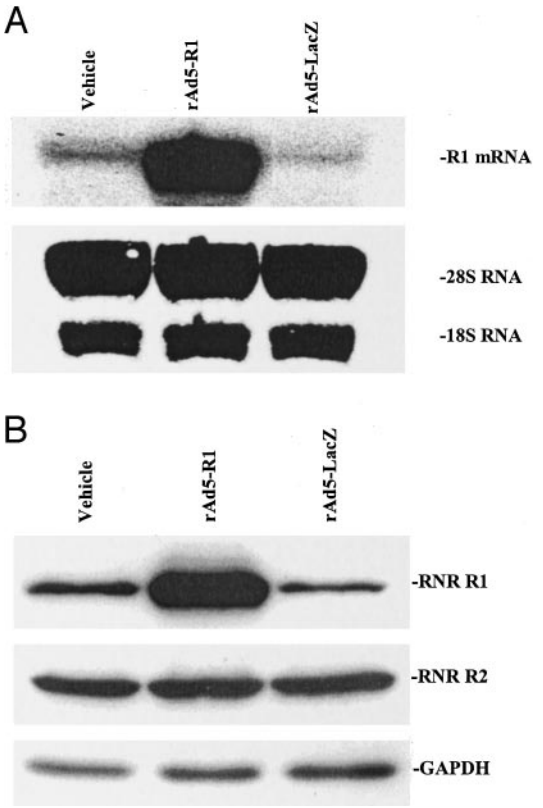

Fig. 2 R1 and R2 expression in Colo320 HRS cells transduced with rAd5-*R1*. *A,* Northern blot analysis of *R1* mRNA expression in Colo320 HRS cells transduced with rAd5-*R1*. Untransduced (*Vehicle*) and rAd5-*LacZ* transduced cells were used as controls. Approximately 40 μg RNA samples were separated on a 1% agarose gel, transferred onto Hybond membrane, and probed with ^{32}P-labeled *R1*, 18S rRNA, and 28S rRNA cDNAs. *B,* Western blot analysis of R1 and R2 protein expression in Colo320 HRS cells transduced with rAd5-*R1*. Total protein lysates were prepared from untransduced cell (*Vehicle*) or cells transduced with rAd5-*R1* or rAd5-*LacZ* 24 h postinfection. The samples were resolved on a 10% SDS-PAGE gel, and immunoblotted with antibodies against human R1 and R2 subunits of RNR. Glyceraldehyde-3-phosphate dehydrogenase was also probed to assess protein integrity and gel loading.

R1 Expression in rAd5-*R1*-transduced Colo320 HRS Cells. Northern blot hybridization was used to determine *R1* mRNA levels in Colo320 HRS cells transduced with either rAd5-*R1* or rAd5-*LacZ*. As illustrated in Fig. 2*A, R1* mRNA levels in rAd5-*LacZ*-tranduced cells were similar to vehicle-treated (no virus control) cells. In contrast, transduction with rAd5-*R1*, at an MOI of 250, induced a significant increase in exogenous *R1* mRNA levels after incubation for 24 h (Fig. 2*A, Lane 2*). The 28S and 18S rRNAs, used as loading controls, were comparable in all of the lanes.

R1 and R2 protein levels in Colo320 HRS cells, transduced with recombinant adenoviral vectors, were determined by Western blotting (Fig. 2*B*). Total cell lysates were prepared from cells, 24 h after transduction with rAd5-*R1* or rAd5-*LacZ* at MOIs of 250. Endogenous human R1 protein was detectable in vehicle-treated (no virus control) cells, with no significant difference in protein levels evident between these control cells and rAd5-*LacZ*-transduced cells (Fig. 2*B, Lanes 1* and *3*). In con-

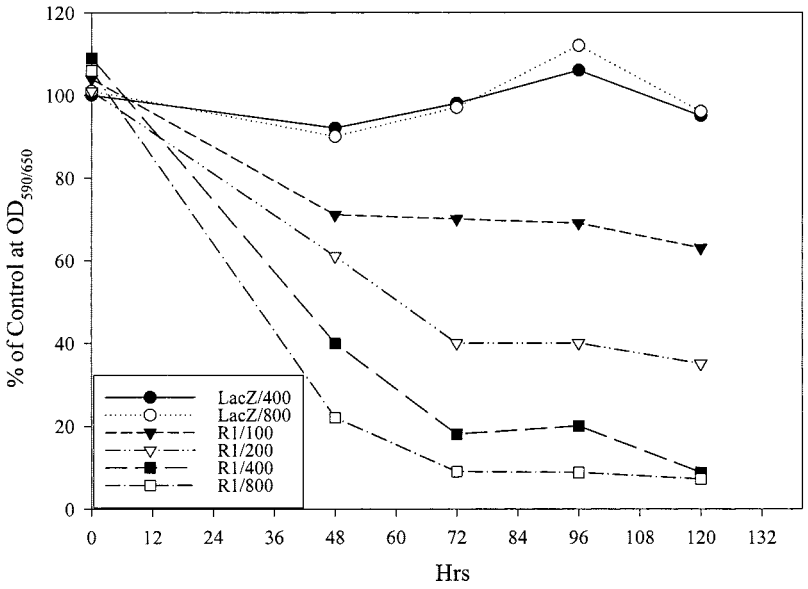

Fig. 3 Inhibitory effects of rAd5-*R1* on growth of Colo320 HRS cells. Colo320 HRS cells were plated at a concentration of 1×10^4 cells/well in 100 μl culture medium, incubated overnight at 37°C, and left untreated or treated with rAd5-*R1* or rAd5-*LacZ* at increasing MOI. After the incubation for indicated duration, the viable cell mass was measured by XTT assay. Cell proliferation was significantly inhibited in a time- and dose-dependent manner.

trast, there was high exogenous R1 protein expression in rAd5-*R1*-transduced cells (Fig. 2*B, Lane 2*). Interestingly, rAd5-*R1* transduction did not result in altered expression of the RNR R2 subunit, suggesting that overexpression of R1 does not affect the pathway regulating R2 expression.

Inhibition of Colo320 HRS Cell Proliferation *in Vitro*. As demonstrated above, transduction of Colo320 HRS cells with rAd5-*R1* dramatically increased expression of the R1 subunit component of human RNR. To examine whether increased expression of R1 affects human colon cancer cell growth, Colo320 HRS cells were treated, *in vitro*, with vehicle (no virus) control, rAd5-*LacZ*, or rAd5-*R1*. To determine the appropriate MOI range for growth inhibition, $\sim 1 \times 10^4$ Colo320 HRS cells were plated onto 96-well microtiter plates, cultured overnight, and fresh media containing recombinant adenoviruses at different MOIs were added in triplicate. After a 3-day incubation, the viable cell mass was measured by XTT assay. The results are expressed as a percentage of the $A_{590/650}$ measured in vehicle-treated cells. Exposure of Colo320 HRS cells to increasing amounts of rAd5-*R1* resulted in a significant decrease in cell proliferation (data not shown). The growth-inhibitory effect was enhanced by increasing the MOI from 100 to 800. In contrast, rAd5-*LacZ* did not show significant growth inhibition, even at the highest MOI of 1200 (data not shown). Subsequently, a range of MOIs, from 100–800, was chosen, and the cells were treated for the durations indicated in Fig. 3. As shown in Fig. 3, rAd5-*R1* clearly inhibited the growth of Colo320 HRS cells in a dose- and time-dependent manner. In contrast, there was no significant growth inhibition of the cells treated with rAd5-*LacZ*, even at an MOI of 800 on day 5 (Fig. 3). These results indicate that rAd5-*R1* specifically suppresses proliferation of human colon tumor cells. rAd5-*R1* treatment of 3 additional colon cancer cell lines, HCT-116, HT-29, and LS513, using a range of MOIs and treatment durations similar to those described for Colo320 HRS, resulted in growth suppression reaching a maximum of 66%, 22%, and 68%, respectively (data not shown). Colo320 HRS growth inhibition reached a maximum of 100% under the same conditions. The observed differences in inhibition may be because of a number of factors including differences in infectivity, differences in the level of R1 expression (relative to R2 or relative to other interacting partners), and differences in the degree of cellular responses to overexpressed R1. The causes of intercell variability will become clearer as the mechanism of R1-induced tumor suppression is elucidated. Treatment of a normal cell line, WI-38, with rAd5-*R1* demonstrated modest inhibition (22%) under conditions that suppress tumor cell growth to a larger extent in all but HT-29 cells, suggesting that R1-mediated tumor suppression acts within a tumorigenic pathway (data not shown).

rAd5-*R1*-mediated Inhibition of Tumor Growth After *ex Vivo* Treatments. To evaluate the effects of R1 overexpression on tumorigenesis *in vivo*, we assessed the ability of Colo320 HRS cells to form tumors in nude mice after *ex vivo* treatment with rAd5-*R1*. Colo320 HRS cells were treated with either rAd5-*LacZ* or rAd5-*R1* at an MOI of 300. The cells were s.c. injected into mice 16 h after transduction and tumor growth monitored over a 3-week period. As shown in Fig. 4, *A* and *B,* rAd5-*R1* dramatically inhibited tumor formation in all of the mice, whereas rAd5-*LacZ*-treated groups showed rapid growth of tumors. The differences in tumor weights between rAd5-*R1* and rAd5-*LacZ*-treated groups (2.14 ± 1.0 *versus* 2281 ± 382.2) were statistically significant with a *P* of 0.0003 (Fig. 4*A, inset*). Furthermore, the average liver, spleen, and body weights of mice were not significantly different between the two groups, indicating no apparent toxicity associated with treatment (data not shown).

rAd5-*R1*-mediated Inhibition of Tumor Growth *in Vivo*. To reflect the clinical application of adenovirus-mediated *R1* gene therapy for colon cancer, the antitumor effects of rAd5-*R1* were evaluated after intratumoral injections of rAd5-*R1* directly into human tumor xenografts implanted in CD-1 nude mice. Approximately 5×10^6 Colo320 HRS cells

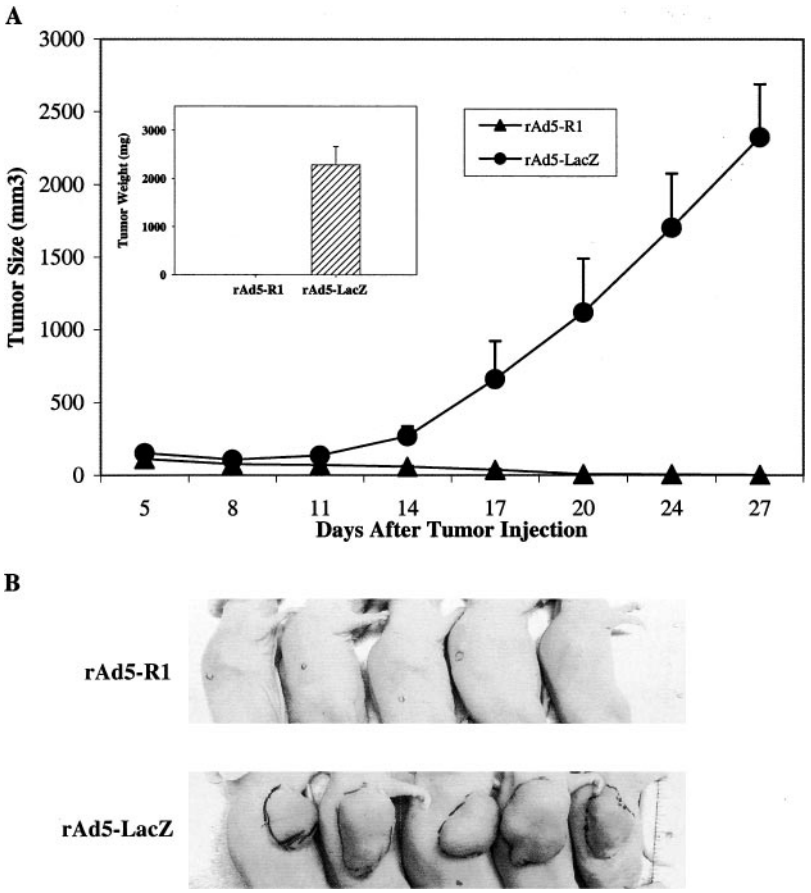

Fig. 4 Growth of human colon adenocarcinoma (Colo320 HRS) in CD-1 nude mice after *ex vivo* treatment with rAd5-*R1*. Colo320 HRS cells were seeded onto plates and transduced overnight with rAd5-*R1* or rAd5-*LacZ* at MOI of 300. Approximately1.5 $\times$ 10^7 cells were s.c. injected into the right flank of CD-1 nude mice. Tumor dimension was periodically measured using calipers over a 3-week period. *A,* tumor growth curves. Each point represents average volume calculated from 7 mice. *Bars,* $\pm$SE. *Error bars* are too small to see in rAd5-*R1* treated group. *Inset,* the differences in tumor weights between rAd5-*R1* and rAd5-*LacZ*-treated groups; *B,* a diagram of Colo320 HRS tumors in the right flank of nude mice after the treatment.

were s.c. injected into the right flank of CD-1 nude mice. When tumors reached a volume of 50–100 mm^3, the mice were injected intratumorally with either rAd5-*R1* or rAd5-*LacZ* once a day for 5 consecutive days. As illustrated in Fig. 5, rAd5-*LacZ*-treated Colo320 HRS tumors grew significantly faster than rAd5-*R1*-treated tumors ($P = 0.0001$). Interestingly, delayed tumor growth was observed well past the 5-day treatment period suggesting that *R1*-based gene therapy could have long-lasting efficacy. Consistent with the results from the *ex vivo* study (Fig. 4), negligible differences in the liver, spleen, and body weights of animals between treatment groups were observed, indicating no apparent toxicity associated with treatment.

DISCUSSION

Previous studies demonstrated that retrovirus-mediated expression of mouse R1 resulted in abrogation or marked suppression of tumorigenicity and metastasis in *ex vivo* experiments. The current study evaluated the clinical applicability of *R1* gene therapy for human colon cancer. In contrast to the earlier study, the human *R1* gene was used to determine whether R1-mediated tumor suppression is a general property of R1. In addition, the retroviral expression system was replaced with an adenoviral system. Modified adenoviruses are one of the most commonly used viral vector systems that offer significant advantages over retroviruses for human gene therapy (31, 32). Finally, the therapeutic potential of R1-mediated gene therapy was investigated in *in vivo* experiments wherein recombinant adenoviruses were administered directly into established tumor xenografts by intratumoral injections. This approach more closely mimics the therapeutically relevant setting, thus permitting evaluation of the potential for R1-mediated adenoviral gene therapy in the treatment of human colon cancers.

A recombinant adenoviral vector, rAd5-*R1*, was produced by FLP-mediated site-specific recombination, and *in vitro* expression studies showed adenovirus-mediated overexpression of R1 compared with vehicle and rAd5-*LacZ* controls. Transduction of rAd5-*R1* into human colon adenocarcinoma cells (Colo320 HRS) induced antiproliferative effects in a dose- and time-dependent manner. More importantly, these inhibitory effects were demonstrated in animal models. Not only did rAd5-*R1* suppress tumorigenesis after *ex vivo* treatment of Colo320 HRS cells but also the growth of Colo320 HRS xenografts when injected intratumorally. In both experiments there was a significant effect on tumor growth compared with treatment with a *lacZ*-expressing recombinant virus, thereby demonstrating sequence specificity. Taken together these results suggest that inhibition of tumor growth, both *in vitro* and *in vivo*, is mediated by overexpression of R1. This is the first published report describing antitumor effects of adenovirus-

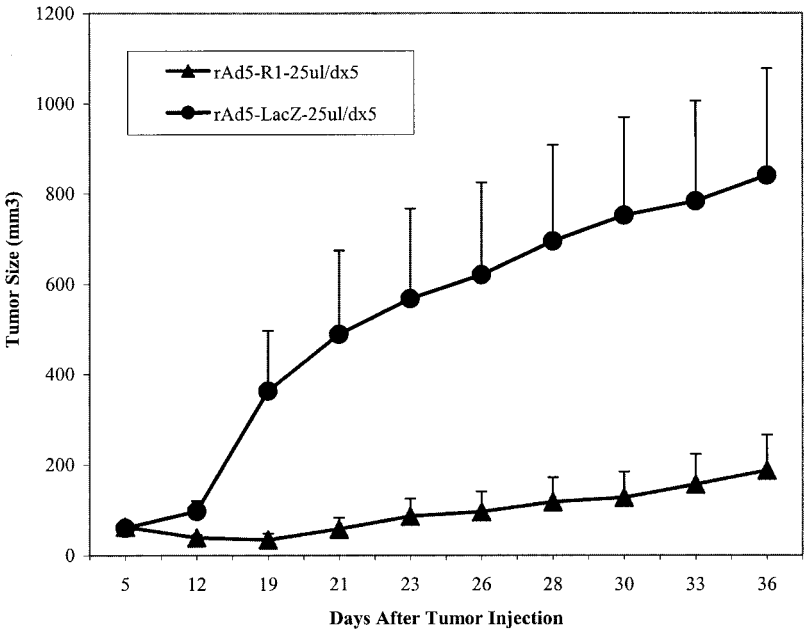

Fig. 5 rAd5-*R1* inhibits human colon tumor growth in CD-1 nude mice. Approximately 5×10^6 Colo320 HRS cells were s.c. injected into the right flank of CD-1 nude mice. When tumors reached a volume of approximately 50–100 mm^3, mice ($n = 10$) were injected with rAd5-*R1* or rAd5-*LacZ* ($\sim 2.8 \times 10^8$ pfu) intratumorally once daily for 5 days. Tumor dimension was periodically measured using calipers over a 4-week period. Compared with the tumor growth after treatment with rAd5-*LacZ*, rAd5-*R1* significantly inhibited tumor growth in CD-1 nude mice ($P = 0.0001$). Each point represents average volume calculated from 10 mice. *Bars,* ±SE.

mediated overexpression of the R1 component of human RNR extending previous observations that suggested the R1 component of RNR is a putative tumor suppressor (7).

Gene therapy has been one of the most exciting and elusive areas of therapeutic research in the past decade (3). Some tumor suppressor genes, such as *p53*, *p16*, and *pRB*, have been investigated for cancer gene therapy. In particular, *p53* tumor suppressor gene therapy has displayed antitumor efficacy against human cancers including colorectal, prostate, breast, cervical, ovarian, and skin cancers (33–38). Here, we have introduced a novel therapeutic approach that uses the tumor-suppressing activity of the R1 component of human RNR. As described in the introduction, allelic loss of *R1* is frequently observed in the LOH11A region on chromosome segment 11p15.5 in a variety of tumors. This, together with our previous data (39), genetic complementation studies (23–27), and clinical correlative studies (14–17, 19, 20) supports the hypothesis that a gene capable of tumor suppressing activity is located in the LOH11A region and that the most likely candidate is the *R1* gene. *R1* appears to be quite different from "classical tumor suppressor genes." For example, loss-of-function mutations in *p53* are usually recessive, and cancer occurs only when both copies are defective. In contrast, complete loss of *R1* is deleterious to cell survival. This is because of RNR activity being essential for *de novo* deoxyribonucleotide synthesis and maintenance of dNTPs pools during DNA replication. The unusual finding that a screen of 117 lung cancer cell lines did not identify any homozygous deletions in the LOH11A region (40) support the hypothesis that at least one functional allele of *R1* gene is required for cell viability, whereas normal or elevated level of *R1* expression results in a suppression of phenotypic characteristics of malignant cells. The malignant phenotype associated with the allelic loss in the LOH11A region likely results from a recessive or null mutation in one allele of *R1* rather than a homozygous recessive mutation, and this categorizes *R1* as a novel tumor suppressor. It would appear that Knudson's "two-mutation" criterion (41) does not sufficiently explain tumor suppressors such as *R1*. This type of "haplo-insufficiency" for tumor suppression has also been observed for $p27^{Kip1}$ (42), a candidate tumor suppressor protein that inhibits cyclin-dependent kinases and blocks cell proliferation (43–47).

RNR is the only enzyme responsible for the reduction of ribonucleotides to their corresponding deoxyribonucleotides. Enzymatic activity of RNR requires both R1 and R2 components that associate to form the active holoenzyme. Different mechanisms control *R1* and *R2* gene expression and enzyme activity during cell proliferation (48, 49). Transcription of both the *R1* and *R2* genes occurs during S phase, but the R2 protein is primarily expressed in S phase and slowly accumulates up to late mitosis when it is rapidly degraded (50–53). The level of R1 protein is constant during the cell cycle in proliferating cells because of its long half-life (52, 54). Therefore, the RNR activity is controlled by the synthesis and degradation of the R2 protein during the cell cycle. In an *in vitro* study, overexpression of R1 in human oropharyngeal carcinoma KB cells had a negligible effect on RNR enzymatic activity and did not alter the R2 expression, although R1 overexpressing cells demonstrated decreased invasive potential than control cells. In contrast, overexpression of R2 in KB cells resulted in a significant increase in the enzymatic activity of RNR and invasive potential (55). When R2 expression in rAd5-*R1*-transduced cells was examined, there was no significant change in the R2 protein expression. Given that the R2 protein is limited in cells, R1 protein overexpression alone would not be expected to increase RNR activity. This suggests that R1-mediated tumor-suppressing activity is not a direct consequence of changes in RNR activity.

The mechanism underlying the tumor-suppressing activity of R1 remains uncertain. There is the possibility that R1 exerts

its tumor-suppressing activity simply through deregulation or competitive depletion of dNTP pools by increasing levels of ATP and dNTP binding to the R1 subunit. This is not likely, given that other tumor cell lines including human metastatic melanoma (A2058) cells were not affected by rAd5-*R1* transduction, despite R1 protein being highly overexpressed (data not shown). Furthermore, in a previous study, tumorigenesis of mutant ras-, p53-, and c-myc-transformed mouse fibroblast 10T ½ cell line, infected with *R1* retroviral construct (RMP/mR1) was not affected in soft agar assays. Although there is no direct evidence, it is attractive to speculate that R1, in excess, modulates growth-signaling pathways, thus affecting cell proliferation, differentiation, and apoptosis. This is supported by the observation that deregulated R2 expression activates the mitogen-activated protein kinase pathway, and alters cell proliferation, differentiation, and apoptosis (39). Given that R1 may be the large subunit for the p53R2 ribonucleotide reductase complex, R1 expression may directly affect DNA repair mechanisms via interaction with p53R2. As with RNR, this pathway has the potential to alter tumorigenesis and growth signaling pathways. Studies are under way to elucidate the molecular mechanism(s) responsible for the tumor-suppressing activity of R1.

In summary, this study demonstrates that intratumorally injected rAd5-*R1* significantly inhibits the growth of human colon carcinoma xenografts in nude mice. These results support the conclusion that human *R1* acts as tumor suppressor and that *R1*-mediated gene therapy may be a novel approach for the treatment of human colon adenocarcinoma. Furthermore these results open up the possibility of genetic complementation of *R1* in tumors harboring an allelic loss at *R1* locus, thereby broadening the potential for gene therapy of some human cancers.

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