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Enzyme-activated Prodrug Therapy Enhances Tumor-specific Replication of Adenovirus Vectors[1]

Kathrin M. Bernt, Dirk S. Steinwaerder, Shaoheng Ni, Zong-Yi Li, Steve R. Roffler, and André Lieber[2]

Division of Medical Genetics, Department of Medicine [K. M. B., D. S. S., S. N., Z-Y. L., A. L.] and Department of Pathology [A. L.], University of Washington, Seattle, Washington 98195, and Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan, Republic of China [S. R. R.]

ABSTRACT

Adenoviruses (Ads) that selectively replicate in tumor cells have shown promising preliminary results in clinical trials, especially in combination with chemotherapy. Here, we describe a system that combines the antitumor synergy of Ads and chemotherapeutic agents with the benefits of enzyme-activated prodrug therapy. In this system, a functional transgene expression cassette is created by homologous recombination during adenoviral DNA replication. Transgene expression is strictly dependent on viral DNA replication, which in turn is tumor specific. We constructed replication-activated Ad vectors to express a secreted form of β-glucuronidase and a cytosine deaminase/uracil phosphoribosyltransferase, which activate the prodrugs 9-aminocamptothecin glucuronide to 9-aminocamptothecin and 5-fluorocytosine to 5-fluorouracil (5-FU) and further to 5-fluoro-UMP, respectively. We demonstrated replication-dependent transgene expression, prodrug activation, and induction of tumor cell toxicity by secreted β-glucuronidase and cytosine deaminase/uracil phosphoribosyltransferase. Furthermore, exposure of cells to activated prodrug or drug at subtoxic concentrations enhanced viral DNA replication. Characteristically, these agents induced changes in the cell cycle status of exposed cells (G_2 arrest), which closely resembled the effect of wild-type Ad infection, and are thought to be favorable for viral replication. We tested a number of cytostatic drugs (camptothecin, etoposide, daunorubicin, cisplatin, 5-fluorouracil, hydroxyurea, Taxol, and actinomycin D) for their effect on viral DNA replication and found considerable differences between individual agents. Finally, we show that the combination of viral and prodrug therapy enhances viral replication and spread in liver metastases derived from human colon carcinoma or cervical carcinoma in a mouse model. Our data indicate that specific vector/drug combinations tailored to be synergistic may have the potential to improve the potency of either therapeutic approach. These data also provide a new rationale for expressing prodrug-activating enzymes from conditionally replicating Ads.

INTRODUCTION

Ads[3] that selectively replicate in tumor cells (CRADs) are promising tools for the treatment of cancer (for a review, see Ref. 1). Several strategies for tumor-selective adenoviral replication have been evaluated, including the deletion of adenoviral E1A and/or E1B functions that are dispensable in tumor cells (2–8) or the regulation of E1A and/or E1B expression with tumor-specific promoters (9–12). An E1B-55k deleted virus, dl1520, also known as ONYX-015 or CI-1042, has been shown to be well tolerated in patients with head and neck cancer after intratumoral injection (13–16) as well as in patients with metastatic solid tumors after intravascular virus administration (16, 17). However, significant antitumor activity was only seen if viral treatment was combined with chemotherapy (14, 17–19). Antitumor synergy of viral oncolysis and chemotherapeutic agents was also reported for other CRADs including those with *E1* genes under the control of tumor-specific promoters (11, 12). The nature of the synergistic effect of viral and chemotherapy is most likely a result of several interactions. Viral infection and gene expression have been shown to sensitize tumor cells to chemotherapeutic agents. The gene products of the *E1A* region are believed to be key players for this effect (20, 21). Furthermore, induction of apoptosis by cytostatic agents may help newly formed virus to escape from infected cells and promote viral spread throughout the tumor (5, 22). In addition, adenoviral infection may cause expression/release of inflammatory cytokines such as tumor necrosis factor α (23), which has been reported to exert a chemosensitizing effect (24, 25). The extent to which each interaction contributes to the observed clinical synergism is unclear. A more detailed understanding of the underlying molecular mechanisms is desirable because it may help to identify virus/drug combinations that are particularly effective.

Based on the synergistic effect of viral and cytostatic therapy, Ads appear to be particularly well suited for the delivery of prodrug-activating enzymes. While confining prodrug activation to the tumor tissue and thus rendering chemotherapy more specific, the viral infection potentially enhances the susceptibility of tumor cells to drug-mediated apoptosis. For systemic application, however, tumor-specific expression of the prodrug-activating enzyme has to be ensured. We have previously described an adenoviral expression system that achieves tumor-specific transgene expression in liver metastases after systemic vector application (26, 27). This new vector system utilizes homologous recombination between inverted repeats to mediate precise rearrangements within the viral genome. As a result of these rearrangements, a promoter is brought into conjunction with a transgene, creating a functional expression cassette (Fig. 1). Genomic rearrangements and transgene expression are dependent on viral DNA replication, which, in turn, efficiently occurs only in tumor cells and not in normal cells.

Here we used this system to express enzymes that convert a nontoxic form of a chemotherapeutic drug (prodrug) to the active drug selectively in tumor cells. Specifically, we generated vectors expressing (*a*) a fusion protein of *Escherichia coli* cytosine deaminase and uracil phosphoribosyl transferase, which activates 5-FC to 5-FU and its intracellularly active metabolite 5-fluoro-UMP (28–30), and (*b*) a secreted form of human β-glucuronidase (SG) (31). The SG converts the water-soluble prodrug 9-ACG into membrane-permeable 9-AC (32). Endogenous β-glucuronidase is a lysosomal enzyme, which is not accessible to the prodrug (31).

In parallel, this system was used to investigate the synergistic activity between viral and cytostatic therapy. From previous studies, it was known that the uptake of viral particles and viral DNA replication are enhanced if the cells are arrested in G_2 (33, 34). Considering that a variety of chemotherapeutic agents induce similar changes to the

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[2] To whom requests for reprints should be addressed, at Division of Medical Genetics, Department of Medicine, University of Washington, Box 357720, Seattle, WA 98195. E-mail: lieber00@u.washington.edu.

[3] The abbreviations used are: Ad, adenovirus; BG, β-galactosidase; SG, secreted β-glucuronidase; CodAupp, cytosine deaminase/uracil phosphoribosyl transferase; GDEPT, gene-directed enzyme-activated prodrug therapy; CRAD, conditionally replicating adenovirus; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide; MOI, multiplicity of infection; pfu, plaque forming unit(s); 5-FC, 5-fluorocytosine; 5-FU, 5-fluorouracil; 9AC, 9-aminocamptothecin; 9ACG, 9-aminocamptothecin glucuronide; DAPI, 4′,6-diamidino-2-phenylindole; RSV, Rous sarcoma virus; SAEC, small airway epithelial cell; HU, hydroxyurea; Topo, topoisomerase; WT, wild-type; X-gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid.

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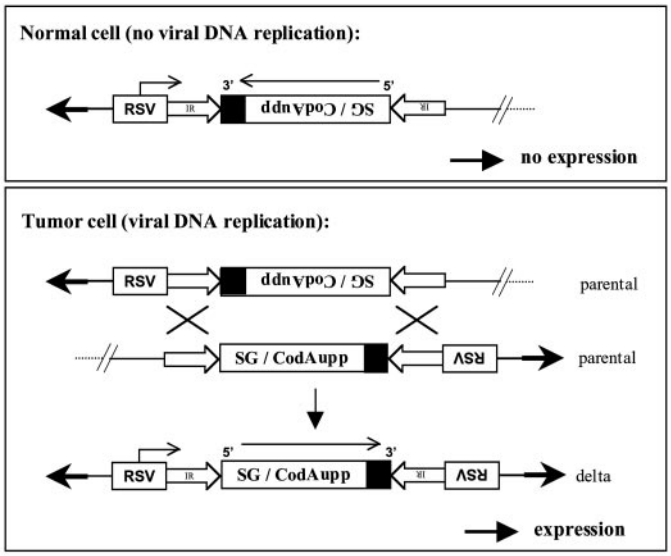

Fig. 1. Replication-dependent expression of prodrug-activating enzymes. In the parental vector, the prodrug-activating enzyme is in 3′-5′ orientation relative to the promoter and is flanked by two introns (*white open arrows*) in inverse orientation. Tumor cells but not normal cells support viral DNA replication (34). During viral replication in tumor cells, homologous recombination between the two inverted repeats within the incoming "parental" viral genomes mediates the formation of a recombined "delta" product carrying two inverted copies of the left half of the parental genome (including the RSV promoter), one at either site of the transgene. In the delta product, the transgene is in "active" 5′→3′ orientation with regard to the RSV promoter, which allows for its expression. *IR*, inverted repeats; *RSV*, Rous sarcoma virus LTR.

cell cycle status, we hypothesized that exposure to these agents should also stimulate adenoviral DNA replication. To test this hypothesis, we studied the effect of a variety of cytostatic agents on the DNA replication of Ad vectors deleted for both the E1A and E1B genes as well as for all E3 genes (Ad.ΔE1). Although they are replication deficient in normal quiescent cells, E1-deleted viral vectors are able to efficiently replicate their DNA in human tumor cell lines (26, 34). Despite efficient DNA replication of Ad.ΔE1 vectors in tumor cells, *de novo* production of infectious particles is inefficient, compared with WT virus (22, 26).

In this study, we analyze the tumor cell-specific replication-dependent expression, prodrug activation, and toxicity to tumor cells by SG and CodAupp. We also show that the activation of prodrugs, in turn, enhances viral replication *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals. Camptothecin, 5-FC, 5-FU, cisplatin, daunorubicin, etoposide, Paclitaxel, HU, actinomycin D, aphidicolin, organic solvents and XTT, phenazine methosulfate, and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). 9AC and 9ACG were synthesized as described elsewhere (32).

Plasmid Constructs and Vectors. pAd.IR-SG was derived from pAd.IR-BG (26) by deleting the BG gene using *Bpu*102/*Avr*II and inserting the cDNA of human glucuronidase fused to a IgG leader sequence (SG; Ref. 31). The cDNA for CodAupp was cut from pORF-codAupp (Invivogen, San Diego, CA) using *Nco*I/*Nhe*I and inserted into the same vector as described above, generating Ad.IR-CodAupp. Shuttle vectors were linearized by *Xmn*I digestion and cotransfected with pBHG10 (Microbix, Toronto, Canada) into 293 cells. The corresponding viruses were named Ad.IR-SG and Ad.IR-CodAupp. As a control virus (Ad.control), we used Ad.IR-BG (26), which has the same structure as Ad.IR-SG and Ad.IR-CodAupp but contains lacZ as the transgene. Recombinant viruses with the correct restriction patterns were propagated in 293 cells, banded in CsCl gradients, dialyzed, and stored in aliquots as described elsewhere (26). Titers were determined by absorbance measurement and plaque titering on 293 cells (35). All of these vectors have the E1A and E1B genes (nucleotides 342-3523) and the E3 genes (nucleotides 28,133–30,818) deleted (Ad.ΔE1). To assess contamination of Ad.ΔE1 vector preparations with $E1^+$ replication-competent Ad, real-time PCR analysis was performed using primers for E1A [GACCGTTTACGTGGAGACTC (sense) and CAGCCAGTACCTCTTCGATC (antisense)] and control primers for a sequence in the E4 region [TAAGCATAAGACGGACTACG (sense) and GTAAGGCTGACTGTTAGGC (antisense)]. Quantitative PCR was performed using the SYBR green kit for the light cycler (Roche, Indianapolis, IN) and external standards for E1 and E4 (15 s at 95°C, 5 s at 57°C, and 17 s 72°C). Only virus preparations that contained less than 1 $E1^+$ (WT) viral genome in 1×10^8 genomes were used in these studies. WT Ad-type 5 (Ad5) was amplified, purified, and titered as described previously (36).

Cells. 293 cells (Microbix) and HeLa cells (ATCC CCL-2) were grown in DMEM with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. LoVo cells (ATCC# CCL-229) were grown in F12K media supplemented with 15% fetal bovine serum, L-glutamine, penicillin, and streptomycin. SAEC cells were obtained from BioWhittaker (Walkersville, MD) and grown in small airway epithelial cell growth medium (SAGM) media supplemented with 0.3 mg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 ng/ml human epidermal growth factor, 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 6.5 ng/ml triiodthyronine, 50 μg/ml gentamicin, 50 ng/ml amphothericin B, and 0.5 mg/ml BSA.

Cytotoxicity Assay. Cytotoxicity was determined by XTT tetrazolium/formazan assays. Cells were seeded in 96-well plates at a density of 10^4 cells/well. HeLa cells were infected with Ad.IR-SG or control virus at a MOI of 100 pfu/cell, and LoVo cells were infected with Ad.IR-CodAupp or control virus at a MOI of 300 pfu/cell. Infections were carried out for 3 h, and cells were washed. Unless stated otherwise, drugs or prodrugs were added 16 h after viral infection at the indicated concentrations. After 72 h, cell viability was assessed by adding 50 μl of XTT reagent (1 mg/ml in DMEM) activated with 0.2% (v/v) phenazine methosulfate (1.53 mg/ml in PBS) and incubation at 37°C for 4 h. Plates were analyzed in a Packard Spectracount microplate reader at a wavelength of 450 nm. Absorbance values were plotted as a function of drug concentration. For synergism analysis, individual dose-response curves for viral and cytostatic treatment were plotted, and calculated MOI-toxicity curves based on isoeffective curves in mode I and II according to the method of Steel and Peckham were computed (11, 63). Data points between the calculated curves I and II indicate an additive effect, and data points below the two curves indicate a supra-additive, or synergistic effect.

Glucuronidase/BG Assays. Replication-dependent expression was shown using an X-gluc or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining assay. Cells were seeded in 6-well plates and infected with Ad.IR-SG or Ad.IR-BG (100 pfu/cell) for 3 h. Then, HU (10 mM final concentration) was added to one set of samples to block viral replication. After 72 h, cells were fixed with 0.5% glutaraldehyde in PBS. Cells or supernatant was stained for glucuronidase with X-gluc (Clontech, Palo Alto, CA) as substrate under acidic conditions [30 mM $K_3Fe(Cn)_6$, 30 mM $K_3Fe(Cn)_6$, 15 mM NaCl, 1.3 mM $MgCl_2$, 100 mM sodium acetate (pH 5.2), 0.1 mg/ml X-gluc, 5 mg/ml stock in n-dimethyl-formamide (DMF)]. Staining for BG was performed with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as the substrate and 50 mM HEPES (pH 7.4) instead of sodium acetate. To determine glucuronidase expression in prodrug/drug-exposed cells, cells were seeded in 96-well plates, infected with Ad.IR-SG (30 pfu/cell) for 3 h, and incubated with 9-ACG (24 h after infection) or camptothecin at the indicated concentrations and time points. X-gluc staining was performed as described above, and glucuronidase activity of individual samples was determined using a standard curve of purified bovine glucuronidase.

To determine BG activity, cells were seeded in 24-well plates and infected with Ad.IR-BG for 3 h or transfected using calcium phosphate with pAd.RSV-BG, the left-handed shuttle plasmid used to generate pAd.BG (replication-independent BG expression from the RSV prompter). Sixteen h after infection or transfection, cells were incubated with subtoxic concentrations of cytostatic agents. Subtoxic concentrations were defined as a drug concentration that caused a 10–30% growth inhibition in the XTT assay and no cell death as determined by trypan blue staining. Seventy-two h after the addition of drug, cells were washed with cold PBS and lysed, and BG activity was determined using a luminometric BG assay kit (Boehringer Mannheim, Mannheim, Germany).

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Replication Assays. For the generation of methylated virus, we amplified the vectors in 293-PMT cells expressing the prokaryotic PaeR7 methyl transferase (37). Cells were infected with methylated virus for 3 h in duplicate. Three h and 72 h after infection, cells were harvested, and total DNA was isolated. To determine the ratio of replicated:nonreplicated viral DNA, the extracted total DNA (10 μg for non-drug-treated control samples) was digested with *Hin*dIII and *Xho*I and subjected to agarose gel electrophoresis followed by Southern blotting (37). To account for the lesser extent of template dilution in drug-treated samples, the amount of DNA loaded/lane was multiplied with a correction factor reflecting the lower amount of cellular DNA and thus the higher viral:cellular DNA ratio in these samples:

$$\text{Total DNA}_{\text{drug}}\ (\mu g) = \text{Total DNA}_{\text{non-drug}}\ (\mu g) \times \text{cell number}_{\text{drug}}/\text{cell number}_{\text{non-drug}}$$

i.e., for a drug-treated sample where the cell number was only 80% of non-drug-treated samples, only 8 μg (10 μg $\times$ 0.8) was loaded. The blots were probed using an 8-kb *Hin*dIII fragment of the Ad5 genome (bp 18319 to bp 26328). Double digestion with *Hin*dIII and *Xho*I yields clearly distinguishable DNA fragments specific for demethylated (replicated) and methylated (nonreplicated) viral DNA because methylation of the viral genome during amplification in 293-PMT cells blocks the *Xho*I site at bp 24796. Therefore, only progeny DNA can be cut by *Xho*I, resulting in two detectable fragments of 1.5 and 6.5 kb after *Hin*dIII and *Xho*I digestion.

Cell Cycle Analysis. Cell cycle analysis was performed as described previously (34). Briefly, cells were presynchronized (12 h in 0.5% serum, 9 h in 10% serum) and synchronized (12 h; 4 μg/ml aphidicolin). At the end of the synchronization period, the cells were infected with Ad.IR-SG or WT Ad5 for 3 h. The cells were washed and placed into medium containing 10% or 0.1% serum (for serum-starved samples). Sixteen h postinfection, camptothecin was added at the indicated concentrations. Thirty-six h after the addition of drug, cells were harvested, washed, and fixed in 70% ethanol for 12 h. Cellular DNA was stained with 50 μg/ml propidium iodine in 4 mM trisodium citrate (pH 7.8), 0.1% Triton X-100, and 1 mg/ml DNase-free RNase for 10 min at 37°C. After adjusting the salt concentration to 13.8 mM, stained cell suspensions were analyzed using a Coulter Epics Elite flow cytometer as described elsewhere (38). Data analysis was carried out using MultiPlus software (Phoenix Flow Systems, San Diego, CA).

Fluorescence-activated Cell-sorting Analysis of Ki-67 Antigen. Cells were synchronized, infected, and serum-starved as described above. After 36 h of serum starvation, the cells were fixed and permeabilized using the Dako intrastain kit (Dako, Glostrup, Denmark). During permeabilization, cells were incubated with 20 μl of FITC-conjugated antibody against the Ki-67 proliferation antigen (clone B56; PharMingen, La Jolla, CA) or isotype control. Ki-67 expression was analyzed in a Becton Dickinson FACScan.

Animals. We performed mouse studies in accordance with the institutional guidelines of the University of Washington. Immunodeficient 8–12-week-old C.B-17/lcrCrl-*scid-bg*/BR (CB17) mice (Charles River, Wilmington, MA) were housed in specific pathogen-free facilities. To establish mouse models with liver metastases, animals were infused with 2×10^6 LoVo or HeLa cells through a permanently placed portal vein catheter (39).

Immunohistochemistry. Liver specimens were frozen in OCT compound (Miles, Elkhart, IN) and sectioned (6 μm). Viral hexon protein was detected with rabbit polyclonal antihexon antibodies (AB1056F; Chemicon, Temecula, CA). Specific antibody binding was visualized with a secondary antirabbit antibody labeled with FITC (Molecular Probes Inc., Eugene, OR). Mouse hepatocytes were stained with Cy3-streptavidin (The Jackson Laboratory, West Grove, PA), taking advantage of the high endogenous biotin expression in liver cells. Cell nuclei were counterstained with 1 μg/ml DAPI (Sigma, St. Louis, MO).

RESULTS

Replication-activated Enzyme Expression Mediates Prodrug Activation and Cytotoxicity in Tumor Cell Lines *in Vitro*. We generated replication-activated Ad vectors containing the cDNAs for the prodrug-activating enzymes SG or CodAupp in 3′-5′ orientation relative to the RSV promoter (Fig. 1), named Ad.IR-SG or Ad.IR-CodAupp, respectively. In these vectors, a functional expression cassette is only generated upon viral DNA replication and replication-dependent homologous recombination between inverted repeats flanking the transgenes. To verify functionality of these constructs, we analyzed replication-activated enzyme expression and subsequent prodrug activation in a cell survival assay (Fig. 2). Because systemic 5-FU chemotherapy is widely used for treatment of metastatic colon carcinoma (40), we selected LoVo cells (human colon carcinoma) to test the Ad.IR-CodAupp/5-FC system. The Ad.IR-SG/9-ACG system was analyzed in HeLa cells (human cervical carcinoma) because TopoI inhibitors are promising agents for the therapy of cervical cancer and are currently being evaluated in Phase II/III studies. Also, the kinetics of viral DNA replication in HeLa cells were known from previous publications (22, 26, 34, 41). Infection of LoVo cells with Ad.IR-CodAupp and exposure to increasing concentrations of 5-FC induced dose-dependent cytotoxicity. Cell death was comparable for equimolar concentrations of Ad.IR-CodAupp + 5-FC and 5-FU, thus demonstrating full activation of the prodrug. In contrast, the control virus (Ad.control) was unable to convert 5-FC to 5-FU and did not increase the toxicity of the prodrug treatment (Fig. 2*A*). Analogous results were observed for the combination of Ad.IR-SG and 9-ACG on HeLa cells. Only cells infected with Ad.IR-SG were able to fully activate 9-ACG and underwent cell death at comparable concentrations of drug and prodrug. However, 9-ACG alone or in combination with Ad.control exhibited substantial cytotoxicity (Fig. 2*B*).

In addition to this functional demonstration of glucuronidase expression, we analyzed glucuronidase activity in Ad.IR-SG-infected cells by X-gluc staining. To demonstrate the dependence of enzyme expression on viral replication, Ad.IR-SG-infected cells were exposed to the DNA synthesis inhibitor HU at concentrations that inhibit viral DNA replication but do not induce apoptosis in the host cells. The dependence on viral DNA replication is critical for the tumor specificity of the system. HU prevented viral replication, recombination, and SG expression (Fig. 2*C*). Furthermore, tumor specificity was demonstrated using primary nontransformed SAECs. As expected, SAECs, which are transducible (BG-expression from Ad.BG) but do not support viral replication (no BG-expression from Ad.IR-BG), also failed to express SG.

Exposure to 9-ACG at Subtoxic Concentrations Enhances Glucuronidase Expression from Ad.IR-SG and Viral DNA Replication. To study whether activation of prodrugs by our Ad.IR vectors affects viral DNA replication, we analyzed glucuronidase expression by X-gluc staining after incubation with the prodrug 9-ACG. Interestingly, exposure to subtoxic concentrations of 9-ACG and subsequent converted to 9-AC acted to increase replication-dependent glucuronidase expression from Ad.IR-SG (Fig. 3, *solid line*). The effect was observed at concentrations that resulted in a moderate growth inhibition of the host cells (Fig. 3, *dashed line*; data not shown). Expectedly, higher concentrations induced host cell death and cytolysis. Enhanced transgene expression was observed upon exposure to 9-ACG prodrug as well as to 9-AC or its analogue, camptothecin. Due to the restricted availability of 9-ACG, and to be able to compare results with other cytostatic agents not available as prodrugs, all of the following *in vitro* experiments were conducted using the 9-AC analogue camptothecin. The best enhancement of SG expression occurred when camptothecin was added before or during viral DNA replication, which starts about 16 h postinfection as assessed in a previous study (34). Therefore, in all of the following *in vitro* experiments, drugs were added 16 h postinfection.

To delineate the mechanism underlying the increase in transgene expression from the Ad.IR-SG vector, we used a site-specific methylation strategy to monitor viral DNA replication (37). Infection with a methylated virus allows one to distinguish incoming (methylated)

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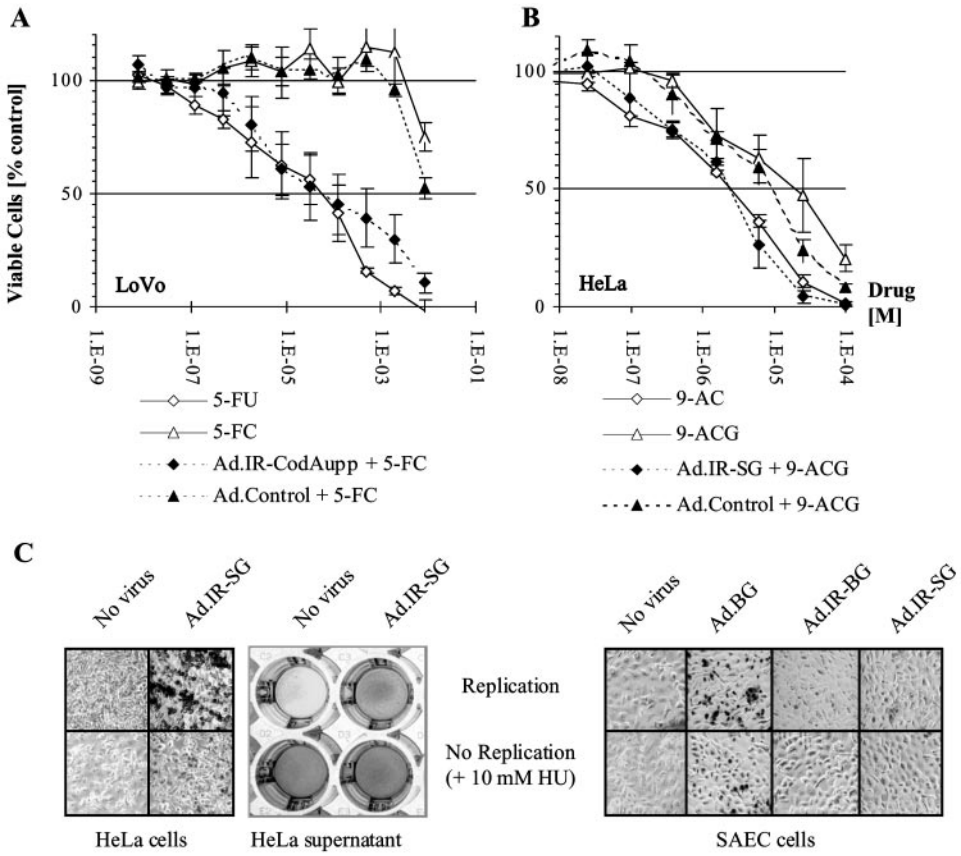

Fig. 2. Replication-dependent expression, prodrug activation, and induction of tumor cell toxicity by SG and CodAupp. *A*, prodrug activation and cell death mediated by CodAupp/5-FC. LoVo cells were infected with Ad.IR-CodAupp or Ad.control at a MOI of 300 pfu/cell [in previous studies, these infection conditions were found to allow for efficient DNA replication of E1-deleted Ads (34)]. Cells were exposed to drugs or prodrugs at the indicated concentrations 16 h after infection. Seventy-two h postinfection, cell viability was assessed using XTT reagent. Cell viability was expressed as the percentage of non-drug-treated controls and plotted as a function of drug concentration. *Error bars* represent SDs from three independent experiments. *B*, prodrug activation and cell death mediated by SG/9-ACG. HeLa cells were infected with Ad.IR-SG or Ad.control at a MOI of 100 pfu/cell [in previous studies, these infection conditions were found to allow for efficient DNA replication of E1-deleted Ads (34)]. Drug treatment and analysis of cell viability were performed as described in *A. C*, analysis of replication-dependent expression of Ad.IR-SG in HeLa and primary nontransformed SAECs. HeLa cells were infected with Ad.IR-SG (100 pfu/cell) and grown for 72 h in the presence or absence of HU, which blocks viral DNA replication. After 72 h, cells or supernatants were stained for glucuronidase. Note that the HU present in the media of HU-treated samples is responsible for the orange color of the supernatant. However, no difference can be found between infected and noninfected samples, indicating the strict replication dependence of glucuronidase expression and excretion. SAECs were infected with Ad.IR-SG, Ad.IR-BG, or Ad.BG (replication-independent, direct expression of lacZ) at 100 pfu/cell. Cells were grown for 72 h in the presence or absence of HU and stained for BG (Ad.BG and Ad.IR-BG) or glucuronidase (Ad.IR-SG).

from replicated progeny (nonmethylated) viral genomes. Southern analysis of viral DNA in drug-treated cells revealed an increase in viral progeny genomes as compared with cells without drug treatment (Fig. 4, 6.5-kb band). Enhanced viral DNA replication was also reflected in an increased amount of recombined (delta) product formation (Fig. 4; 5-kb band), which in turn results in elevated glucuronidase expression in these cells. In conclusion, exposure of virally infected cells to subtoxic concentrations of camptothecin enhances viral DNA replication.

Synergistic Toxicity of Viral and Cytostatic Treatment. We further analyzed whether drug exposure acted synergistically with the viral infection to enhance tumor cell death. Fig. 5 shows the MOI-toxicity curves for drug-treated and non-drug-treated cells. At subtoxic drug concentrations (1×10^{-7} to 6×10^{-9} M), the observed toxicity was higher than the calculated toxicity, assuming an additive effect of both treatment modalities (*dashed line*). This indicates a superadditive, or synergistic, effect of viral and cytostatic treatment on tumor cell death.

Cells Exposed to Subtoxic Doses of Camptothecin Accumulate in S-G_2. We next investigated the cellular basis for the drug-mediated stimulation of viral DNA replication. We hypothesized that treatment with camptothecin elicits changes in the cell cycle status of exposed cells to create favorable conditions for viral replication. Our group and

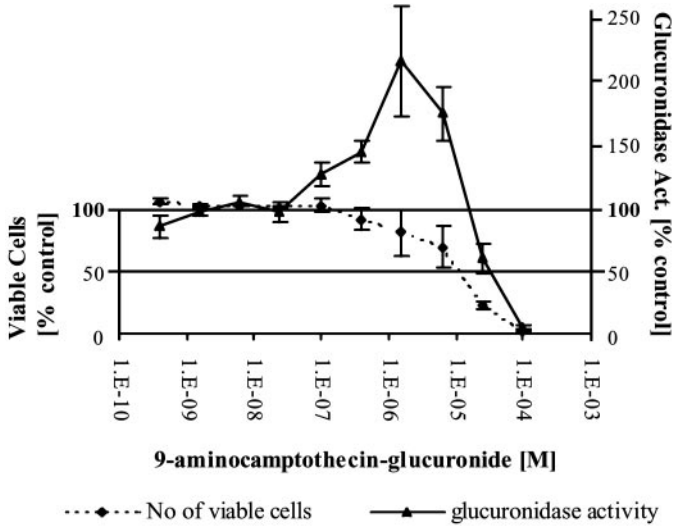

Fig. 3. Incubation with subtoxic amounts of 9-ACG prodrug increased glucuronidase expression from HeLa cells infected with Ad5.IR-SG. HeLa cells were infected with Ad.IR-SG at a MOI of 30 pfu/cell for 3 h in duplicate plates. Twenty-four h after infection, cells were exposed to 9-ACG at the indicated concentrations. Seventy-two h after infection, glucuronidase activity levels were determined using X-gluc substrate (*solid line*). The replicate plate was analyzed for cell viability using XTT reagent (*dashed line*). Glucuronidase activity and cell viability were expressed as a percentage of non-drug-treated controls.

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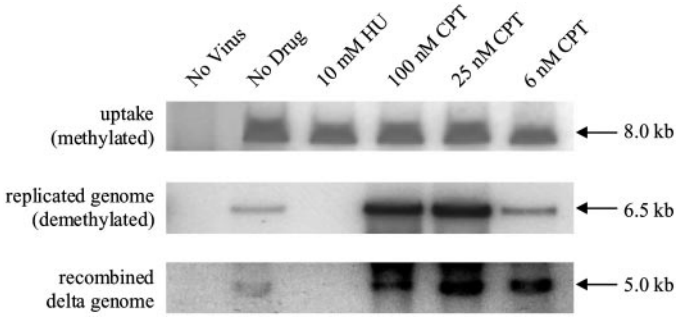

Fig. 4. Subtoxic amounts of camptothecin increased viral DNA replication. A demethylation assay was used to analyze viral DNA replication (see "Materials and Methods"). HeLa cells were infected with methylated Ad.IR-SG for 3 h in duplicate for each condition. Three h after the infection, samples were collected to show equal viral uptake for all conditions (*top panel*; 8-kb band). Sixteen h after viral infection, camptothecin was added at the indicated concentrations. Seventy-two h after infection, cells were harvested, and the amount of replicated viral DNA (*middle panel*, 6.5-kb band) and delta product formation (*lower panel*, 5-kb band) were analyzed by Southern blotting. To one set of infected cells, 10 mM HU was added 1 h after infection to block replication. The blots were probed using the 8-kb *Hin*dIII fragment from the Ad5 genome. Note that treatment with the DNA synthesis inhibitor HU prevents the formation of demethylated (replicated) genomes and recombined delta products.

others have previously shown that adenoviral infection causes a slow-down in cell cycle progression and eventual arrest in G_2 (33, 34, 42, 43). Cell cycle analyses (Fig. 6) demonstrated that infection of HeLa cells with Ad.IR-SG (Ad.ΔE1) at a MOI of 30 caused a slight increase in the percentage of cells in S-G_2 compared with uninfected cells (Fig. 6, *a* and *b*). Exposure to camptothecin also resulted in a larger fraction of cells in S-G_2 (Fig. 6*c*). Importantly, the combination of Ad.ΔE1 infection and camptothecin treatment induced a more pronounced increase than did either agent alone (Fig. 6, *b*−*f*). The extent of cell cycle arrest after Ad.ΔE1/drug treatment was comparable with the effect of WT (E1-positive) Ad on cell cycle progression (compare Fig. 6, *e/h* and *f/i*). This demonstrates that drug treatment enhances the partial Ad.ΔE1-mediated G_2 cell arrest. The induction of favorable cell cycle conditions represents a potential mechanism for the drug-mediated stimulation of Ad.IR-SG viral DNA replication.

G_0 Arrest Fails to Enhance Viral DNA Replication. Subtoxic amounts of camptothecin caused a moderate delay in cell cycle progression (see Fig. 3). This may have resulted in a higher intracellular concentration of replication templates and thus in a higher number of replicated genomes at the time of analysis than in actively proliferating cells, where episomal adenoviral genomes would be distributed between the daughter cells. If the camptothecin-induced decrease in dilution of viral replication templates was the mechanism underlying the enhanced viral DNA replication, then this effect should be independent of the phase at which the cell cycle arrest occurred. To test this, we arrested cells in G_0 by serum starvation. Serum starvation effectively decreased cell growth to about 70% of an untreated control, a reduction comparable with that seen with drug treatment. Analysis of Ki-67 staining showed that serum-starved cells were

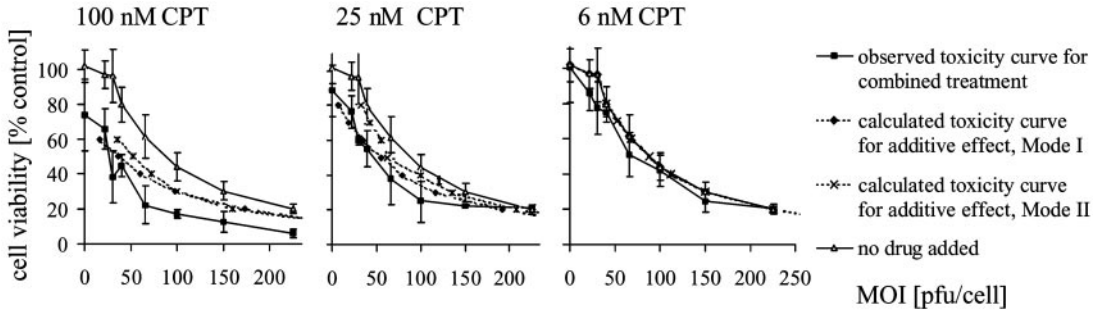

Fig. 5. Synergistic cytotoxicity of viral and cytostatic treatment. HeLa cells were infected with an E1-deleted virus (Ad.IR-BG) at MOIs ranging from 20 to 225 pfu/cell and exposed to drugs at the indicated concentrations 16 h after infection. Seventy-two h postinfection, cell viability was assessed using XTT reagent. Cell viability was expressed as a percentage of noninfected, non-drug-treated controls and plotted as a function of viral MOI. Calculated toxicity curves based on the assumption of an additive effect (mode I and II) were computed based on the method of Steel and Peckham (63). The observed cytotoxicity exceeds the calculated curve, indicating a supra-additive, or synergistic, effect. $n = 3$; *error bars*, SD.

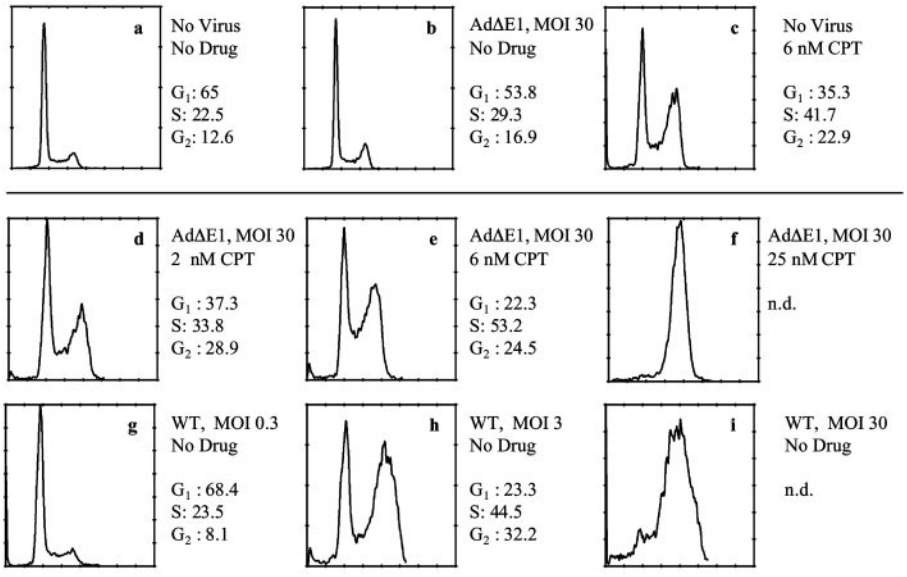

Fig. 6. Cell cycle analysis of virus-infected and drug-exposed cells. Synchronized HeLa cells were infected with Ad.IR-SG or WT Ad5 and exposed to camptothecin as indicated. Forty-eight h after infection, cell cycle analysis was carried out as described in "Materials and Methods." *Top panels*, cell cycle distribution of cells that are untreated (*a*), infected with Ad.IR.BG at a MOI of 30 (*b*), or exposed to 6 nM camptothecin (*c*). *Middle panel*, Ad.IR.BG-infected cells exposed to increasing concentrations of camptothecin (*d*−*f*). *Bottom panel*, HeLa cells infected with increasing MOIs of WT Ad5 (*g*−*i*).

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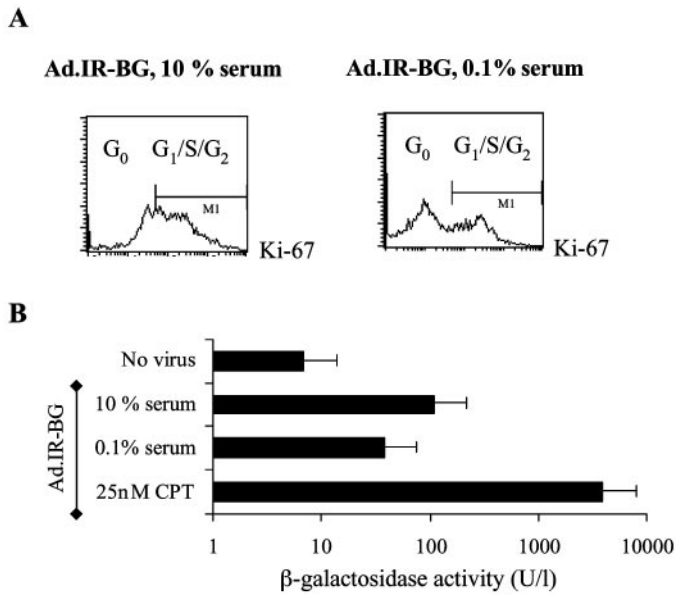

Fig. 7. G_0-G_1 arrest by serum starvation does not enhance viral replication. Cells were synchronized and infected with Ad.IR-BG at a MOI of 30 pfu/cell. Sixteen h after infection, cells were serum-starved using 0.1% serum. After 36 h, cells were fixed, permeabilized, and stained with FITC-conjugated antibody against the Ki-67 proliferation antigen to demonstrate the partial G_0 arrest in serum-starved samples (*A*). After 72 h, cells lysates were analyzed for BG expression (*B*). For comparison, BG expression of cells exposed to 25 nM camptothecin is also shown (see Fig. 8). *Error bars* represent the SD of at least three independent experiments.

partially arrested in G_0 (Fig. 7*A*). Viral DNA replication was assessed based on the level of BG activity in lysates of cells infected with the replication-activated Ad.IR-BG vector (Fig. 7*B*). The BG luminometric assay using BG as reporter gene was chosen because it is more sensitive and linear over a broader range of activity levels than the X-gluc stain. In contrast to camptothecin treatment, the partial G_0 arrest failed to stimulate replication-activated transgene expression.

Disparate Cytostatic Agents Differ in Their Ability to Enhance Adenoviral Replication. The fact that induction of G_2, but not G_0-G_1, arrest increased viral replication led us to assume that different cytostatic drugs might exert distinct effects on viral replication and transgene expression. Furthermore, some cytostatic agents have been shown to interfere with the viral life cycle and are therefore likely to decrease rather than increase replication-activated transgene expression. Such agents would not be good candidates for combined viral/cytostatic therapy. We analyzed the effect on replication-dependent transgene expression of a panel of cytostatic agents varying in their mechanism of action and cell cycle specificity (Fig. 8, *black bars*). To account for a potential drug mediated up-regulation of RSV promoter activity, we also transiently transfected cells with a RSV-BG-containing expression plasmid (replication-independent expression, *striped bars*). Camptothecin and etoposide, inhibitors of TopoI and TopoII, are both very specific for S phase and induce G_2 arrest in exposed cells (44, 45). These two drugs were most effective in stimulating replication-activated transgene expression, closely followed by daunorubicin. Daunorubicin also inhibits TopoII, but other intracellular targets have also been described that are not cell cycle dependent (46). Cisplatin, one of the drugs used in the ONYX studies, also enhanced replication-activated transgene expression (47). The other agent used in trials by ONYX Pharmaceuticals, the antimetabolite 5-FU, shows a more complex pattern. Compared with the drugs discussed thus far, the toxicity curve of 5-FU is much less steep (compare Fig. 2, *A* and *B*; data not shown). A broad range of concentrations enhanced replication-activated transgene expression (range, 0.25–50 μM; 4 μM 5-FU is shown). However, very high doses ($\geq$0.25 mM, resulting in $\leq$15% cell viability) seemed to increasingly interfere and finally inhibit viral replication. A similar biphasic pattern was observed for HU. The replication-inhibiting properties of HU are well known, and 10 mM HU, which led to a near complete inhibition of viral replication and cell growth while permitting a cell viability of 80% as assessed by trypan blue staining, was used as blocking agent in our replication studies (Fig. 2, *C* and *D*). Paclitaxel induced an only moderate increase in BG expression that is almost exclusively due to promoter up-regulation (58). For all other drugs, however, we can exclude a major contribution of drug-enhanced transcriptional activity from the RSV promoter to the observed increase in transgene expression. Finally, the RNA and protein synthesis inhibitor actinomycin D failed to enhance replication-activated transgene expression. Because actinomycin D affects the expression of proteins required for S-phase entry, exposed cells therefore accumulate in G_1 (48). The failure to provide favorable conditions for viral DNA replication and direct interference with viral RNA and protein synthesis are likely explana-

Virus / drug	Mechanism of action	Cell cycle spec./arrest (reference)
(No virus)		n.a.
(No drug)		n.a.
Camptothecin 25 nM	Inhibition of topoisomerase I	S/G_2 (45)
Etoposide 1.5μM	Inhibition of topoisomerase II (topo II)	S/G_2 (44)
Daunorubicin 100 nM	Intercalation, inhib. of topo II + helicase, oxygen radical format.	G_2 (46)
Cisplatin 2μM	Covalent binding of DNA, strand breaks during replication	G_2 (47)
5-FU 4 μM	Inhib of thymidylate synthetase, incorporation into RNA + DNA	S (64)
5-FU 250 μM		
Hydroxy urea 1 mM	Inhib of ribonucleotide reductase and other enzymes in DNA replication	S (65)
Hydroxy urea 10 mM		
Paclitaxel 10 nM	Stabilization of microtubule formation, mitotic block	M (66)
Actinomycin D 10 nM	Inhibition of RNA and protein synthesis	G_1 (48)

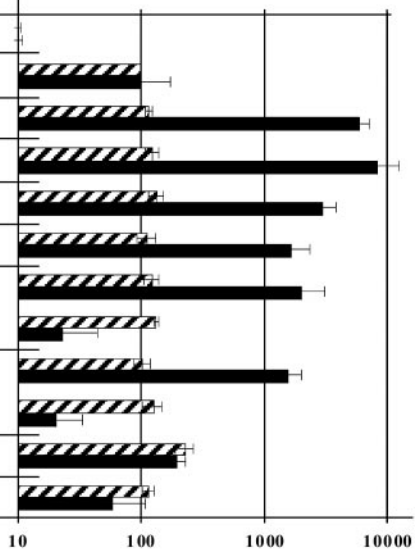

Fig. 8. Subtoxic amounts of different cytostatic agents have distinct effects on viral replication. HeLa cells were infected with Ad.IR.BG for 3 h. Sixteen h after infection, cells were exposed to subtoxic amounts of cytostatic agents. In preliminary studies, the toxicity profiles of drugs on HeLa cells were established (data not shown). Seventy-two h after infection, cell lysates were analyzed for BG expression (*black bars*). To account for drug-induced promoter up-regulation, cells were also transfected with the pAd.RSV-BG shuttle plasmid (replication-independent expression) and exposed to drugs at the same dosage and schedule (*striped bars*). *Error bars* represent the SD of at least three independent experiments.

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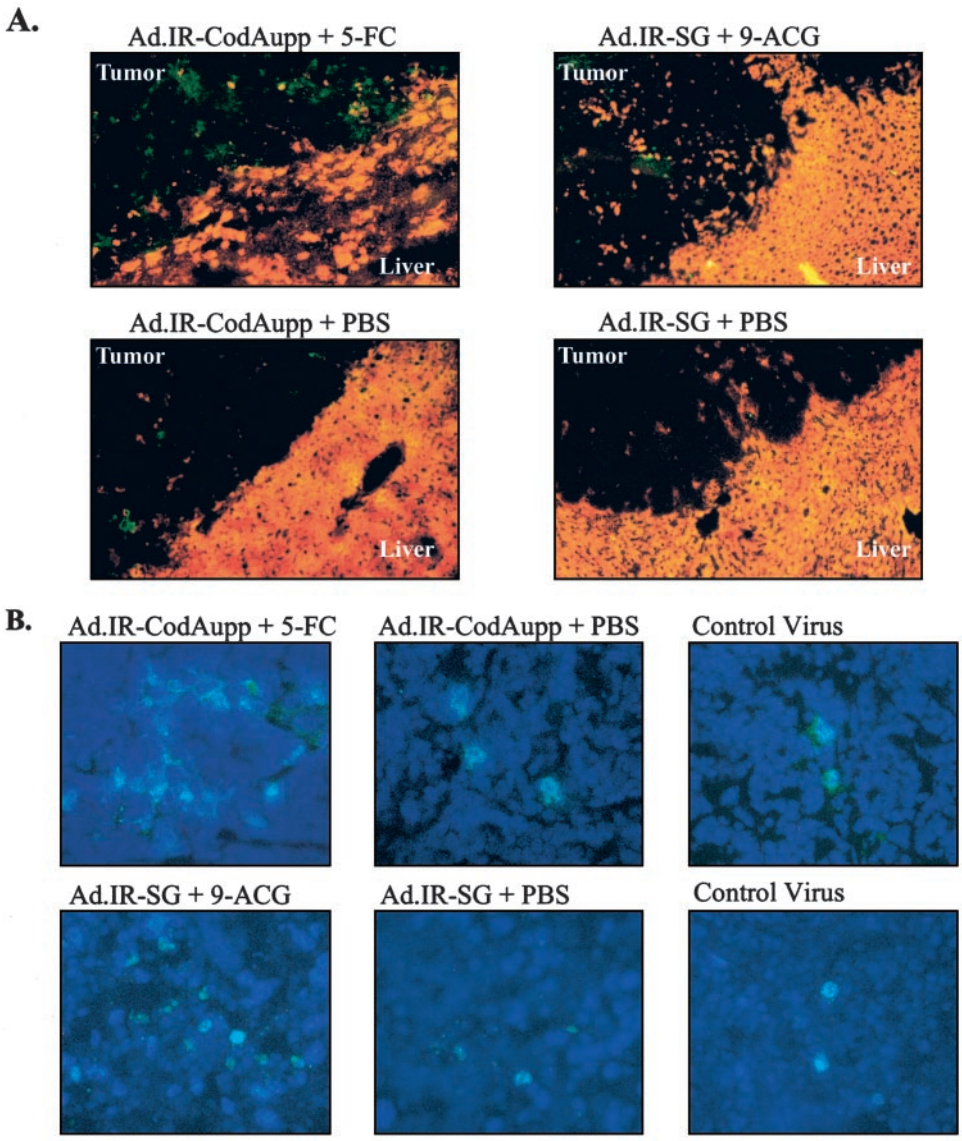

Fig. 9. Enzyme-mediated prodrug activation enhances viral replication in liver metastases *in vivo*. Groups of 7 mice received 2×10^6 HeLa or LoVo cells via the portal vein catheter. Fourteen days after transplantation, mice were given i.v. injection of Ad.IR-SG, Ad.IR-CodAupp, or Ad.IR.BG control on two consecutive days. Mice received 2×500 mg/kg 5-FC/day on days 4–14 after Ad.IR-CodAupp virus injection, 2 mg/kg 9-ACG on day 4 and 10 after Ad.IR-SG injection, or PBS as solvent control. In mice bearing the slower growing LoVo cell metastases, a second cycle of virus/prodrug treatment was administered during weeks 6 and 7. *A*, for histological analyses, mice were sacrificed after one (HeLa) or two (LoVo) virus/drug cycle(s). Frozen liver sections were stained for viral hexon and detected with a secondary FITC-conjugated antibody (*green*). Hepatocytes were stained with Cy3-streptavidin against hepatocellular endogenous biotin (*red*). *B*, high-power magnification of frozen liver section after one (HeLa) or two (LoVo) virus/drug cycle(s). Sections were stained for viral hexon and detected with a secondary FITC-conjugated Ab (*green*), and cell nuclei were counterstained with DAPI (*blue*).

tions for the observed decrease in transgene expression. These results show that different cytostatic agents vary greatly in their effect on viral replication.

Combined Virus/Prodrug Treatment Enhances Viral Replication/Spread *in Vivo*. To evaluate whether the increased viral DNA replication upon drug treatment *in vitro* reflected in an increased replication *in vivo*, mice bearing HeLa liver metastases received i.v. injection with Ad.IR-SG or control virus and subsequently received several injections of 9-ACG or solvent control. In parallel, mice bearing LoVo colon carcinoma liver metastases were treated with Ad.IR-CodAupp and 5-FC or the respective controls. Virally transduced cells were identified by immunohistochemistry for viral hexon. Notably, hexon expression is only initiated late in infection, upon viral DNA replication (49). The amount of viral hexon present in tumors from mice treated with Ad.IR-SG/9-ACG or Ad.IR-CodAupp/5-FC was higher than that in tumors from mice treated with control virus/prodrug or therapeutic virus/solvent control (Fig. 9*A*). Furthermore, viral hexon was only present in tumor cells and not in surrounding hepatic tissue, thus demonstrating the tumor specificity of the combined treatment. The high-power magnification showed clusters of virus-containing cells in the treatment group, which indicated enhanced viral replication and/or enhanced viral spread of progeny virus to neighboring cells. In contrast, only single cells and scarce small clusters ($\leq$4 cells) of hexon-positive cells could be found in all control animals (Fig. 9*B*).

In conclusion, after systemic application, replication-activated Ad.IR vectors allowed for enzyme expression specifically in liver metastases. Combined virus/prodrug treatment enhanced viral replication/spread in liver metastases.

DISCUSSION

CRAD-mediated GDEPT is a promising new strategy for tumor treatment (50, 51). However, to avoid side effects, the expression of prodrug-activating genes from CRADs must be restricted to the tumor. The classical approach to provide tumor-specific gene expression is the use of tumor-specific promoters. However, expression from these promoters is generally lower than that from viral promoters, such as the RSV or cytomegalovirus promoters (52). More importantly, heterologous promoters exhibit considerable background transcriptional activity when placed into an adenoviral context due to interference with adenoviral enhancers (53, 54). We have designed an Ad.ΔE1-based expression system in which a functional transgene cassette is created by homologous recombination during viral DNA

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replication (26). Transgene expression is therefore strictly dependent on viral DNA replication, which, in turn, is tumor specific. This Ad.IR system allowed us to use the strong viral RSV promoter to drive transgene expression. Replication-activated expression of SG or CodAupp was able to activate the prodrugs 9-ACG to 9-AC and 5-FC to 5-FU, respectively, and thus induce tumor cell toxicity. However, the cell survival curves for both systems also point toward the main problems associated with the use of these compounds. Very high concentrations of 5-FU or Ad.IR-CodAupp-activated 5-FC are needed to kill 90% of the tumor cells. In addition, a multi-(pro)drug therapy is not possible with this approach, and tumors resistant to 5-FU will not respond to 5-FC + CodAupp. SG, on the other hand, can theoretically activate a variety of drug glucuronides, allowing for multi-(pro)drug chemotherapy. Glucuronide conjugates have been described for a variety of drugs, including 9AC (32), *p*-hydroxyaniline mustard (55), and doxorubicin (56). Unfortunately, the 9-ACG used in this study induced considerable toxicity even without activation, which could be due to leakage of endogenous glucuronidase resulting in activation of 9-ACG. A similarly narrow window between drug and prodrug toxicity was reported for the doxorubicin-glucuronide used by Weyel *et al.* (31). Clearly, a strategy to improve CRAD-mediated GDEPT involves the development of better prodrug systems. Ideally, such a system (*a*) would be nontoxic without activation; (*b*) should allow for the conjugation and subsequent activation of a variety of cytostatic agents, preferably highly toxic agents such as Taxols or calicheamicins; and (*c*) could be activated with high specificity and high catalytic turnover. Further improvements are also required with regard to productive replication and spread of Ad.IR vectors. To address this, we have recently generated Ad.IR vectors with replication-activated bicistronic expression of *E1A* and transgenes, which demonstrate greatly enhanced viral replication, spread, and antitumor efficacy.[4] We are also attempting to selectively target viral vectors to tumor cells (57), which may increase the number of initially transduced tumor cells and the number of viral genomes/tumor cell and hence viral replication. Clearly, data on tumor-specific replication obtained in our xenograft model do not necessarily predict the outcome in patients.

In addition to demonstrating tumor-specific, replication-activated prodrug conversion leading to tumor cell toxicity, we also delineated a mechanism that may contribute to the synergy of adenoviral and cytostatic therapy observed in clinical trials. We found that the combination of viral infection with several cytostatic agents induced an increase of viral DNA replication in exposed cells *in vitro* and enhanced viral replication and spread to neighboring cells *in vivo*. This is particularly interesting in the case of 5-FC/5-FU because the effect of 5-FU on viral replication *in vitro* was concentration dependent, and inhibition of viral replication at high concentrations [around the IC_{90} (the drug concentration at 90% cytotoxicity)] was observed. *In vitro* studies demonstrated that the cytotoxic effect of combined virus/drug treatment is greater the sum of both agents, indicating synergism. Our finding that a number of cytostatic agents increase viral replication may also explain the recently reported phenomenon that the burst size of a hepatoma-specific CRAD was increased in cells treated with doxorubicin (11). In these studies, the combined viral and cytostatic effect also proved to be synergistic.

From a number of cytostatic drugs tested, camptothecin and etoposide, both highly cell cycle-specific drugs inducing G_2 arrest, were most efficient at increasing viral DNA replication. There are a number of reports indicating that infection with WT Ad as well as with E1 mutants causes a dose-dependent G_2 arrest that is favorable for viral DNA replication (33, 34, 42, 59). S phase (and G_2) ensures ample supply of nucleotides and other cellular factors needed for viral replication. In this context, a p21 binding site has recently been identified in the E1A 243R and 289R proteins (60). Inactivation of p21 may inhibit a "premature" arrest in G_1 that would more poorly support viral replication. This is supported by our observation that drugs that affect the cell cycle in phases other than S and/or G_2 were not as effective at enhancing viral DNA replication. Along this line, our group has recently reported that viral replication is increased in cells arrested in G_2 with nocodazole (34). The increased viral yield after nocodazole treatment has also been noted by Seidman *et al.* (61), who reported that an up-regulation of the primary Ad5 attachment receptor, CAR, and α_v integrins resulting in subsequent higher viral uptake during G_2 was primarily responsible for the effect. However, in all our studies, drugs were added after internalization (16 h postinfection), excluding differences in receptor density, attachment, internalization, or trafficking as major determinants for the level of viral replication. Theoretically, camptothecin could also act to inhibit viral replication because Ads rely on cellular TopoI to unwind their DNA. However, camptothecin does not induce toxicity by completely blocking TopoI activity in the sense of traditional pharmacological inhibition of an essential enzyme. Rather, cytotoxicity results from signaling events elicited by strand breaks, which are induced by TopoI-DNA complexes during cellular DNA replication (45). We hypothesize that although camptothecin-TopoI-DNA complexes might also induce sporadic strand breaks in adenoviral DNA, the effect is likely to be outweighed by the observed increase in viral DNA replication.

Our data demonstrate a broad range of possible interactions between cytostatic agents and the viral life cycle. For a successful combined adenoviral/cytostatic therapy or GDEPT, it may be crucial to choose agents that enhance or at least do not inhibit viral replication. The potential significance of drug-induced inhibition has also been recognized for a vaccinia virus-based GDEPT approach using cytosine deaminase, where 5-FC was shown to significantly decrease vaccinia virus replication *in vitro* and *in vivo* (62). Based on our *in vitro* data, we would like to tentatively delineate the following groups of cytostatic agents with respect to their interference with viral DNA replication: (*a*) agents that strongly increase viral replication, including camptothecin, etoposide, daunorubicin and cisplatin; (*b*) agents that moderately increase viral replication such as paclitaxel; (*c*) agents that increase viral replication at low doses and inhibit viral replication at high doses (5-FU and HU); and (*d*) agents that inhibit viral replication at all dose levels, *i.e.*, actinomycin D. Agents from the last group would be expected to be poor candidates for a combined viral and cytostatic therapy.

Considering the fact that many cytostatic agents are cell cycle specific and that a variety of mutations resulting in conditionally replicating viral vectors involve cell cycle active proteins encoded in the Ad *E1A* and *E1B* regions, our observation does not exclude that some Ad mutants may be more effective in achieving synergistic antitumor effects in combination with chemotherapy than others. Delineating specific vector/drug combinations that are tailored to be particularly effective in different types of tumors may have the potential to greatly improve the already encouraging results seen with this new approach in cancer therapy.

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[4] Kathrin M. Bernt, Tumor targeting decreases systemic toxicity and increases tumor-specific replication of adenovirus vectors, submitted for publication.

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