Inhibition of the Multidrug-Resistant Phenotype by Targeting YB-1 with a Conditionally Oncolytic Adenovirus: Implications for Combinatorial Treatment Regimen with Chemotherapeutic Agents

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Abstract

Bearing in mind the limited success of available treatment modalities for the therapy of multidrug-resistant tumor cells, alternative and complementary strategies need to be developed. It is known that the transcriptional activation of genes, such as MDR1 and MRP1, which play a major role in the development of a multidrug-resistant phenotype in tumor cells, involves the Y-box protein YB-1. Thus, YB-1 is a promising target for new therapeutic approaches to defeat multidrug resistance. In addition, it has been reported previously that YB-1 is an important factor in adenoviral replication because it activates transcription from the adenoviral E2-late promoter. Here, we report that an oncolytic adenovirus, named Xvir03, expressing the viral proteins E1B55k and E4orf6, leads to nuclear translocation of YB-1 and in consequence to viral replication and cell lysis in vitro and in vivo. Moreover, we show that Xvir03 down-regulates the expression of MDR1 and MRP1, indicating that recruiting YB-1 to the adenoviral E2-late promoter for viral replication is responsible for this effect. Thus, nuclear translocation of YB-1 by Xvir03 leads to resensitization of tumor cells to cytotoxic drugs. These data reveal a link between chemotherapy and virotherapy based on the cellular transcription factor YB-1 and provide the basis for formulating a model for a novel combined therapy regimen named Mutually Synergistic Therapy.

Introduction

Although during the past decades progress has been made in the treatment of cancer by improvement of surgical intervention, radiation, and chemotherapy, these methods seem to have reached a “working plateau.” Particularly, the development of the multidrug-resistant phenotype remains a significant obstacle to successful chemotherapy in clinical settings. Among other factors, resistance to therapy has been correlated to the overexpression of ATP-binding cassette transporters, such as MDR1/P-glycoprotein (P-gp) and MRP1, which actively expel chemotherapy drugs from the interior (1, 2). Therefore, the determination of MDR1 and MRP1 gene expression levels along with studies of their regulatory mechanisms will be useful in developing improved therapeutic strategies. The MDR1 and MRP1 genes are regulated by promoters that contain inverted CCAAT sequences, so-called Y-boxes (3). The human transcription factor YB-1 binds within these promoters and it has been shown that nuclear localization of YB-1 induced by environmental stress, such as chemotherapy, UV light, and hyperthermia, is followed by an enhanced expression of multidrug-resistant genes (4–8).

YB-1 is a member of a family of DNA-binding proteins, characterized by a cold shock domain, a highly conserved nucleic acid recognition domain, and interacts with a specific sequence motif, termed Y-box, which contains an inverted 5′-CCAAT-3′ sequence (9, 10). Apart from its role in transcription, YB-1 is a multifunctional protein that affects splicing, translational control, and repair of damaged DNA by interacting with several repair proteins (11, 12).

Results from the literature suggest that overexpression as well as nuclear localization of YB-1 is predictive of drug resistance and tumor progression in breast, ovarian, lung, synovial, and prostate cancers (13–18). cDNA array hybridization and reverse transcription-PCR analysis revealed that YB-1 is one of the strongest up-regulated genes in resistant cell lines compared with nonresistant counterparts (19). In addition, YB-1 interacts with p53 and functions as a transcriptional repressor of the cell death–associated fas gene (20, 21). Direct evidence for the role of YB-1 in cell growth and survival (22) gives the observation that adenocarcinoma, hepatoma, fibrosarcoma, and colon cancer cells die when its expression is inhibited with antisense RNA against YB-1 (23).

Conditionally replicating adenoviruses are a novel class of therapeutic agents in cancer treatment. Currently, there are three major approaches to achieve preferential replication of adenoviruses in cancer cells: deletion or mutations of viral genes, such as E1B55k and E1A, to promote viral replication in p53- or pRB-defective tumor cells (24, 25), the use of tumor-selective promoters to control the expression of early viral genes (26), and, a strategy followed in this article, the up-regulation and nuclear localization of YB-1 (27, 28).

Adenovirus replication depends on viral proteins that are encoded by the E2 genes (29). Expression of the E2 genes is controlled by E2-early and E2-late promoters during the viral life cycle (30). Activity of the E2-early promoter is controlled by E1A and the host cell factor E2F (31). Interestingly, we could show previously that the E2-late promoter is activated by YB-1 (27).
In addition, we showed that the viral protein E1B55k is involved in translocation of YB-1 into the nucleus in infected cells. The significance of YB-1 for adenoviral replication has been proven in another study using dl520, an E1A mutated adenovirus, which does not replicate unless YB-1 is located in the nucleus (28).

Although oncolytic adenoviruses led to inhibition of tumor growth in xenograft mouse models, complete regression was rarely observed. Therefore, the antitumor activity of several oncolytic adenoviruses in combination with chemotherapeutic drugs or radiation has been examined (32). These studies have shown that the therapeutic effect of oncolytic adenoviruses is strongly enhanced by chemotherapy without increasing toxicity. This dual mode of therapy can even exert synergistic effects in some instances. Nevertheless, the reason for this effect is still unknown.

Considering these observations, we were interested in determining the replication capacity of a novel YB-1-triggered self-promoting adenoviral vector named Xvir03, expressing the adenoviral proteins E1B55k and E4orf6, and to evaluate its influence on the expression of MDR1 and MRP1 gene expression. Our results show for the first time that a YB-1-associated oncolytic adenovirus possesses dual functions: YB-1-associated oncolytic activity and inhibition of the multidrug-related genes MDR1 and MRP1 leading to a potent antitumor effect in combination with chemotherapy. Owing to its remarkable dual role in modulating drug resistance and adenoviral replication, Xvir03 offers new strategies in developing novel combinatorial treatment regimens.

**Materials and Methods**

Cell lines and culture. HEK293 cells (embryonic kidney cells), Hs68 dermal fibroblasts, and U2OS [osteosarcoma; American Type Culture Collection (ATCC)], Manassas, VA) cells were maintained in DMEM. The multidrug-resistant cell lines EPG8S-257RDB (gastric carcinoma) and EPP65-181RDB (pancreas carcinoma) were maintained in L-15 medium (33, 34). All of the drug-resistant P-gp-positive cell lines were treated with 100 ng/mL daunomycin every 4 weeks to ensure P-gp expression. DU145 and PC3 derived from prostate carcinoma (ATCC) were maintained in RPMI and a 1:1 mixture of RPMI and F-12, respectively. HeLa (ATCC), a cervical carcinoma, was maintained in DMEM. EJ28 is a metastatic bladder carcinoma cell line and was cultured in RPMI. All of the media (Cambrex) were supplemented with 10% fetal bovine serum, 1-glutamine (200 mg/mL), penicillin (100 mg/mL), and streptomycin (25 mg/mL). All cell lines were of human origin.

Adenoviral vector construction. For a schematic outline of the cloned constructs and genomes, see Fig. 2A. The MfeI/EcoRI [cytomegalo-virus (CMV), multiple cloning site, and polyadenylate signal] from pShuttle (Clontech) was inserted into the blunted XbaI site of pShuttle AdEasy (Qiogene) after deletion of the EcoRI site to create pShuttle CMV-AdEasy. The expression cassette E4orf6-internal ribosomal entry site (IRES)-E1B55k-3' untranslated region (UTR) was inserted into the multiple cloning site [E4orf6 BamHI fragment from pGEX-E4orf6 (kindly provided by Matthias Dobbelstein, Center of Molecular Bioscience, Gottingen, Germany), and E1B55k-3' UTR BamHI fragment from pCGNE1B (kindly provided by Matthias Dobbelstein)] joint sequence for a sequence from IRES to pCTE 4a(+), (Novagen)]. Xviri03 pAdEasy was generated by homologous recombination in B5J183 Escherichia coli after cotransformation of 800 ng Bst 1107I and Mro1 linearized Xviri03 pShuttle and 100 ng pAdEasy following the manufacturer's protocol. The recombinant plasmid Xviri03 pAdEasy was validated by PCR and restriction digest. Adenovirus particles were produced by transfection of PacI-digested Xviri03 pAdEasy into HEK293 cells using calcium phosphate (Clontech). Virus plaques developed 11 days later and were amplified in HEK293 cells. Viruses were banded in CsCl gradients, dialyzed, and stored in aliquots. The viral titer of the E1A-deleted adenovirus Xviri03 was determined by plaque assays using HEK293 cell monolayers.

**Infection procedure.** Subconfluent cells were infected by addition of indicated plaque-forming units (pfu)/cell of adenoviral vectors to infection medium (Opti-MEM containing 2% FCS) after incubation for 1 hour at 37°C in a 5% CO2 atmosphere with brief agitation every 15 minutes. After infection, the medium was replaced by medium containing 10% FCS.

**PCR analysis.** To exclude any contamination with wild-type (WT) adenoviruses, purified DNA virus of Xviri03 was subjected to PCR. Taq DNA polymerase (5 units; Fermentas GmbH, St. Leon-Rot, Germany) 0.25 mmol/L sense and antisense primers, and PCR buffer were combined to make a final volume of 50 μL. PCR was then carried out for 30 cycles. The primers used for these experiments were as follows: (a) E1A sense 5'GATGGCCGCAGTCTTTTGT-3' and antisense 5'AAAAGGCCCCCGCAAACTTTAC-3' and (b) E2A sense 5'CCGGAGACGGGCGGTC-3' and antisense 5'TGGTGCAGCGCACCACCA-3'. The PCR products were electrophoresed on 1% agarose gels and visualized after ethidium bromide staining of the gel.

**Cell cycle analysis.** Cells were washed once with PBS/1% FCS and once with PBS and subsequently fixed in 85% ethanol. Following fixation, cells were washed once with PBS/1% FCS, resuspended in PBS/1% FCS containing 10 μg/mL propidium iodide and 250 μg/mL RNase, and incubated for 30 minutes at 37°C. Samples were analyzed using Coulter Epics XC-MCL (Beckman Coulter) and Expo32 software.

**Replication analysis via Southern blotting.** For viral replication analysis in vitro, cells were infected at indicated multiplicity of infection (MOI). DNA was isolated 72 hours after infection using the Qiagen Purification System (DNeasy Tissue kit) according to the manufacturer's instructions (Qiagen, Hilden, Germany). DNA (2 μg) from each sample was digested with the restriction endonuclease KpnI, size fractionated on a 1% agarose gel, transferred to a nylon membrane using the Turboblotter Rapid Downward Transfer System (Schleicher & Schuell, Dassel, Germany), and hybridized against a 32P-labeled adenovirus E2A-cDNA probe. Blots were visualized by a BASReader (Raytest, Straubenhardt, Germany).

**Northern blot analysis.** Total RNA of infected cells was isolated using the Trizol system (Life Technologies, Inc.) according to the manufacturer's instructions. Total RNA (10 μg) was size fractionated on 1% agarose formaldehyde gels, transferred to a nylon membrane (Amersham), and hybridized using indicated 32P-labeled cDNA probes.

**Generation of cDNA probes.** Radioactive labeled cDNA probes for MDR1 gene (785 bp) and MRP1 gene (527 bp) were generated by PCR. The primers were (a) MDRI sense 5'-AAGCTTCAAGACAGAGGCTCCGTG-3' and antisense 5'-GGCTAAGAAAAATAGTGAAGAAAC-3' and (b) MRP1 sense 5'-AGAACCTCTAGTGTCGGGAGCC-3' and antisense 5'-TGCAAGATCTGGT-CTCTCCGGG-3'. The E2A-late probe (1,502 bp) was used to analyze viral replication and E2 gene activation (kinetic studies) and the E2A-early probe (240 bp), which is located in the E2A-early promoter, were generated by PCR and WT adenovirus DNA as template. The primers were E2A-early sense 5'-AGCTGATCTTTCCGTTTG-3' and antisense 5'-GGATACGAGACTCTGACAA-3' and E2A-late sense 5'-GTCGGAGATACGGTTCG-3' and antisense 5'-GTCCTCGTCTGCTTGCCTT-3'. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe for the loading control was generated by PCR using the primers hG3PD-F300 5'-CTCTGGAGGAGAAAGAGGCTCC-3' and hG3PD-B600 5'-GGCCCATCACGAGAGCTTTT-3'. Cycling conditions were 30 cycles consisting of 60 seconds at 95°C, 60 seconds at 55°C, and 60 seconds at 72°C. The 18S probe for the loading control was a kind gift from A. Krueger (Technical University of Munich, Munich, Germany).

**Immunoblots.** Cells were mock-infected or infected with 10 pfu/cell dl312, AdE1-minus, Ad-WT, or Xviri03. At 4 days postinfection, the cells were washed thrice with cold PBS and harvested by scraping. Protein concentrations were determined using the BCA Protein Assay kit (Pierce). Protein (10 μg) was electrophoresed on 10% SDS polyacrylamide gels and electroblotted onto Hybond-ECL nitrocellulose membrane (Amersham). The membrane was incubated overnight at 4°C in TBS (50 mmol/L Tris-HCl pH 7.6, 150 mmol/L NaCl, 0.2% Tween 20) containing 5% fat-free dry milk (Applichem, Darmstadt, Germany) and then probed with the P-gp specific monoclonal mouse antibody C219 (Alexis, Lausen, Switzerland; 1: 10,000), the MRPI-specific monoclonal rat antibody MAB4124 (Chemicon; 1:5,000), or the rabbit anti-actin antiserum A5060 (Sigma; 1:10,000). All primary antibodies were incubated with the membrane in TBST containing...
mice were randomized into four groups with eight animals in each group.

Two-tailed Student’s t-test was used to compare all treatment groups; control and pairwise comparisons were done with the significance of the data was examined by one-way ANOVA test with Xvir03. Purified virus DNA was analyzed by PCR and Northern blot analysis that the E2-late promoter is highly active at 3 to 6 hours after infection (35) but too weakly to be detected by Northern blot analysis. Nevertheless, the early promoter acts as soon as 1 to 3 days after infection, Opti-MEM was replaced by full medium. Cells were incubated for 24 hours with docetaxel. Cells were subsequently infected with Xvir03 or d312 at indicated MOI in 400 μL Opti-MEM. One hour after infection, Opti-MEM was replaced by full medium. Cells were fixed after 5 to 7 days and stained with 1% crystal violet in formaldehyde followed by washing with water to remove excess color. Quantifications were done photometrically at 590 nm after dissolving dried crystal violet with 0.1% SDS in PBS.

Analysis of adenovirus Xvir03 cytotoxicity alone and in combination with daunorubicin or docetaxel in PC3 and DU145 cells. For determination of virus-mediated cytoxicty, cells were seeded in six-well plates at a density of 1 × 10^4 per well. DU145 cells were incubated at indicated concentrations for 12 or 48 hours with daunorubicin; PC3 cells were incubated for 24 hours with docetaxel. Cells were subsequently infected with Xvir03 or d312 at indicated MOI in 400 μL Opti-MEM. One hour after infection, Opti-MEM was replaced by full medium. Cells were fixed after 5 to 7 days and stained with 1% crystal violet in formaldehyde followed by washing with water to remove excess color. Quantifications were done photometrically at 590 nm after dissolving dried crystal violet with 0.1% SDS in PBS.

Tumor xenograft experiments. Xenografts were established in 6-week-old BALB/c nu/nu mice (Charles River, Sulzfeld, Germany) by infecting 3 × 10^4 DU145 prostate carcinoma cells s.c. into the right flank. Cells were suspended in 200 μL PBS. Tumor measurement was done twice weekly in two dimensions by external caliper. Volume was estimated by the formula: 4/3π × a × b × c/2. When the tumors reached a volume of ~100 mm³, mice were randomized into four groups with eight animals in each group. The first group received 50 μL PBS on days 1 and 5. The second treatment group received Xvir03 on days 1 and 3 with a dose of 1 × 10^8 pfu in each injection in a volume of 50 μL. The third group received 3 mg/kg docetaxel i.v. on days 1 and 5 with a total dose of 6 mg/kg. The last group received a treatment combination. On days 1 and 5, all mice of the group received docetaxel (3 mg/kg) i.v. with a total dose of 6 mg/kg; on days 2 and 6, they received additional injections of Xvir03 at a total dose of 2 × 10^8. The tumor volume was measured twice weekly. After 25 days, the animals were sacrificed, and tumor and liver samples were isolated for further histologic analysis. The significance of the data was examined by one-way ANOVA test for all treatment groups; control and pairwise comparisons were done with two-tailed Student’s t-test.

Results

E2-late promoter regulation plays a significant role in E2 gene expression at early stage of infection. To verify the contribution of the E2-early and E2-late promoter in E2 expression (Fig. 1A), we did kinetic analyses using different probes. For this purpose, U2OS cells were infected with WT adenovirus and RNA were isolated at indicated time points. As shown in Fig. 1B, the amount of RNA expressed from the E2-late promoter was higher after 6 to 12 hours than the expression by the E2-early-driven promoter. Nevertheless, the early promoter acts as soon as 1 to 3 hours after infection (35) but too weakly to be detected by Northern blot analysis. However, we revealed for the first time by Northern blot analysis that the E2-late promoter is highly active at early times in WT adenovirus-infected cells.

No E1A contamination is responsible for the effects seen with Xvir03. Purified virus DNA was analyzed by PCR and restriction digest (see Materials and Methods) to confirm modifications to the adenoviral genome as well as the presence of the expression cassette (data not shown). To exclude E1A traces in our Xvir03 preparations, which may occur in the phase of propagation in HEK293 cells through homologous recombination with the E1 region of 293 cells, we did E1A-PCR to confirm absence of E1A contamination (Fig. 2B). Because there was no sign of E1A contamination in our preparation, we concluded that the effects seen with Xvir03 are independent from E1A.

Xvir03 does not drive HeLa cells into S phase. It is known that E1A interacts with pRb, releasing E2F and forces entry into the S phase of the cell cycle that supports viral replication (36). Because Xvir03 is E1A deleted, we asked how it might influence the cell cycle of HeLa cells. The diagrams (Fig. 2C) of noninfected cells and Xvir03-infected cells look very similar, whereas there is a clear shift from G1 to S phase in WT adenovirus-infected cells. Because the distribution of Xvir03-infected cells between G1 and S phase is nearly identical to uninfected cells, it can be concluded that Xvir03 was not contaminated with E1A. Another important finding is that the translocation (Fig. 3A of YB-1 by Xvir03 into the nucleus does not lead to S-phase induction (Fig. 2C).

In contrast to WT adenovirus, Xvir03 does not induce cell lysis in fibroblast cells. As shown in Fig. 2D, we used human dermal fibroblasts to test the effect of Xvir03 on nonmutogenic cells. Using 100 pfu/cell to ensure efficient infection, WT adenovirus lyses fibroblasts. At the same pfu/cell, Xvir03 does not kill the nonmutogenic cells nor does it affect the morphology of the fibroblasts. This illustrates together with Fig. 3C the selectivity of Xvir03.

YB-1 is translocated into the nucleus on Xvir03 infection. We have shown previously that infection with WT adenovirus causes nuclear translocation of YB-1, thereby permitting E2-late promoter

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Figure 1. A, a schematic outline of the adenoviral E2-gene regulation. The E2-late promoter is activated by YB-1, whereas the E2-early promoter is regulated by E2F. B, kinetic analysis of E2 gene expression. U2OS cells were infected with WT adenovirus (Ad5) and RNA was isolated at 3 to 24 hours after infection. E2-late expression level early in infection is higher than E2-early expression (a). Scheme showing the location of the probes regarding E2 promoters (b).
activation. In addition, we showed that the E1B55k protein is involved in the process of YB-1 translocation (27). Because E1B55k and E4orf6 proteins exist in a physical complex, we assumed that they act jointly. To prove the hypothesis that translocation of YB-1 into the nucleus is linked to the expression of E4orf6 and E1B55k, we used U2OS cells, where YB-1 is present predominantly in the cytoplasm in the perinuclear space (28). As shown in Fig. 3A, YB-1 is preferentially located in the nucleus after infection with Xvir03.

**Xvir03 replicates in tumor cells.** The ability of Xvir03 to replicate in tumor cells was evaluated in tumor cell lines of different origins. For this purpose, we infected U373, PC3, DU145, EJ28, HeLa, and U2OS cells with 20 pfu/cell Xvir03 and assessed viral replication (Fig. 3B). The Southern blot analysis revealed a clear signal for virus DNA in all tumor cell lines tested, indicating that E1A-independent replication is associated with the expression of E1B55k and E4orf6, translocation of YB-1, and its binding to the E2-late promoter (27). U2OS cells infected with WT-Ad5 served as positive control; infection with d312 adenovirus as negative control.

**Xvir03 induces strong cytopathic effect in PC3 and DU145 cancer cells.** To investigate whether Xvir03 induces a cytopathic effect (CPE) in infected tumor cells, CPE assays were done on PC3 and DU145 prostate carcinoma cells. As shown in Fig. 3C (plates A and B), Xvir03 caused complete cytolysis of both infected cancer cell lines within 7 days. Xvir03-infected PC3 cells were completely lysed at a pfu of 30; DU145 cells were killed at a pfu of 40. Our results show that the E1A/E3-deleted adenovirus Xvir03 is able to eliminate both prostate cancer cell lines and U2OS and EJ28 (data not shown).

**Cell killing of PC3 and DU145 by Xvir03 is enhanced by incubation with daunorubicin or docetaxel.** Enhanced killing of cells after adenoviral infection and cytostatic drugs has been reported repeatedly. Therefore, we sought to evaluate the cytotoxicity of the virus in combination with daunorubicin and docetaxel. For this purpose, PC3 and DU145 prostate carcinoma cells were treated with 1 to 18 nmol/L daunorubicin or 0.1 to 1 nmol/L, docetaxel for indicated hours before infection. As seen in Fig. 4, incubation with daunorubicin or docetaxel or Xvir03 alone had only a slight effect on cell density. The combination of daunorubicin or docetaxel with Xvir03 caused enhanced cell lysis, indicating that a combination of chemotherapy and Xvir03 infection is more efficient in cell killing than both treatments alone.

**In vivo growth inhibition of prostate xenograft with Xvir03 and docetaxel in a mouse model.** Because the in vitro results using docetaxel or irinotecan were encouraging, we decided to extend our studies in vivo (Fig. 5). To imitate the current clinical treatment protocols for prostate cancer, we applied docetaxel in our xenograft model. DU145 prostate tumors were generated in 6-week-old male BALB/c nu/nu mice. Tumors were treated with...
PBS (control) or Xvir03/docetaxel alone or with a combination of virus and drug (see Materials and Methods). Whereas by day 21 the single treatments already inhibited the tumor growth compared with PBS control by 56%, the combination of Xvir03 and docetaxel inhibited tumor growth more efficiently (76.5%). There was a significant difference between the combination group and the single-treatment groups (Xvir03/docetaxel versus docetaxel, $P = 0.012$; Xvir03/docetaxel versus Xvir03, $P = 0.02$). Although complete tumor regression was not achieved, the in vitro results were verified by our in vivo outcome.

Multidrug resistance–related genes $MDR1$ and $MRP1$ are down-regulated on WT adenovirus and Xvir03 infection. To study the enhanced effect of the combined treatment regimen, we studied three cell lines (DU145, EPP85-181RDB, and EPG85-257RDB) with high expression levels of known multidrug resistance–related genes (i.e., $MDR1$ and $MRP1$) by Northern blot and Western blot analysis (Figs. 6 and 7). After infection, the expression levels of the $MDR1$ and $MRP1$ genes were drastically down-regulated in WT adenovirus-infected cells and Xvir03-infected cells. In contrast, infection with dl312, an E1A-deleted virus, which does not express the viral proteins E1B55k and E4orf6 (37), and infection with an E1-minus adenovirus had no effect on $MDR1$ and $MRP1$ gene expression, respectively. The results indicate that the expression of E1B55k and E4orf6 seems to be a prerequisite for the down-regulation of genes that are regulated by Y-box-containing promoters.

Discussion
The development of drug resistance is a major problem for the clinical therapy of malignant diseases. Although certain mechanisms that cause a multidrug-resistant tumor phenotype in vitro are understood, a translation of this knowledge into successful therapeutic regimens in the clinic is still a major challenge (38).

Therefore, it is evident that the development of new concepts for overcoming the resistant phenotype in tumor therapy is important. Such therapies have to be highly selective and should not interfere with present standard therapies. Thus, a strategy to attack tumor cells on several fronts without increasing toxicity would be a key advancement in the treatment of cancer. Replication-competent oncolytic adenoviruses show just such qualities and hold promise for the treatment of cancer. Especially in combination with current standard therapies, such as radiation and chemotherapy, oncolytic adenoviruses showed additive or even synergistic effects on cytotoxicity in vitro and in vivo (39, 40). Although the mechanisms underlying this effect are still unknown, several hypothesis exist, such as that adenoviruses may increase the cell-killing effects of chemotherapy through induction of apoptotic pathways (41). This study investigates the replication capacity of a novel YB-1-associated oncolytic adenovirus and explores the possible molecular mechanisms underlying the observed effects in combination with chemotherapy.

The Y-box-binding protein YB-1 has multiple functions, including regulation of gene expression. Of primary interest for us is

Figure 5. Tumor growth inhibition by Xvir03 in combination with docetaxel in a xenograft prostate model. Mice bearing s.c. DU145 prostate carcinoma xenografts were randomized to four groups: control animals received injections of PBS (●); treated animals received Xvir03 at $10^8$ pfu/d on 2 days (●), 3 mg/kg docetaxel (Chx) on 2 days (▲), or virus and drug (●). Points, mean of seven tumors.
targeting the adenoviral E2-late promoter in multidrug-resistant
cancer cells. In addition, we showed that the adenoviral protein E1B55k is involved
in nuclear translocation of YB-1. Given that complexes containing adenoviral proteins E4orf6 and E1B55k
play critical roles in productive infection as well as in nuclear translocation of YB-1, we hypothesized that targeting YB-1 by a
recombinant E1/E3-deleted adenoviral vector expressing E1B55k and E4orf6 will be capable of translocating YB-1 into the nucleus
and in consequence to replicate and destroy tumor cells.

In a first step, we did a kinetic analysis of adenoviral E2 expression
using two different probes to discriminate between the activity of the
E2-early and E2-late promoter in E2 gene expression in Ad-WT-infected cells. Despite the activation of the E2-early promoter at
early times postinfection, our results indicate that the E2-late
promoter plays a predominant role in the later course. It seems that
E2-early is only needed in little amounts at the very early state of
infection, whereas E2-late acts throughout the whole cycle in quite
considerable quantities. Our findings correspond to published data,
showing that the E2-early promoter is active at early times
postinfection (35). Because YB-1 acts through the E2-late promoter,
it represents a suitable target for oncolytic adenovirus development.

To investigate whether E1B55k and E4orf6 facilitates adenoviral
replication, we constructed a recombinant adenoviral vector
expressing E1B55k and E4orf6. PCR analysis showed no sign of
E1A contamination. In addition, cell cycle analysis showed that no
progression from G1 to S phase occurs, whereas WT adenovirus
efficiently induced S phase in infected HeLa cells.

Next, we compared the relative CPE of the E1A-deleted
adenovirus Xvir03 with those of WT adenovirus at high MOI in
fibroblast cells. We used high titer to ensure viral infection in

the observation that nuclear localization of YB-1 regulates MDR1
and MRP1 gene expression that is known to confer the multidrug-
resistant phenotype in tumor cells (4). These data show that YB-1 is
involved in several aspects of drug resistance, and it was shown that YB-1 is an important regulator of pleiotropic resistance (12).

YB-1 overexpression with increased nuclear localization occurs
in many malignant diseases (e.g., breast, non–small cell lung,
osteosarcoma, ovarian, and colon carcinoma; refs. 19–23). Fur-
thermore, YB-1 mRNA is highly abundant in glioblastomas (42)
and malignant melanomas (43) and is involved in the progres-
sion of prostate cancer (17). Several studies indicate that YB-1
positively regulates cell proliferation and it was shown that YB-1
translocates from the cytoplasm to the nucleus at the G1-S-phase
transition (44). Recent evidence suggests that activated Akt is
involved in nuclear translocation of YB-1 and blocking of phos-
phatidylinositol 3-kinase/Akt pathway may therefore be helpful for
overcoming chemoresistance (45).

The use of replication-competent adenoviruses for cancer
therapy receives widespread attention, especially for the treatment
of tumors insensitive to current treatments. Previously, we showed
that YB-1 facilitates E1-independent adenoviral replication by
targeting the adenoviral E2-late promoter in multidrug-resistant

Figure 6. Down-regulation of multidrug resistance–related genes after
adenoviral infection. Northern blot analyses of cells expressing multidrug
resistance–related genes. Cells were infected with 50 pfu/cell of indicated
adenovirus. A, DU145, a prostate carcinoma cell line, strongly expressing the
MRP1 is down-regulated after infection with Xvir03 but not after infection
with an E1A-deleted adenovirus dl312. GAPDH served as loading control. B,
EPP85-181RDB, a multidrug-resistant pancreatic carcinoma cell line strongly
expressing the P-gp, was infected with indicated adenovirus. MDR1 gene
expression is down-regulated after infection with Xvir03 but not after infection
with an E1-minus adenovirus. 18S served as loading control. C, EPP85-
181RDB, also expressing MRP1, was infected with indicated adenovirus. MRP1
gene expression is down-regulated after infection with Xvir03 but not after
infection with an E1-minus adenovirus. 18S served as loading control.

Figure 7. Down-regulation of multidrug resistance–related proteins MDR1
and MRP1 after adenoviral infection. Western blot analyses of cells infected with
50 pfu/cell of indicated adenovirus. A, Xvir03 down-regulates MRP1 gene
expression like WT adenovirus does in infected EPP85-181RDB cells. B, Xvir03
down-regulates MDR1 gene expression like WT adenovirus does in infected
EPP85-257RDB cells. C, Xvir03 down-regulates MDR1 gene expression in
EPP85-181RDB but not after infection with an E1-minus adenovirus. Amounts of
loaded protein were reflected by hybridization with an antibody against β-actin.

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fibroblast cells, which otherwise are highly resistant to adenoviral infection. Whereas WT adenovirus infection causes strong induction of CPE and cell killing, no effects were observed in Xvir03-infected fibroblast cells. These results correspond with published data showing that E1A mutant adenoviruses with reduced S-phase induction show tumor-selective replication (46).

To verify YB-1 localization after Xvir03 infection, immunohistochemistry analysis was done. The results clearly indicate that adenoviral expression of E1B55k and E4orf6 mediates nuclear translocation of YB-1 in tumor cells. Nuclear translocation of YB-1 only takes place when cells are infected with WT adenovirus or Xvir03 but does not occur with an E1B55k-deleted adenoviral vector (27) or an E1-deleted adenoviral vector (and therefore lacking E4orf6 expression) expressing E1B55k under CMV control (data not shown). Efficient replication of an E1A-minus adenovirus (d312) at high MOI has been reported (37) and we could show previously that high MOI of d312 causes nuclear localization of YB-1 (27). Although binding studies were done, no interaction of the complex with YB-1 was detectable. Therefore, further studies are definitely required to clarify nuclear transport of YB-1 by the complex E1B55k/E4orf6.

To determine Xvir03 replication and its ability to destroy tumor cells in a replication-dependent manner, Southern blot analysis and CPE assays were done on a panel of human cancer cell lines that varied in their tissues of origin. Xvir03 was capable to replicate and induce CPE in all cell lines tested thus far.

Conditionally replicating adenoviruses have shown enhanced antitumor activity when combined with chemotherapy or radiation and it has been suggested in several reports that irradiation or chemotherapy creates an environment that is more conducive to adenoviral infection or replication (26, 47, 48), including our own data showing that cytostatic drugs cause an increase in nuclear YB-1 and concomitant viral replication (49).

Therefore, we investigated Xvir03 in combination with chemotherapy. In vitro experiments showed the potentiating effects of daunorubicin or docetaxel on Xvir03-induced cytotoxicity (Fig. 4). For in vivo studies, docetaxel was selected because it is used to treat hormone-resistant prostate cancer. However, docetaxel in combination with Xvir03 causes increased growth delay of DU145 xenograft compared with treatment with either docetaxel or virus alone. Considering the fact that Xvir03 does not express the adenoviral death protein (50) and E1A proteins suggests that it should be possible to design adenoviral mutants that are even more effective in achieving enhanced antitumor effects in combination with chemotherapy.

As mentioned earlier, YB-1 regulates, besides other factors, the expression of the drug-related transport proteins MDR1 and MRP1. This prompted us to test the hypothesis whether YB-1-associated oncolytic adenovirus Xvir03 causes inhibition of these genes. Northern blot analysis in prostate, pancreatic, and gastric cancer-derived cell lines infected with Xvir03 revealed clear down-regulation of these genes (Figs. 6 and 7). Our studies show that Xvir03 inhibits the expression of multidrug resistance–related genes MDR1 and MRP1. Because both genes are regulated by YB-1, this indicates that the recruitment of YB-1 via the complex E1B55k/E4orf6 to the adenoviral E2-late promoter is responsible for this effect. This is supported by the observation that the expression level of MDR1 was unaffected (data not shown) by an E1-deleted adenoviral vector expressing E1B55k under CMV control (kindly provided by Matthias Dobbelstein).

Nuclear translocation of YB-1 by Xvir03 leads to resensitization of tumor cells to cytostatic drugs; thus, the potential of radiation and chemotherapy is restored. Chemotherapy, on the other hand, yields an increased expression and nuclear localization of YB-1 and in consequence enhances YB-1-associated adenoviral replication (Fig. 8A).

This is the first study that reveals a link between chemotherapy and virotherapy based on the cellular transcription factor YB-1. Xvir03 is to our knowledge the first oncolytic vector that possesses as a single agent the potential of down-regulating two multidrug resistance–related genes MDR1 and MRP1. Due to their low homology, it is unlikely that a potent, nontoxic inhibitor for both proteins could be developed.

Based on our results, we would like to tentatively propose a model termed Mutually Synergistic Therapy (MUST; Fig. 8B), which...
highlights the existing relationship between YB-1-associated virotherapy on the one hand and chemotherapy on the other. Thus, combining YB-1-dependent virotherapy with chemotherapy is beneficial for both treatments. This theoretical model may be helpful in developing new combined strategies involving YB-1-associated virotherapy and chemotherapy, a novel combination for the treatment of non-small cell lung cancer. Clin Cancer Res 2001;7:3151–5.


Inhibition of the Multidrug-Resistant Phenotype by Targeting YB-1 with a Conditionally Oncolytic Adenovirus: Implications for Combinatorial Treatment Regimen with Chemotherapeutic Agents

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