Multidrug-resistant Cancer Cells Facilitate E1-independent Adenoviral Replication: Impact for Cancer Gene Therapy

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ABSTRACT

Resistance to chemotherapy is responsible for a failure of current treatment regimens in cancer patients. We have reported previously that the Y-box protein YB-1 regulates expression of the P-glycoprotein gene mdr1, which plays a major role in the development of a multidrug resistant-tumor phenotype. YB-1 predicts drug resistance and patient outcome in breast cancer. Thus, YB-1 is a promising target for new therapeutic approaches to defeat multidrug resistance. In drug-resistant cancer cells and in adenosivirus-infected cells YB-1 is found in the nucleus. Nuclear accumulation of YB-1 in adenosivirus-infected cells is a function of the E1 region, and we have shown that YB-1 facilitates adenovirus replication. Here we report that E1A-deleted or mutant adenovirus vectors, such as Ad312 and Ad520, replicate efficiently in multidrug-resistant (MDR) cancer cells and induce an adenovirus cytopathic effect resulting in host cell lysis. Thus, replication-defective adenosviruses are a previously unrecognized vector system for a selective elimination of MDR cancer cells. Our work forms the basis for the development of novel oncolytic adenosivirus vectors for the treatment of MDR malignant diseases in the clinical setting.

INTRODUCTION

The development of clinical drug resistance is a major limitation for effective cancer chemotherapy. The classical multidrug-resistant (MDR) phenotype is associated with increased transcription and translation of the mdr1 gene, which encodes P-glycoprotein, a multifunctional drug transporter (1, 2). Certain environmental stresses, for instance chemotherapy, UV light irradiation (3, 4), and hyperthermia (5), cause nuclear accumulation of Y-box protein (YB-1). Nuclear localization of the transcription factor YB-1 is associated with transcriptional activation of the human mdr1 gene (3, 6). Results from the literature suggest that YB-1 is involved in pleiotropic resistance to different classes of DNA-targeting drugs (7). YB-1 interacts with p53 (8) and functions as transcriptional repressor of the cell death-associated fas gene (9), indicating that YB-1 is involved in certain processes that control cell survival. Y-box proteins are characterized by a highly conserved nucleic acid recognition domain, the so-called cold region, and they are acting as transcriptional, translational, and developmental regulators (10–12). Y-box proteins interact specifically with a sequence motif termed Y-box, which is characterized by the presence of an inverted 5′-CCAAAT sequence.

Adenosviruses have attracted considerable attention for their use as vectors for gene therapy. First-generation, replication-deficient adenovirus gene therapy vectors have been constructed with a deletion of the E1 region consisting of the E1A and E1B genes. These viral early genes are required for efficient adenovirus replication (13–15). Because of differential splicing, the E1A region of human adenosirus type 5 encodes for two major proteins with a length of 289 and 243 amino acid residues (16, 17). The small E1A protein induces transcription of viral and cellular genes less efficiently than the large E1A protein (18). Thus, E1A mutant adenosviruses, which produce just the small E1A protein, are replication-defective like E1A-deleted adenosviruses (19). However, a number of reports demonstrated low-level viral DNA replication and formation of viral particles in cultured cells, and it was hypothesized that as yet unidentified viral or cellular proteins can substitute functionally for E1A in viral promoter regulation (15, 20–23). Adenosirus DNA replication depends on viral replication factors, which are encoded by the E2 genes. Expression of the E2 genes is controlled by E2 early and late promoters during the viral life cycle (24). Activity of the E2 early promoter is controlled by E1A and the host cell factor E2F (25). In contrast, activity of the E2 late promoter does not depend on E1A or E2F. We have reported recently that an infection with adenosirus causes nuclear accumulation of YB-1 as a function of the E1 region, and in consequence E2 late promoter activation, which facilitates adenosirus replication (26).

Because YB-1 is mainly located in the nuclei of MDR tumor cells (3), we investigated whether E1A-deleted adenosviruses (Ad312) and the E1A mutant adenosirus Ad520 (originally termed dl520; Ref. 27), which does not express the large 289 amino acid long E1A protein, can replicate in MDR cancer cells. Here we show that both the E1A-deleted and E1A mutant adenosirus vectors replicate efficiently in MDR cancer cells causing an adenosirus cytopathic effect (CPE) with concomitant cell lysis. Our findings demonstrate that replication-defective adenosviruses are a promising vector system for the treatment of MDR malignant diseases.

MATERIALS AND METHODS

Cell Lines. Cells (293; Ref. 28) were kindly provided by Frank Graham (McMaster University). The MDR cell lines EPP85–257RDB, EPP85–181RDB (29, 30), MCF-7Adr, and HCT-15 (5) were maintained in L-15 medium with 10% FCS. HeLa, 293, U2OS, and A549 cells were maintained in DMEM containing 10% FCS. The cell lines HBL-100 and HBL-100/YB-1 were cultivated in RPMI 1640 with 10% FCS. All of the drug-resistant P-glycoprotein-positive cell lines were treated with 100 ng/ml daunomycin every 4 weeks to ensure P-glycoprotein expression. All of the media were supplemented with l-glutamine (200 μg/ml), penicillin (100 μg/ml), and streptomycin (25 μg/ml).

Recombinant Adenosirus. The viral titer of the E1A-deleted adenosirus Ad312 and Ad520 was determined by plaque assays using 293 cell monolayers. To exclude any contamination with wild-type adenosviruses with an intact E1A region in Ad312 virus stocks, PCR was performed using specific primers (26). Subconfluent cells were infected by addition of 50–100 plaque-forming units/cell of adenosirus vectors to infection medium (OPTIMEM containing 2% FCS) after incubation for 2 h at 37°C in a 5% CO2 atmosphere with brief
agitation every 15 min. After infection, the medium was replaced by medium containing 10% FCS.

Preparation of Nuclear Extracts and Western Blot Analysis. Cells (10⁶) were washed twice in ice cold PBS and permeabilized by incubation for 5 min in 3 ml of ice cold hypotonic lysis-buffer [10 mM Tris–Cl (pH 8.0) supplemented with Complete protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany)]. For the preparation of nuclear extracts, we followed our published procedure (31). Ten μg of proteins per lane were subjected to electrophoresis on 10% SDS-PAGE. For immunoblotting, standard procedures were used. For the detection of P-glycoprotein we used the monoclonal antibody C219 (Roche Diagnostics). Western blots were developed with the enhanced chemiluminescence system (Amersham).

Electrophoretic Mobility Shift Assay (EMSA). Preparation of nuclear extracts and conditions for EMSA were described previously (26, 32). To detect nuclear expression of YB-1, the following recognition oligonucleotide was used: mdr1 promoter (−86 to −67): TGAGGCTGTA TTGGCTGGGCA (the Y-box is italicized). For competition experiments, unlabeled oligonucleotides from the Y-box of the mdr1 promoter and human cyclin E promoter (5) were used in 50-fold and 100-fold excess, respectively.

Determination of Viral Yields. Cells (10⁶) were infected with Ad312 at a multiplicity of infection (MOI) of 50−100. After infection, the medium was replaced with medium containing 10% FCS. At 72 h after infection, cells were scraped into the culture medium and centrifuged at 2500 rpm for 10 min. The cell pellets were extensively washed with PBS and centrifuged again. PBS was removed, and cells were suspended in 1 ml OPTIMEM. Virus was harvested by multiple cycles of freezing and thawing, and the supernatants were tested for virus production by a plaque assay in 293 cells.

Northern Blot Analysis. Total RNA of infected cells was isolated using the Trizol system (Life Technologies, Inc.) according to the manufacturer’s instructions. Ten μg of total RNA were size fractionated on 1% agarose-formaldehyde gels, transferred to a nylon membrane (Amersham), and hybridized using a 32P-labeled adenovirus E2A-cDNA probe as described previously (26).

Viral DNA Analysis. For viral replication analysis the cells were infected at a MOI of 10−50, and DNA was isolated 72 h after infection using the Qiagen Purification System (DNaseasy Tissue kit) according to the manufacturer’s instructions (Qiagen, Hilden, Germany). Two μg of DNA were digested with the restriction endonuclease Kpn I and size fractionated on 1% agarose gel, transferred to a nylon membrane, and hybridized using a 32P-labeled E2A-cDNA probe.

Generation of an E2A-specific cDNA Probe. A radioactively labeled cDNA probe for the adenovirus E2A gene was generated by PCR using wild-type Ad5 DNA and specific primers for the E2A gene (33). Primers used for the amplification of the cDNA were 5’-CCGGACAGGCCGCGTCA-3’ and 5’-TTGGCTGGGCAACACCCA-3’. Cycling conditions were 30 cycles consisting of 60 s at 95°C, 60 s at 55°C, and 60 s at 72°C.

Electron Microscopy. For ultrastructural analysis, confluent monolayers of infected cells were washed three times with 0.1 M sodium phosphate buffer (pH 7.2) and fixed with 2.5% glutaraldehyde in the same buffer for 30 min at room temperature. Cell layers were washed again with buffer and postfixed for 30 min with 1% osmium tetroxide. For additional processing, the fixed cells were scraped from the culture dishes, collected by centrifugation, and embedded in Epon resin according to standard procedures. Finally, thin sections were cut from resin blocks, mounted on 200 mesh copper grids, and stained with uranyl acetate and lead citrate. Sections were examined on a Zeiss EM10CR transmission electron microscope at 60 kV.

Analysis of Adenovirus Cytotoxicity. For determination of virus-mediated cytotoxicity assays were performed in 6-well-plates and then infected with either Ad312 or Ad520 at a MOI of 1–50 pfu/cell. One h after infection the medium was replaced. After 7 days, the cells were fixed and stained with 1% crystal violet in formaldehyde, followed by washing with water to remove excess of color. All of the experiments were performed in duplicate wells.

Analysis of in Vivo Adenovirus DNA Replication and Oncolytic Activity. Xenografts were established in 8-week-old BALB/c-nu/nu mice by injecting 5 × 10⁵ 257RD/B- or HeLa cells suspended in 100 μl of PBS s.c. When tumor volume reached between 200 and 500 mm³ mice were randomized into groups of 4 animals. The first group received Ad520 at a dose of 2.5 × 10⁵ pfu in a volume of 50 μl daily for 3 days. As a control, a second group was treated with normal saline. Tumors were measured every 3–4 days in two dimensions by external caliper, and volume was estimated by the formula 4/3π × a² × (b/2)². After 30 days animals were sacrificed, and tumor DNA was isolated using a Qiagen purification system (DNaseasy Tissue kit) according to the manufacturer’s instructions. To detect adenovirus replication, 2 μg of tumor DNA were digested with the restriction endonuclease Kpn I, and size fractionated on 1% agarose gels, transferred to a nylon membrane, and hybridized using a 32P-labeled adenovirus E2A-cDNA probe.

RESULTS

P-Glycoprotein and YB-1 Expression in MDR Cancer Cells. To detect P-glycoprotein expression in cancer-derived MDR cell lines we performed Western blot analysis using the monoclonal antibody C219. For this purpose, membrane extracts of several MDR and chemosensitive cell lines were isolated. As shown in Fig. 1, the MDR cell lines 181RDB (pancreatic cancer), 257RD (gastrointestinal cancer), and MCF-7Adr (breast cancer) produced large amounts of P-glycoprotein, whereas the drug-sensitive cell lines HeLa and U2OS did not synthesize any detectable P-glycoprotein. As a next step YB-1 expression was analyzed by an EMSA using nuclear extracts and a radiolabeled Y-box oligonucleotide from the mdr1 gene promoter as a probe (3). Substantial amounts of YB-1 were detected in nuclear extracts of the three MDR cell lines (Fig. 2, lanes 257RD, 181RDB, and MCF-7Adr). In contrast, no or trace amounts of YB-1 were detected in drug-sensitive HeLa and U2OS cells, respectively (Fig. 2, lanes HeLa and U2OS). The presence of YB-1 in retarded DNA:protein complexes was confirmed by competition with a molar excess of unlabeled Y-box oligonucleotides from the mdr1 gene promoter and an unrelated promoter fragment from the cyclin E gene (Ref. 5; data not shown).

Efficient Lysis of MDR Cancer Cells by a Replication-defective E1A-deleted Adenovirus Mutant. MDR cells 181RDB, 257RD, and chemosensitive control cells (U2OS and A549) were infected at a MOI of 50 with the E1A-deleted adenovirus mutant d312 referred to as Ad312 throughout the text. To detect replicated adenovirus DNA in infected cells, total DNA was isolated, digested with the restriction endonuclease Kpn I, and processed for Southern hybridization (26). Fig. 3 shows efficient adenovirus DNA replication in MDR pancreatic and gastric cancer cells (Fig. 3, lanes 181RDB and 257RDB). In contrast, viral replication was barely detectable in chemosensitive A549 and undetectable in U2OS cells. To exclude the possibility that any difference in replication efficiency was attributable to a difference in infectivity, we performed an infectivity assay using Ad5CMVlacZ. The assay revealed that there was no difference in Ad5CMVlacZ
infectivity for U2OS, A549, 181RDB, and 257RDB cells (data not shown). Next we examined whether Ad312 could induce CPEs in the MDR cell lines. Therefore, the drug-resistant and drug sensitive cells were infected at various MOIs (20–200 pfu/cell), and monitored for the appearance of an adenovirus CPE. As shown in Fig. 4, induction of a CPE occurs 4–7 days after infection in the MDR cell lines (Fig. 4, panels 181RDB and 257RDB). In contrast, drug-sensitive Ad312-infected HeLa and U2OS cells did not develop any sign of an adenovirus CPE. Thus, the E1A-deficient Ad312 vector has the potential to selectively eliminate MDR cancer cells.

The Adenovirus E2 Genes Are Transcribed in Ad312-infected MDR Cancer Cells. We have constructed a recombinant E1-minus adenovirus vector expressing YB-1 as a transgene (AdYB-1) and have shown that YB-1 activates transcription of the E2 genes through the E2 late promoter (26). The E2 genes encode the three viral replication factors (34). Next, we investigated whether nuclear localization of YB-1 in MDR cancer cells facilitates E2 gene transcription. To test this issue we analyzed E2 gene activity in Ad312-infected cells by Northern analysis using an E2A-cDNA as a probe (Fig. 5). As a control, HeLa cells were infected with Ad5 viruses (Fig. 5, panel Ad5). It is recognizable that the E2 gene was not expressed in drug-sensitive Ad312-infected U2OS and HeLa cells (Fig. 5, lanes U2OS and HeLa). In contrast, infected 257RDB and 181RDB cell expressed considerable amounts of E2A mRNA. In MCF-7Adr cells E2A mRNA levels were almost as high as in Ad5-infected HeLa cells. Note that the level of nuclear YB-1 protein was higher in MCF-7Adr cells than in 257RDB and 181RDB cells (Fig. 2). Thus, the nuclear levels of YB-1 in MDR cancer cells correlate directly with adenovirus E2 gene activity.

Efficient Ad312 Replication in Breast Epithelial Cells with Constitutive Nuclear Overexpression of YB-1. To rule out a contribution of unrecognized transcription factors in E2 late promoter regulation in Ad312-infected MDR cancer cells, we investigated...
whether nuclear overexpression of YB-1 in chemosensitive cells permits viral replication. To this end, we used a cell line with constitutive high level expression of YB-1 (5). High level expression of YB-1 in a normal diploid breast epithelial cell line (HBL-100) was achieved by transfection with a YB-1 cDNA under control of the cytomegalovirus promoter (5). For detecting exogenous YB-1 protein, the YB-1 cDNA was fused to a cDNA encoding the V5-epitope (5). The V5-epitope is derived from a paramyxovirus protein (35). In HBL-100 cell clones overexpressing the V5 epitope-tagged YB-1 cDNA (HBL-100/YB-1), YB-1 protein was located predominantly in the perinuclear region and in the nucleus (data not shown). In HBL-100 parental cells YB-1 expression is low, and in these cells YB-1 is located in the cytoplasm in the perinuclear region (3). To confirm nuclear localization of the V5-tagged YB-1 protein in HBL-100/YB-1 cells using a biochemical approach, we analyzed nuclear extracts by immunoblotting with a V5-specific antibody (Fig. 6). The V5 tag does not affect the function of YB-1, as it is able to specifically interact with the Y-box.9 In addition, the V5 tagged YB-1 has the capacity to transactivate a mdr1 promoter-driven reporter gene (5). Next, we infected HBL-100/YB-1 and HBL-100 cells with Ad312, and monitored the cells for the appearance of an adenovirus CPE. Fig. 7 shows an adenovirus CPE in Ad312 infected HBL-100/YB-1 cells 4 – 6 days after infection. The infected HBL-100/YB-1 cells exhibited rounded morphology and loss of adherence, whereas the Ad312-infected HBL-100 cells appeared normal. To exclude a potential difference of infectivity between parental HBL-100 and HBL-100/YB-1 cells we performed an infectivity assay using a recombinant adenovirus expressing the lacZ gene. The assay revealed that stable YB-1 transfectants have the same infectivity as the parental HBL-100 cells.4 Thus, YB-1 is important for the completion of an adenovirus life cycle and the development of CPE.

**FIG. 6.** Overexpression of V5-tagged YB-1 in nuclei of transfected HBL-100 cells. Thirty μg of nuclear extracts from HBL-100 cells and HBL-100/YB-1 cells were size fractionated on SDS-PAGE, transferred to nitrocellulose, and incubated with a monoclonal antibody directed against the 14 amino acid long V5 epitope. The position of V5-tagged YB-1 protein is indicated by an arrow.

**Production of Infectious Progeny Virus Particles in Ad312-infected MDR Cancer Cells.** To test whether infectious virus particles are generated in Ad312-infected MDR cancer cells, we determined the amount of infectious virus progeny particles in supernatants of infected cells by a plaque assay using 293 cells, which allows replication of E1A-minus adenoviruses. We considered it necessary to control for the presence of E1A-positive adenoviruses in these experiments. To test for contaminating E1A-positive adenoviruses we used three different approaches: (a) E1A-specific PCR (PCR conditions and primer were published elsewhere; Ref. 26); (b) E1A-specific immunofluorescence; and (c) a plaque assay on A549 cells. We were unable to detect any E1A-positive contaminants by PCR, we did not detect E1A protein in infected cells, and we were unable to detect an adenovirus CPE even at high MOI.4 A low titer of infectious virus particles could be detected in supernatants of infected drug-sensitive HeLa, U2OS, SKOV3, and A549 cells, however, the Ad312 titers in supernatants of infected MDR cancer cells were 3 – 4 orders of magnitude higher (Fig. 8). This result indicates efficient Ad312 replication and progeny particle formation in MDR cancer cells (Fig. 8).

**FIG. 7.** YB-1 is important for adenoviral DNA replication. The appearance of a CPE in HBL-100/YB-1 cells indicates adenoviral DNA replication.
E1A-deleted and E1A Mutant Adenoviruses Induce a Strong CPE in MDR Cancer Cells. To investigate whether Ad312 and Ad520 induce a CPE in MDR and drug-sensitive cancer cells, CPE assays were performed. As shown in Fig. 10, Ad312 and Ad520 caused complete cytolysis of infected MDR 257RDB cancer cells within 7 days. Ad520-infected 257RDB cells were completely lysed at a MOI of 2.5, whereas Ad312-infected 257RDB cells were lysed at a MOI of 30. In contrast, a CPE could not be detected in Ad520- and Ad312-infected drug-sensitive HeLa cells, even at a MOI of 50–100 pfu/cell (data not shown). Our results demonstrate that the E1A mutant adenovirus Ad520 is able to eliminate MDR cancer cells with a much higher efficiency than the E1A-deleted adenovirus Ad312.

Oncolytic Activity of Ad520 in Xenotransplanted MDR Tumors. To evaluate a potential oncolytic activity of Ad520 we established a mouse xenograft model system using MDR 257RDB- and drug-sensitive HeLa cells. Once tumors had developed in the transplanted mice, they were infected with the E1A mutant adenovirus Ad520, and the tumor volume was monitored over time. The tumor volume data presented in Fig. 11A show that there was a significant decrease in tumor volume in the Ad520-treated group of mice, which carried MDR 257RDB tumors (Fig. 11A, filled rectangles). Four weeks after treatment the relative volume of 257RDB tumors had decreased 72.5% (average of 4 mice) compared with the saline treated group (Fig. 11A, open rectangles). Thus, intratumorally delivered Ad520 displays oncolytic activity in the 257RDB mouse model. In contrast, Ad520 infection had no effect on tumor volumes in mice transplanted with drug-sensitive HeLa cells (Fig. 11A, filled triangles).

To determine viral DNA replication of Ad520 in the transplanted tumors, DNA was isolated 30 days after treatment. Fig. 11B shows that all three of the established 257RDB tumors treated with Ad520 allow efficient adenovirus DNA replication (Fig. 11B, Lanes 2–4). In contrast, viral replication was not detectable in Ad520-treated tumors, which were derived from drug-sensitive HeLa cells (Fig. 11B, Lane 5–7). The data show that the E1A mutant adenovirus Ad520 is able to efficiently replicate in MDR 257RDB tumors. These results explain, at least in part, how the oncolytic activity of Ad520 in MDR tumors is brought about.

DISCUSSION

The development of clinical drug resistance is a major problem for the therapy of malignant diseases. Although certain mechanisms that cause a MDR tumor phenotype are understood, a translation of this knowledge into successful therapeutic regimens in the clinic is still a major challenge. Recently, results obtained in pilot studies suggest that YB-1 could serve as a new tumor biological marker involved in...
we think that E1A-defective adenoviruses can ultimately be used to treat MDR tumors in the clinical setting.

We are of course aware that it is now necessary to determine physiological expression patterns of YB-1 in the different tissues of the human body. In this context it is interesting to note that YB-1 mRNA levels are high in fetal tissues for example muscle, brain, lung, adrenal gland, heart, and liver (52). However, in adult tissues, YB-1 is expressed at much lower levels e.g., adult kidney and breast epithelial cells (3). To investigate this issue in detail we have begun a systematic analysis using an immunohistochemistry approach.

In cancer gene therapy, the failure to achieve sufficient tumor transduction led to the strategy to use replication-competent adenoviruses. Viral replication seems to be of benefit in the treatment of human cancer, because this may improve transgene delivery and cancer cell killing (53, 54). Our data suggest that in a therapeutic setting it is of advantage to induce nuclear accumulation of YB-1 to achieve a more efficient elimination of drug-resistant tumor cells in vivo. We have reported that heat shock leads to a rapid nuclear accumulation of YB-1 in colon cancer cells (5), and it was shown that heat shock enhances the oncolytic effect of replicative adenoviruses (55). It was reported that treatment of carcinoma cells with oncolytic adenoviruses in combination with DNA-damaging agents results in enhanced cell killing (56, 57). Therefore, it is a therapeutic option to combine adenoviral cancer therapy with other treatment modalities, which cause nuclear accumulation of YB-1 in vivo. Thus, our findings delineate a novel therapeutic approach for MDR malignant tumors.

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