Pharmacologic Indicators of Antitumor Efficacy for Oncolytic Virotherapy

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ABSTRACT

Central to the development of oncolytic virotherapies for cancer will be a better understanding of the parameters that influence the outcome of virotherapy to treat disseminated cancer by i.v. administration versus regional disease by local treatment. Intratumoral administration of 01/PEME, an oncolytic adenovirus, required >1000-fold less dose than i.v. administration to induce similar tumor growth inhibition. Despite the short (<10 min) circulating half-life of the virus DNA, we could monitor virus distribution to the tumor site and observed virus replication by 01/PEME for which the virus DNA concentration in the tumor increased over time but did not result in antitumor efficacy. Oncolytic virus replication at a tumor site may not be a relevant indication of antitumor efficacy. Efficient distribution to the tumor site may be one of the most critical parameters for antitumor efficacy with oncolytic virotherapy.

INTRODUCTION

Oncolytic adenoviruses are currently undergoing clinical trials as a strategy to treat cancer. For example, the oncolytic adenovirus dl1520 (Onyx-015), an E1b-55k deleted adenovirus, has been evaluated by a variety of routes of administration (1) including i.v. administration (2). Whereas adenovirus administration was well tolerated by cancer patients, only limited responses were noted. Other oncolytic adenoviruses also have been used in preclinical models to demonstrate antitumor efficacy. It is expected that virus replication in tumor cells will spread the virus throughout the tumor, causing oncosysis and tumor regression. To treat a variety of tumor types, an infusion of adenovirus by i.v. administration will need to distribute a sufficient quantity of virus to the tumor to cause a tumor response. Small concentrations of virus may be sufficient to initiate spread throughout the tumor.

The utility of nonreplicating adenovirus vectors to transfer genes to tumors after i.v. administration is limited, because adenovirus localizes primarily to the liver (3). It is estimated that after i.v. administration of adenovirus, 90% of the adenovirus is found in the liver, and virus DNA is rapidly cleared (4). The advantage of oncolytic replicating adenoviruses would be their ability to replicate in tumor tissue from initially low quantities and increase over time causing oncosysis. i.v. administration of oncolytic adenoviruses has been shown to control tumor growth in animal models. Engineered viruses with deletion of E1b (5), E1 region genes under control of tissue-specific promoters (6, 7), and modified E1a (8) were reported as being effective at inhibiting tumor growth after i.v. administration.

Modified adenoviruses for oncolytic virotherapy have been suggested to attenuate replication in normal cells. dl1520 is an E1b-55K deleted adenovirus and, therefore, does not express a viral gene that interacts with p53. Growth of this virus is attenuated in normal cells; however, studies suggest that it is also attenuated in tumor cells (9). CN706 (10) and CV787 (6) use prostate-specific expression of the E1 region to limit replication to prostate cancer cells. CV890 (7) and AvE1a04i (11) contain the α-fetoprotein promoter controlling expression of E1, which is proposed to restrict replication to hepatocellular carcinoma cells. These strategies are designed to function in a subset of tumors.

Our approach is to use a strategy in which virus replication is actively antagonized by the cellular pathways that remain intact in normal cells but are dysregulated in tumor cells. 01/PEME (12) is an adenovirus modified to attenuate replication in normal cells by: (a) a deletion in the E1a gene derived from dl1101 that prevents viral inactivation of p53 and impairs virus-induced cell cycle progression (13); (b) a deletion in the E3 region derived from d327 that prevents viral interference with immune response (14, 15); (c) insertion of a p53 responsive promoter driving an E2F antagonist, E2F-Rb (16), that blocks viral replication in normal cells; and (d) insertion of a major late promoter regulated E3–11.6K that enhances virus spread in tumor cells (17, 18). 01/PEME was shown to be attenuated in normal cells and to be more effective in inhibiting tumor growth than dl1520, requiring ~1000-fold fewer particles to inhibit growth by 50% by intratumoral administration (12).

Demonstration of antitumor efficacy of oncolytic adenovirus via i.v. administration in addition to intratumoral administration would improve the utility of a virus-based oncolytic therapy for cancer. Successful drug development with oncolytic viruses will depend on their pharmacologic properties. Understanding the parameters that influence antitumor efficacy will be critical to identifying the clinical situations with the greatest likelihood of success. Virus replication in the tumor is an important component of the mechanism of action of an oncolytic virus therapy. A number of studies have demonstrated the efficacy of oncolytic adenovirus by i.v. administration in xenograft tumor models (5, 6, 8). The purpose of this study was to evaluate the pharmacologic parameters that influence the antitumor efficacy of the oncolytic adenovirus 01/PEME after i.v. administration. We observed antitumor efficacy of 01/PEME by i.v. administration in four s.c. tumor models in nude mice. 01/PEME virus DNA increased over time in tumor tissue, and tumor sections were positive for proteins associated with late gene expression, confirming virus replication after i.v. administration. The circulating half-life of the virus was <10 min. Dose-response studies comparing intratumoral and i.v. administration suggested that the initial concentration at the tumor site was the key indicator of antitumor efficacy.

MATERIALS AND METHODS

Adenovirus Constructs. rAd-p53 is an E1-deleted adenovirus vector encoding human p53 cDNA expressed from the cytomegalovirus immediate early promoter (Ref. 19; Fig. 1). 01/PEME is an oncolytic adenovirus with a deletion of the region encoding the domain of E1a that binds to p300/CPB (amino acids 4–25) and inserted into the deletion in E3 region were fragments encoding an inhibitor of virus replication, E2F-RB fusion protein, expressed from a p53-responsive promoter and the E3–11.6K cytolytic protein expressed under control of the adenovirus major late promoter (Ref. 12; Fig. 1). Viruses were purified by column chromatography (20), and particle concentration was determined (21).

Animal Models. Efficacy of intratumoral administration was evaluated in nude mice with s.c. PC3 prostate tumors. Nude mice were implanted with...
5 × 10^6 PC3 cells in the flank to initiate tumor growth. After 7 days, groups of 8 mice were treated with 1 × 10^6, 1 × 10^7, 1 × 10^8, or 1 × 10^9 particles of 01/PEME or rAd-p53 on 5 consecutive days by intratumoral administration.

Antitumor efficacy of i.v. administration was evaluated in four s.c. tumor models in nude mice: PC3, prostate; C33A, cervical; A549, lung; and SW620, colorectal. Treatment was initiated after 7, 7, 21, or 5 days for each tumor model, respectively, by i.v. administration of 1 × 10^10 particles on 5 consecutive days (total dose, 5 × 10^10 particles). The PC3 tumor model was also dosed at 1 × 10^10, 1 × 10^11, or 1 × 10^12 particles on 5 consecutive days by the i.v. route of administration. There were 8 mice/treatment group.

Tumor volume was calculated assuming spherical geometry using measurements taken in 3 dimensions with calipers. Mean tumor size for each treatment group ± SE means was plotted versus time after cell injection. For statistical analysis, one-way ANOVA was performed on the tumor sizes on the last day of the study and additionally analyzed by Dunnett’s multiple comparisons analysis, one-way ANOVA was performed on the tumor sizes on the last day versus indicating modifications to the adenovirus genome.

Virus Quantification by PCR. DNA was extracted from ~100 mg of each tissue or 100 μl whole blood using Tri-Reagent (Molecular Research Center, Inc.) per the manufacturer’s protocol. Quantification of adenovirus DNA was performed using Real Time Quantitative PCR (22) using the Taqman Universal PCR master mix (Applied Biosystems, Foster City, CA). PCR reactions were performed in a total volume of 25 μl containing 1 μl of test sample, 12.5 μl of Universal PCR master mix, 120 nM of probe, and 300 nM of primer. The following thermal cyclers conditions were optimized: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 62°C. The sequence of the oligonucleotides used were derived from the hexon coding region of adenovirus type 5: forward primer 5’-ACT ATA TGG ACA ACG TCA ACC CAT T-3’: reverse primer 5’-AAC TTC TGA GCC ATG TGG ATG T-3’, probe 5’-carboxyfluorescein-ACC ACC GCA ATG CTG GCC TGC-6-carboxytyramethylrhodamine-3’. The number of virus DNA copies was determined based on a standard curve derived from purified adenovirus. The lowest limit of detection was 250 01/PEME DNA copies/mg tissue.

RESULTS

The antitumor efficacy of the oncolytic adenovirus 01/PEME was compared with that of rAd-p53 (19), an E1-deleted adenovirus vector encoding human p53, to evaluate the relative potency of the two constructs (Fig. 1A). The PC3 cell line, which is null for p53, has been shown to be responsive to p53 gene-replacement strategy (23). Doses of 1 × 10^4, 1 × 10^5, 1 × 10^6, or 1 × 10^10 particles 01/PEME or rAd-p53 were administered on 5 consecutive days by the intratumoral route. The highest dose, 1 × 10^10 particles, of rAd-p53 resulted in tumor growth inhibition with 1 of 8 animals being tumor-free at the end of the experiment. Treatment with lower doses of rAd-p53 did not cause significant tumor growth inhibition. In contrast, treatment with 01/PEME resulted in significant tumor growth inhibition across all of the dose levels (Fig. 1B). Each of the 8 animals treated with 01/PEME at the highest dose level of 1 × 10^10 particles were tumor-free, and 3 of 8 animals treated at 1 × 10^9 particles were tumor-free. Even doses as low as 1 × 10^7 particles 01/PEME resulted in significant tumor growth inhibition (P < 0.01).

The efficacy of 01/PEME via intratumoral administration at very low doses led us to evaluate the ability of 01/PEME to control tumor growth when administered i.v. The PC3 tumor model was used in a dose response study with doses of 1 × 10^8, 1 × 10^9, or 1 × 10^10 particles on 5 consecutive days using the i.v. route of administration (Fig. 2A). In the PC3 tumor model, i.v. administration of 1 × 10^8 particles 01/PEME inhibited tumor growth 52%, and dosing with 1 × 10^10 particles inhibited tumor growth 82% (P < 0.01). The dose of 1 × 10^8 particles had no effect on tumor size compared with vehicle treatment in this study. Two of the 8 animals treated with 1 × 10^10 particles 01/PEME and 1 of 8 animals treated with 1 × 10^9 particles 01/PEME by i.v. administration were tumor-free.

Comparing the intratumoral and i.v. routes of administration in the PC3 tumor model, dose response studies revealed that the efficiency of i.v. administration was ~1000 fold less than intratumoral admin-
Dose-dependent PEME DNA was detected in the liver and D3 decreased to 3 % of the initial level after treatment with 10^10 particles PEME by i.v. administration. The concentration of PEME DNA in the circulation of nude mice after injecting 10^10 particles PEME by i.v. administration was below the limit of detection. The dose of 10^10 particles PEME by i.v. administration resulted in 20% tumor growth inhibition on day 1, whereas treatment with 10^3 particles PEME by i.v. administration inhibited tumor growth of each of the tumor types.

Antitumor efficacy of PEME by the i.v. route of administration was evaluated by the tumor growth inhibition percentage and the number of tumor-bearing nude mice after administration. Treatment with 10^3 DNA copies/mg tumor as 10^10 particles PEME but not 10^3 particles PEME inhibited tumor growth by 52%, whereas treatment with 10^3 particles PEME by intratumoral administration caused a similar degree of growth inhibition.

Three additional tumor models derived from lung (A549), cervical (C33a), and colorectal (SW620) cells, along with PC3, were challenged with i.v. administration of 10^10 particles PEME for 5 consecutive days. Treatment with 10^3 DNA copies/mg tumor (Fig. 2B) by the i.v. route of administration inhibited tumor growth of each of the tumor types.

The concentration of virus in the blood may be one factor that influences antitumor efficacy. We determined the concentration-time profile of PEME in the circulation of nude mice after injecting 10^10 particles PEME by the i.v. route of administration. Virus DNA copies were quantified by PCR from whole blood samples collected over time. As shown in previous studies (24), the half-life in the circulation was <10 min (Fig. 3A). The virus DNA concentration in the blood decreased 95% between 1 and 30 min after administration.

Despite the short half-life in circulation, the observation of antitumor efficacy with i.v. administration suggested that sufficient quantities of virus were distributed to the tumor to cause tumor responses. A dose-response study to quantify the virus DNA over time in PC3 tumor-bearing nude mice was undertaken. Virus DNA from tumor (Fig. 3B), liver (Fig. 3C), and whole blood (Fig. 3D) from mice treated with increasing doses of PEME was analyzed by PCR. In mice bearing PC3 tumors, the concentration of virus DNA in tumor homogenates harvested 3 h after dosing 10^10 or 10^9 particles PEME was below the limit of detection. The dose of 10^10 particles PEME by i.v. administration resulted in 2 x 10^3 DNA copies/mg distributed to the tumor at 3 h after administration. However, for each dose, by 2 days after administration the PEME DNA concentration increased, and by day 22 reached a plateau of ~10^7 PEME DNA copies/mg tumor. The plateau concentration of virus DNA copies was not dose-dependent. In contrast, 10^3 DNA copies rAd-p53 injected i.v. achieved the same initial tumor concentration of 3 x 10^7 DNA copies/mg tumor as 10^10 particles PEME but decreased to <100 DNA copies/mg tumor tissue by day 22.

As opposed to the observations in the tumor that showed increasing PEME DNA concentration over time, PEME DNA concentration decreased over time in the liver (Fig. 3C) and in the blood (Fig. 3D). Dose-dependent PEME DNA was detected in the liver and blood. The ratio of concentration of virus DNA initially distributed in the liver compared with the tumor was ~1000 at the 1 x 10^10 particles dose. The concentration of the replication-defective virus, rAd-p53, DNA detected in the liver and blood was similar to the concentration of PEME at the 3-h time point and then decreased to a similar concentration as PEME by day 22.

Increasing concentration of virus DNA over time suggested that viral replication was ongoing in the tumor. To confirm that the PCR results were indicative of virus replication, viral late protein (hexon) expression was evaluated by immunohistochemistry (Fig. 4). Only tumors from PEME-treated animals had a positive staining with the antibody. Hexon-positive areas were not evenly distributed. Pockets of infiltrating cells were observed near areas that stained positive with the antihexon antibody. Histopathological evaluations confirmed that the tumor cells were positive for the hexon antibody, and areas of necrosis were present in the tumor sections, along with infiltrates of neutrophils and lymphocytes, an indication of an acute inflammatory response. Tumors derived from mice treated with a nonreplicating vector had minimal cellular infiltrates and were negative for late virus protein. Tumors derived from the vehicle group had no cellular infiltrate, and antibody staining was negative as well.

We performed additional studies to corroborate the relationship between virus delivery to the tumor site and antitumor efficacy in another tumor model. In this study we used the C33A cervical xenograft tumor model to determine the dose response with a single intratumoral administration compared with a single i.v. administration. The tumors from 3 animals in each dose group were harvested 3 h after administration to quantify the virus delivered to the tumor. The remaining cohort of animals in each dose group was assessed for antitumor efficacy. The group of animals treated by the i.v. route with

Fig. 3. Virus DNA concentrations in blood and tumor tissue after i.v. administration. A, 1 x 10^10 particles PEME were injected into nude mice by i.v. administration. Virus DNA copies in whole blood samples were quantified by PCR using hexon primers. The mean 01/PEME DNA copies per ml blood was plotted over time after administration. Nude mice bearing PC3 tumors were treated with 1 x 10^10 particles rAd-p53 (•), 10^3 (□), 10^2 (■), or 1 x 10^10 (○) particles PEME by i.v. administration. Virus DNA in tumor (B), liver (C) homogenates, or blood (D; n = 3) was measured by PCR using primers from the hexon region. The mean 01/PEME DNA copies/mg tissue or copies/ml blood was plotted over time after virus administration; bars, ±SD.
a single bolus administration of $1 \times 10^{11}$ particles 01/PEME had elevated transaminase levels, and their body weights were 7% less than the control treatment group. As observed in the PC3 tumor model, dose-dependent tumor growth inhibition (Fig. 5A) and virus delivery (Fig. 5B) was observed with 01/PEME treatment in the C33A tumor model. There was a good correlation with the extent of tumor growth inhibition and the quantity of virus delivered to the tumor as measured 3 h after virus administration (Fig. 5C) independent of the route of administration. In this model with a single intratumoral administration, 3 of 6 animals treated with $1 \times 10^{10}$ particles 01/PEME were tumor-free.

**DISCUSSION**

The mechanism of action of oncolytic viruses is based on the replication of virus in tumor cells, and the spread of newly formed virus to nearby and distant tumor cells. 01/PEME is an oncolytic adenovirus that has been demonstrated to have attenuated replication in normal cells, whereas retaining replication capacity in tumor cells (12). This study focused on determining which pharmacologic parameters of 01/PEME correlated with antitumor efficacy in established xenograft tumors. The efficiency of distribution to the tumor was the key indicator for antitumor efficacy.

We demonstrated the ability of 01/PEME to inhibit tumor growth at very low doses after intratumoral administration. Five consecutive days of intratumoral administrations of $1 \times 10^4$ particles of 01/PEME were shown to inhibit growth of s.c. PC3 tumors. In contrast, the nonreplicating p53 adenovirus vector, rAd-p53, inhibited tumor growth only at a dose of $1 \times 10^{10}$ particles. There was a dose response for which the dose of 01/PEME corresponded to the degree of tumor control. This suggested that the initial concentration of virus in the tumor site was responsible for the degree of tumor control. The demonstration that doses as low as $1 \times 10^4$ particles 01/PEME administered directly to the tumor inhibited tumor growth supplied a good rationale for i.v. administration. i.v. administration of 01/PEME was effective in controlling tumor growth in four different tumor models. The design of 01/PEME takes advantage of dysregulated growth control pathways in tumor cells (12), in contrast with other strategies designed for a particular type of tumor. Therefore, despite differences in the mutation status of each cell line, 01/PEME was effective in each of the tested tumor types. Importantly, one of the tumor lines tested, A549, was not mutated in the p53 gene. Despite having wild-type p53, A549 cells were as effectively controlled as the other tumor types carrying mutated p53 genes. This is especially notable because 01/PEME uses p53-dependent expression of an E2F antagonist as an inhibitor of viral gene replication, E2F-Rb fusion, to attenuate replication in normal cells. It is hypothesized that 01/PEME virus can replicate in the tumor cell environment because of the higher transactivation potential of E2F relative to the transactivation potential of p53 (12) in the tumor cells (25, 26). This imbalance does not exist in normal cells. As a consequence, 01/PEME can overcome the p53-mediated expression of the E2F-Rb inhibitor even in wild-type p53 tumor cells.

The PC3 tumor model was used to examine the effects of both, intratumoral and i.v., routes of administration. i.v. administration of $1 \times 10^{10}$ particles 01/PEME caused ~80% tumor growth inhibition. To achieve 80% tumor growth inhibition required a dose of $\sim 1 \times 10^7$ particles by intratumoral administration. This suggested that i.v. administration was ~1000-fold less efficient than intratumoral administration. Despite this decreased efficiency, i.v. administration was effective at inhibiting tumor growth when high doses of 01/PEME were used.

Evidence of 01/PEME replication in PC3 tumors after i.v. administration was observed by quantification of virus DNA over time. The comparison between the E1-deleted replication-deficient rAd-p53 vector and the oncolytic 01/PEME virus demonstrated 01/PEME replication in the tumor over time. Concentrations of virus DNA in the tumor increased over time with each of the three doses of 01/PEME. The concentration of the virus DNA increased and reached a plateau.

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**Fig. 4.** Immunohistochemical detection of virus capsid proteins in tumor tissue. Sections from PC3 tumors harvested on day 12 from mice treated with 01/PEME (A and B), treated with vehicle (C), or treated with a nonreplicating vector (D) were stained with a control isotype matched antibody (A) or with antihexon antibody (B-D). Only tumors that were treated with 01/PEME stained positive for antihexon antibody, red brown color as shown in B. Magnification ×20.
...tumor. C, tumor growth inhibition was plotted delivery ( ) administration. The mean tumor growth inhibition was plotted versus the mean 01/PEME DNA copies per mg tumor. C, data points from measured responses versus virus DNA in the tumor were plotted.

of ~1 × 10^7 01/PEME DNA copies/mg. Interestingly, even the 1 × 10^8 particles dose, which had no antitumor efficacy, eventually reached the same plateau as the 1 × 10^10 particles dose. The basis for the plateau of ~1 × 10^7 DNA copies/mg tumor is not understood, but doses of virus that were not efficacious achieved the same concentration of DNA copies/mg tumor as doses that inhibited tumor growth. These data suggest that the initial concentration of virus DNA distributed to the tumor strongly correlated with the degree of tumor control.

By directly comparing the i.v. and intratumoral routes of administration in the C33A model, we observed dose-dependent antitumor efficacy and dose-dependent delivery of the virus to the tumor. Using C33A as an additional tumor model corroborated the observations that were made in the studies with PC3 tumor model. In this study we used a single administration so that we could measure the distribution of the virus to the tumor site without the complication of the increases in DNA copies because of replication. For those doses that had antitumor efficacy, there was a good correlation between the measurement of the delivered dose to the tumor and antitumor efficacy that seemed to be independent of the route of administration.

Although replication of 01/PEME was detected in the tumor at all of the dose levels, it appeared that the quantity of 01/PEME virus initially distributed to the tumor correlated with the degree of tumor control. We might predict that complete tumor control should occur even at very low doses if viruses were able to replicate in tumor cells and then infect nearby tumor cells through multiple rounds of replication. However, because there was a dose response based on initial dose, there must be some mechanism that limits the spread of virus throughout the tumor or perhaps the rate of tumor growth may exceed virus replication. Alternatively, the observed cellular infiltrate that colocalized with virus-infected cells may balance or abrogate viral replication. In the future, understanding the kinetics of virus replication and cell proliferation in the tumor may permit the design of dosing regimens to enhance the oncolytic effects of virotherapy.

As virotherapies develop in the clinic, evaluating the initial distribution of the virus to the tumor site will likely be one of the best bioanalytical indicators of clinical response. On the basis of these preclinical models, it will be necessary to distribute sufficient quantities of the virus to control the tumor. The concentration of 01/PEME DNA copies in the tumor increased >100-fold from 3 h to 2 days after treatment. However, we observed increases in virus DNA copies with doses that did not inhibit tumor growth in the time course of these studies. Therefore, it will be necessary to assess virus distribution very close in time to the initial administration to distinguish the administered virus DNA from replicated virus DNA. A number of factors may influence the outcome of therapy based on predictions from virus concentration in the tumor such as tumor size, tumor site, tumor type, and immune responses. Efforts to increase distribution to tumor after i.v. administration have included modifications of the virus using tumor-targeted ligands and/or masking with polymers (27, 28). These strategies attempt to limit uptake in the liver, hide from the immune system, or re-target the adenovirus to the tumor. Whereas changing the transduction of adenovirus in cell culture has been achieved, successful implementation of these strategies in animal models with i.v. administration has not been demonstrated as yet. Future iterations of virotherapies should continue to strive toward improving pharmacologic properties to increase the distribution of the virus to tumor sites.

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