Macrophage Depletion Combined with Anticoagulant Therapy Increases Therapeutic Window of Systemic Treatment with Oncolytic Adenovirus

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Abstract

Liver tropism of systemically delivered adenoviruses (Ad) represents a considerable challenge for their use as anticancer therapeutics. More than 90% of i.v. injected Ad is rapidly taken up by the liver leading to hepatotoxicity, reduced virus uptake by target tumor tissue, and diminished therapeutic efficacy. The lack of clinical activity of systemically given oncolytic Ad demands for better understanding and improvement of virus pharmacokinetics. We studied the effects of Ad “detargeting” from liver macrophages (Kupffer cells) and hepatocytes on toxicity and anticancer efficacy using a nonattenuated oncolytic Ad expressing enhanced green fluorescent protein–luciferase fusion protein (Ad-EGFP-Luc). Kupffer cell depletion before i.v. injection of Ad-EGFP-Luc increased transgene expression in the liver 40.7-fold on day 3 after the injection indicating compensatory enhancement of hepatocyte transduction due to increased bioavailability of the virus. Pretreatment of mice with the anticoagulant drug warfarin to block blood factor–dependent binding of the virus to hepatocytes markedly reduced luciferase expression in the liver and mediated the corresponding decrease of hepatotoxicity in mice with intact and depleted liver macrophages. Combined depletion of Kupffer cells and pretreatment with warfarin before a single i.v. injection of Ad-EGFP-Luc significantly reduced tumor growth and prolonged survival of nude mice bearing subcutaneous xenografts of aggressive human hepatocellular carcinoma. The improved antitumor activity correlated with enhanced transgene expression and virus spread in the tumors. These data suggest that detargeting oncolytic Ad from liver macrophages and hepatocytes is an effective strategy to increase the therapeutic window for therapy against disseminated tumor sites. [Cancer Res 2008;68(14):5896–904]

Introduction

Oncolytic viruses are a novel class of self-amplifying therapeutic agents with anticancer activity derived from the natural ability of viruses to replicate in and kill cells (1–4). Cancer-selective replication of genetically engineered oncolytic viruses amplifies initial viral dose inside the tumor. Spread of virus progeny results in farther infection and lysis of an increasing number of cancer cells. The first engineered oncolytic adenovirus (Ad) was recently approved for treatment of head-and-neck cancer patients through intratumoral (i.t.) administration in combination with chemotherapy (5).

Treatment of metastatic or inaccessible tumors requires systemic delivery of an anticancer agent. To date, clinical trials with systemically given oncolytic Ad have not shown significant anticancer efficacy (6). This route of administration proved to be challenging for Ad as >90% of systemically delivered dose is rapidly taken up by the liver (7, 8). The massive loss of virus to the liver reduces the virus uptake by tumors and diminishes therapeutic efficacy. In addition, Ad infection of hepatocytes results in dose-dependent hepatotoxicity (9).

Kupffer cells are the resident macrophages of the liver that represent the first line of host defense against circulating pathogens (10). The vast majority of i.v. injected Ad is taken up and destroyed by Kupffer cells within 24 hours after injection (7). Ad interaction with platelets (11, 12) was recently implicated as a mechanism of the virus sequestration by Kupffer cells (13). Ad doses of 1011 viral particles (vp)/kg or higher cause rapid necrosis of Kupffer cells after systemic administration, preventing further uptake of second Ad dose by these cells (14). Depletion of Kupffer cells was found to increase transduction of hepatocytes with i.v. injected Ad (15–19).

Recently, the role of blood factors as mediators of Ad transduction of hepatocytes was shown (20). It was proposed that blood coagulation factors IX and X and complement protein C4BP bind to Ad capsid and allow for hepaticocyte infection through cell surface heparin sulfate proteoglycans or low-density lipoprotein receptor–related protein (21, 22). Anticoagulant treatment with warfarin that reduces plasma concentration of vitamin K–dependent blood factors was shown to decrease levels of transgene expression in the liver after i.v. administration of Ad (23). Total virion accumulation in the liver, however, was not changed at early time points, suggesting that Ad uptake by Kupffer cells, which is not resulting in significant transgene expression, is not dependent on blood factors.

Given that the liver is a major organ sequestering i.v. injected Ad, in this study, we have tested the utility of detargeting oncolytic Ad from the liver. We hypothesized that depletion of Kupffer cells and anticoagulant treatment will result in detargeting of i.v. injected Ad from both liver macrophages and hepatocytes and therefore will reduce hepatotoxicity and increase antitumor efficacy of single i.v. injection of oncolytic Ad.

Materials and Methods

Cell culture. Human cancer cell lines LNCaP (prostate carcinoma) and Hep3B (hepatocellular carcinoma) were purchased from American Type Culture Collection. Human embryonic kidney cells HEK 293 were from...
Liver Detargeting Improves Systemic Efficacy of Ad

Figure 1. Oncolytic Ad-EGFPLuc transduces LNCaP tumors, spreads, and inhibits the tumor growth after i.v. administration. A, schematic of Ad-EGFPLuc genome. Open arrows, early Ad regions; closed arrows, late Ad regions; gray or green arrow, inserted transgenes; closed boxes, inverted terminal repeats (ITR). Ad-EGFPLuc is a replication-competent Ad vector that overexpresses AdP (E3-11.6K) for increased spread and expresses EGF-firefly luciferase fusion protein for imaging virus transduction, spread, and persistence. B, nude mice with established s.c. LNCaP tumors (average tumor volume, 280 mm³) were injected with 3 × 10¹⁰ vp of Ad-EGFPLuc on day 0. At days 1, 8, and 20, tumor size was measured (left; n = 3) and luciferase expression was quantified in the excised tumors (right; n = 3). C, representative bright field and fluorescence microphotographs of tissue sections of the tumors from B. Original magnification, 20x. Area enclosed in dotted line, areas of EGFP expression.

Microbix. All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; HyClone). Suspension NS3 cells (Microbix) and KB cells (kindly provided by WSM Wold) were maintained in HyQ MEM/EBSS (HyClone) supplemented with 10% FBS and were used for propagation of replication-defective Ad-DsRed-RD or replication-competent Ad-EGFPLuc, respectively.

Viruses. Ad-DsRed-RD and Ad-EGFPLuc-RD are E1 and E3 deleted replication-defective Ad type 5 (Ad5) viruses that have cytomegalovirus (CMV) expression cassettes in the place of E1 deletion to express either DsRed or EGFP-Luc, a fusion protein of the enhanced green fluorescent protein (EGFP) and firefly luciferase (Clontech). Ad-EGFPLuc is a derivative of Ad5 that has wild-type (wt) E1 and that has the immunomodulatory E3 genes deleted. Ad-EGFPLuc overexpresses the Ad Death Protein (ADP; E3-11.6K) for improved virus spread (24). EGF-firefly luciferase fusion gene (EGFPLuc) under the control of CMV promoter (Clontech) was inserted into HpaI site in the E1 region of Ad5 in pXC1 (Microbix) downstream of E1A open reading frames and upstream of E1A polyadenylation signal resulting in early and late expression of the transgene (data not shown). The resulting plasmid pKD855 was cotransfected with pBHGD3E3 (kindly provided by Jackson Laboratory).

Animals. Outbred ICR female mice (6–9 wk old) were purchased from National Cancer Institute. Female nude mice (4–6 wk old) were purchased from Harlan Sprague-Dawley. Female C57BL/6 mice (4–6 wk old) were from National Cancer Institute. Female nude mice (4–6 wk old) were purchased from Harlan Sprague-Dawley. Female C57BL/6 mice (4–6 wk old) were from Harlan Sprague-Dawley.

Ad predosing and warfarin pretreatment. To deplete Kupffer cells, mice were injected i.v. with 3 × 10¹⁰ vp of Ad-DsRed-RD in 100 µL of PBS 4 h before Ad-EGFPLuc injection (18). Anticoagulant treatment was done as described previously: mice were injected s.c. with 133 µg of warfarin (Sigma-Aldrich) in 100 µL of peanut oil 3 and 1 d before Ad injection (22).

Bioluminescence imaging. Mice were anesthetized with ketamine and xylazine and injected i.p. with 100 µL of α-luciferin (20 mg/mL; Molecular Imaging Products) and were imaged on Kodak In vivo F system (Kodak) for 5 min. Images were processed and analyzed using Kodak Imaging Software (Kodak).

Hepatotoxicity studies. Mice were injected i.v. via the tail vein with 3 × 10¹⁰ vp of Ad-EGFPLuc on day 0. Blood was collected at day 3 and analyzed for serum levels of alanine aminotransferase (ALT) by colorimetric end point reaction method according to manufacturer's instructions (Biotron Diagnostics).

LNCaP tumor model. LNCaP tumors were established s.c. in the hind flank of nude mice by injecting 1 × 10⁶ LNCaP cells in 100 µL of medium containing 50% Matrigel (BD Biosciences). At day 54 after cell injection (average tumor volume, 280 mm³) mice were injected i.v. via the tail vein with 3 × 10¹⁰ vp of Ad-EGFPLuc in 100 µL of PBS. Tumor dimensions and luciferase expression were measured at days 1, 8, and 20 after virus injection. At each time point, three animals were euthanized and excised tumors were frozen in optimal cutting temperature (OCT) compound (Sakura Finetechncial). Tumor sections with a thickness of 7 µm were prepared and analyzed for EGFP expression by fluorescent microscopy.

Hep3B tumor model. Hep3B tumors were established s.c. in the hind flank of nude mice by injecting 5 × 10⁶ Hep3B cells in 100 µL of medium containing 50% Matrigel. At 19 d later, the mice with Hep3B tumors were randomized and assigned into groups with an average tumor volume of 187 mm³. The animals received single i.v. injection via the tail vein of PBS or 3 × 10¹⁰ vp of Ad-EGFPLuc in 100 µL of PBS (n = 6 or 7). Some animals were pretreated with warfarin and/or predosed with 3 × 10¹⁰ vp of Ad-DsRed-RD. Tumor dimensions were measured twice a week with a caliper, and tumor volumes were calculated as width² × length × 0.5. Mice were euthanized when the tumor volume reached 2,000 mm³.

In a second experiment, subcutaneous Hep3B tumors were established and mice were injected i.v. via the tail vein with 3 × 10¹⁰ vp of Ad-EGFPLuc on day 21 after cell injection (average tumor volume, 358 mm³), some mice were pretreated with warfarin and/or predosed with 3 × 10¹⁰ vp of Ad-DsRed-RD. Mice were euthanized at day 29 after Ad-EGFPLuc injection, tumors were excised and frozen in OCT compound. Tumor sections of 7-µm thickness were prepared and analyzed for EGFP expression.
Real-time PCR and luciferase expression. Nude mice with Hep3B tumors (day 22 after cell injection; average tumor volume, 250 mm³) were injected i.v. with 3 × 10¹⁰ vp of Ad-EGFPLuc-RD; with/without warfarin and predosing (n = 3 or 4). At 24 h later, the tumor, spleen, and left lobe of the liver were flash frozen in liquid nitrogen and DNA was purified with Qiagen DNAeasy Blood and Tissue kits. For quantitative PCR, Qiagen Quantitect SYBR Green PCR Master Mix was used with primers against firefly luciferase (GGATTCTAAAACGGATTACCAGGG and CAGTTCTATGAGGCAGAGCGAG) and human GAPDH (GGATTCTAAAACGGATTACCAGGG and CAGTTCTATGAGGCAGAGCGAG). Standard curves were set up using plasmid DNA containing the EGFPLuc expression cassette “spiked” into purified DNA from the same organs of an untreated mouse. Real-time PCR was performed with an ABI 7900HT in absolute quantification mode, and genome quantity was determined with ABI analysis software.

To quantify luciferase expression, organs were lysed using Reporter Lysis 5× buffer and luciferase activity was measured using Luciferase Assay System (Promega). Protein concentration was determined using BCA Protein Assay kit (Pierce).

Statistical analyses. Data are presented as mean ± SE. Statistical analyses were performed using SPSS software. The statistical significance was assessed using General Linear Model followed by Tukey’s HSD test for pairwise comparisons between groups. Survival data were analyzed with the log-rank test. P < 0.05 was considered significant.

Results

Systemic antitumor activity of oncolytic Ad in a prostate cancer model. It was shown previously that antitumor activity of replication-competent Ad vectors was improved by overexpression of ADP (E3-11.6K) due to enhanced vector spread both in cell culture and in vivo after direct i.t. injections (28–30). For studying the anticancer activity of systemically delivered Ad, we constructed a nonattenuated oncolytic Ad5 that overexpresses ADP. This virus was modified to express the EGFPLuc reporter protein to enable imaging of the vector distribution, spread, and persistence (Fig. 1A).

It has been shown previously that replicative Ad expressing E3 genes, including the gene for ADP, spread efficiently in LNCaP tumors (31). We studied whether Ad-EGFPLuc virus, which has E3 region engineered to overexpress ADP but lacks other E3 genes, would spread efficiently in LNCaP tumors after systemic i.v. delivery. A single dose of 3 × 10¹⁰ vp of Ad-EGFPLuc was injected i.v. into mice bearing established subcutaneous LNCaP tumors. The tumors were excised at three time points after virus injection, and tumor volumes and luciferase expression were measured. We found that tumor volumes did not change at day 8 after virus injection (P = 0.87) but decreased at day 20 (P = 0.04) compared with tumor volumes at day 1 (Fig. 1B, left). Low levels of luciferase expression were detected in the tumors at day 1 after the virus injection. Luminescence signal was slightly, but not significantly, increased at day 8 (P = 0.17) and dramatically increased at day 20 (P = 0.04) relative to the levels detected at day 1 (Fig. 1B, right). The inverse correlation between tumor volume and the level of luciferase expression suggested that tumor regression was caused by cell lysis due to intratumoral replication and spread of Ad-EGFPLuc.

Tumor sections were analyzed for EGFP expression (Fig. 1C). We found small areas of dim EGFP expression in tumor sections at day 1. EGFP expression was brighter and occupied larger areas of the tumors at day 8. At day 20, intense EGFP signal was detected over entire tumor section demonstrating efficient spread of the virus in this tumor model.

Anticoagulant treatment detargets Ad from hepatocytes and reduces liver toxicity. Replication-competent Ad5 cause dose-dependent hepatotoxicity mediated by virus replication in the animal models and humans (9, 32–35). Given that the anticoagulant warfarin inhibits blood factor–dependent infection of hepatocytes by Ad5 (22, 23), we tested whether warfarin could reduce hepatocyte transduction and toxicity produced by oncolytic Ad-EGFPLuc (Fig. 2). Nude mice bearing s.c. LNCaP tumors were pretreated with warfarin or the vehicle followed by i.v. injection of 3 × 10¹⁰ vp of Ad-EGFPLuc. Imaging of luciferase expression was performed on day 3. Blood from mice in A was collected at day 3 after virus injection and analyzed for serum ALT levels (n = 4).
i.v. injection of Ad-EGFPluc into immunocompetent C57BL/6 mice ($P < 0.01$, data not shown) suggesting that the detargeting effect of warfarin on Ad was not mouse strain–specific.

Warfarin efficiently restores hepatocyte transduction with Ad-EGFPluc in mice depleted of Kupffer cells. Previous studies showed that saturation of Kupffer cells with i.v. injected Ad prevents uptake of subsequent Ad dose by these cells (18). We studied the effect of Kupffer cell depletion on the kinetics of luciferase expression by Ad-EGFPluc by “predosing” the mice i.v. with $3 \times 10^{10}$ vp of replication-defective Ad-DsRed-RD 4 h before Ad-EGFPluc. Imaging at day 1 after i.v. Ad-EGFPluc injection showed that predosing increased luciferase expression in the livers 7.7-fold relative to control mice that were not predosed with Ad-DsRed-RD ($P = 0.03$; Fig. 3A and B). Interestingly, luciferase expression increased in Ad-predosed group at day 3 compared with expression level at day 1 ($P = 0.04$). In contrast, the already lower luciferase activity in nonpredosed mice decreased over 3 d ($P = 0.05$). These differing expression kinetics resulted in overall 40.7-fold difference between the control and predosed groups at day 3 ($P < 0.01$).

This suggested that predosing prevented subsequent uptake of injected Ad-EGFPluc by Kupffer cells, allowing more virions to infect hepatocytes. Whereas this might release more virions to infect tumor cells, diverting virus into hepatocytes might also increase liver damage. To test this and to determine if warfarin could protect hepatocytes, mice were predosed and pretreated with warfarin or vehicle before Ad-EGFPluc injection. Luciferase imaging showed strong luminescence signal in the livers of all mice that were not pretreated with warfarin (Fig. 3C). In contrast, five of six mice pretreated with warfarin had undetectable levels of luciferase. When serum ALT was measured on day 3, the levels in warfarin-treated mice were not significantly different from those of mock-treated mice ($P = 0.59$; Fig. 3D). As expected, Ad-EGFPluc injection increased ALT levels significantly (Ad-EGFPluc versus Mock; $P = 0.01$) and warfarin significantly reduced this ALT increase (Ad-EGFPluc versus Ad-EGFPluc + warfarin; $P = 0.02$) to near control levels (Ad-EGFPluc + warfarin versus Mock; $P = 0.18$).

Ad predosing before Ad-EGFPluc injection produced slightly higher, but not significantly increased ALT levels relative to mice injected with Ad-EGFPluc alone ($P = 0.12$). Warfarin again significantly reduced ALT levels in the combination treatment group (Ad-EGFPluc + predosing versus Ad-EGFPluc + predosing + warfarin; $P < 0.01$). These levels were not significantly different from the levels in mock-treated mice (Ad-EGFPluc + predosing + warfarin versus Mock; $P = 0.10$). Effects of warfarin and predosing on transduction by replication-competent Ad-EGFPluc in a mouse model of human hepatocellular carcinoma. We examined the effects of Kupffer cell depletion and anticoagulant treatment on distribution and persistence of Ad-EGFPluc after systemic administration into nude mice bearing s.c. established Hep3B tumors. Unlike LNCaP tumors that are readily killed by oncolytic Ad, Hep3B tumors are rarely cured even by multiple direct i.t. virus injections (9, 36, 37). Therefore, this model could allow assessing if predosing and pretreatment with warfarin mediate improvements in systemic oncolytic therapy.

Mice bearing s.c. Hep3B tumors were treated with or without predosing and with or without warfarin and then given a single i.v. injection of $3 \times 10^{10}$ vp of Ad-EGFPluc (Fig. 4). Imaging of luciferase expression at day 3 after virus injection showed high luminescence signal mostly in the livers of Ad-EGFPluc–injected mice, but with some signal in tumors (Fig. 4A). Warfarinpretreatment reduced luciferase expression in the liver 7.3-fold ($P = 0.04$). Predosing significantly increased luminescence in the liver relative to Ad-EGFPluc–injected mice (2.2-fold; $P = 0.02$). Warfarin pretreatment was able to reduce liver infection in predosed mice 5.8-fold ($P < 0.01$) and produced distinct luminescence signal in the tumors of all animals.

Luciferase levels in the liver were highest on day 3 after virus injection and decreased in all groups by days 7 to 10 (Fig. 4B). There was no detectable luminescence in the liver on day 29 after injection (data not shown), suggesting clearance of the virus from this organ below detection by imaging.
Anticancer activity of a single i.v. injection of Ad-EGFPLuc is improved by depletion of liver macrophages combined with warfarin pretreatment. Hep3B cells form aggressive, fast-growing tumors in nude mice and are a rigorous model to assess the anticancer activity of oncolytic Ad (9, 36, 37). To test utility of predosing and warfarin, mice bearing s.c. established Hep3B tumors were pretreated with warfarin or vehicle and predosed with Ad-DsRed-RD or vehicle before injection of $3 \times 10^{10}$ vp of Ad-EGFPLuc and the tumor growth was monitored. Mean tumor volume for each group is shown in Fig. 5A. At day 21 after injection, the last day when all animals in each group had tumors of <2,000 mm$^3$ volume, only mice in Ad-EGFPLuc + predosing + warfarin group had significantly smaller tumors compared with those from buffer-treated group ($P = 0.04$; Fig. 5B). There was no statistically significant difference in tumor volumes between other groups ($P > 0.05$). Up to day 34, mice treated with Ad-EGFPLuc + predosing + warfarin had significantly smaller tumors relative to Ad-EGFPLuc + predosing group ($P = 0.01$). Treatment with Ad-EGFPLuc + warfarin produced intermediate antitumor activity with tumor volumes not significantly different from those in either Ad-EGFPLuc + predosing ($P = 0.15$) or Ad-EGFPLuc + predosing + warfarin ($P = 0.33$) groups.

To assess the long-term effects of the studied interventions, we analyzed the differences between survival times in the treated groups (Fig. 5C). Warfarin pretreatment alone did not have an effect on survival of the mice compared with survival of buffer-treated mice ($P = 0.44$). Treatment with Ad-EGFPLuc alone increased survival time relative to that of buffer + warfarin-treated group (median survival 48 days versus 34 days; $P = 0.02$) but this was not significantly different from the overall survival of buffer-treated mice (median survival 48 days versus 24 days; $P = 0.24$). Combination of Ad-EGFPLuc with predosing and warfarin produced significantly prolonged survival compared with Ad-EGFPLuc treatment alone ($P = 0.02$). In contrast, treatment with Ad-EGFPLuc + warfarin or Ad-EGFPLuc + predosing did not increase survival relative to Ad-EGFPLuc treatment [median survival time, 48 days versus 48 days ($P = 0.95$) and 48 days versus 48 days ($P = 0.21$), respectively]. Ad-EGFPLuc + predosing + warfarin treated mice had prolonged survival compared with that of Ad-EGFPLuc + warfarin treated group ($P = 0.05$); however, the difference with survival of Ad-EGFPLuc + predosing treated group was not significant ($P = 0.31$).

Spatial distribution of the virus inside Hep3B tumors. Mice with established Hep3B tumors were pretreated with warfarin or vehicle and predosed with Ad-DsRed-RD or vehicle before i.v. injection of Ad-EGFPLuc. Tumors were excised at day 29 after virus injection and analyzed for EGFP fluorescence. Tumors treated with Ad-EGFPLuc had small scattered areas of dim EGFP expression (Fig. 6A). Treatment with Ad-EGFPLuc and warfarin produced more areas infected by the virus; however, they were isolated from each other. In contrast, treatment with Ad-EGFPLuc + predosing with or without warfarin resulted in intense EGFP expression distributed over the entire tumor sections. The difference in fluorescence patterns suggests that predosing may either deliver more virus to the tumor initially or increase intratumoral spread and persistence of i.v. delivered oncolytic Ad.

Effects of warfarin and predosing on transduction by replication-defective Ad in a mouse model of human hepatocellular carcinoma. Mice are generally not thought to support replication of Ad5 well. Therefore, relative distributions of replication-competent Ad-EGFPLuc may favor detection of the virus in the tumor versus other sites. Whereas it is true that mice are not perfect models, previous work has shown that Ad5 actually does replicate in mouse liver (32–34). Indeed, virus titers are observed to increase 10 to 100,000-fold after i.v. injection (32). Therefore, comparison of liver and tumor virus distribution has
some merit in mouse models. To better compare the effects of warfarin and predosing on tumor targeting, we repeated the experiment in Hep3B tumor-bearing mice, but in this case injected replication-defective Ad-EGFP-Luc-RD to correct for the effects of virus amplification in the liver and tumor (Fig. 6B). Mice were injected with Ad-EGFP-Luc-RD and tissue and tumor samples were harvested 24 hours later for real-time PCR of viral genomes and luciferase activity. Consistent with previous reports on early time points (14, 23), warfarin and predosing had only modest effects on viral genome deposition in the liver. Viral genomes in the spleen were also generally similar. In contrast, both predosing and warfarin individually increased viral genomes in the tumor. When viral delivery was assessed by luciferase assay, similar results were observed. Liver and spleen transduction generally mimicked genome distributions with the exception that warfarin markedly reduced luciferase activity relative to the other groups. Luciferase activity in the tumor also generally paralleled genome distributions by the different treatments, with highest levels in the warfarin alone and predosing alone groups and lower activity in the combined group. These data suggest that predosing or warfarin by

Figure 5. Anticancer activity of single i.v. injection of Ad-EGFP-Luc is improved by Ad predosing and pretreatment with warfarin. A, nude mice bearing established Hep3B tumors (average tumor volume, 187 mm$^3$) were i.v. injected once with $3 \times 10^{10}$ vp of Ad-EGFP-Luc with or without prior predosing and/or warfarin pretreatment ($n = 6$ or 7). Tumor volumes were measured twice a week. B, average tumor volumes are shown up to the last day when all animals were alive in a group. Mice were euthanized when tumor volume reached 2,000 mm$^3$. C, Kaplan-Meyer survival curves for mice from A were plotted.
themselves both increase virus delivery to tumors after i.v. injection. When the two were combined, viral genomes and transduction were not as high as in the individual treatment groups, perhaps due to more complex and competing effects on systemic distribution at this short time point. Given that the combined treatment was most effective at enabling the replication-competent virus to control tumor growth, it may well be that the two interventions enhance virus deposition over time periods after the 24-hour time point.

Discussion

Work has been done to increase oncolytic potency of the vectors by arming them with therapeutic genes and has produced promising results (30, 37–41). However, most of the preclinical and clinical studies with oncolytic Ad have been performed by direct i.t. injection. Systemic administration of oncolytic Ad to treat metastatic tumors poses more challenges due to a nonfavorable biodistribution profile of the vectors with a majority of the injected dose being sequestered by the liver (7). The liver tropism of Ad results in significant nontarget loss of injected bolus and cause dose-dependent hepatotoxicity (9). Engineering Ad with cancer-specific features allows for reduced liver toxicity (42). However, results from clinical trial with i.v. given prostate-specific oncolytic Ad CG7870 showed liver damage as evidenced by grades 2 to 3 transaminitis in patients receiving highest dose of CG7870 (35). Additionally, systemic virus leakage from the tumor after

Figure 6. Ad distribution. A, Ad predosing increases intratumoral spread of replication-competent Ad-EGFPLuc. Nude mice bearing s.c. Hep3B tumors were i.v. injected with $3 \times 10^{10}$ vp of Ad-EGFPLuc with or without prior predosing and with or without pretreatment with warfarin. Mice were euthanized on day 29 after virus injection and bright field and fluorescence microphotographs of tumor tissue sections were taken. Original magnification, 20×. Area enclosed in dotted line, areas of EGFP expression. B, effects of warfarin pretreatment and/or predosing on biodistribution of replication-defective Ad-EGFPLuc-RD. Nude mice bearing subcutaneous Hep3B tumors were injected i.v. with $3 \times 10^{10}$ vp of Ad-EGFPLuc-RD with or without prior predosing and with or without pretreatment with warfarin. Mice were euthanized 24 h after virus injection and virus genomes and luciferase activity were quantified in the liver, spleen, and tumor.
i.t. injection was shown (43–45). These results underscore the need for additional strategies capable of decreasing hepatotoxicity of oncolytic Ad viruses.

Given the need to treat metastatic cancer and the problems of hepatotoxicity, this study tested approaches to mitigate uptake of oncolytic Ad in the liver. Kupffer cells are in large part responsible for Ad clearance from the blood (7). Previous work showed that saturating Kupffer cells with a “predose” of Ad allows subsequent injections of virus to evade these phagocytic cells to more effectively transduce hepatocytes (18). We hypothesized that predosing might also free up oncolytic Ad after i.v. injection to allow more virus to reach distant tumor sites. Whereas this might be useful, this also was likely to increase liver damage. Given this and recent observations that Ad transduction of hepatocytes is due to virus binding to coagulation factors (20), we tested the hypothesis that depletion of Kupffer cells combined with anticoagulant pretreatment could reduce hepatotoxicity, increase virus delivery to distant tumors, and increase antitumor efficacy of systemically given oncolytic Ad.

We tested this with a fully replication-competent Ad named Ad-EGFPLuc that expresses EGFPLuc reporter to track virus distribution. This virus is oncolytic (i.e., cancer killing), but is not genetically engineered for more cancer selectivity than native Ad. Therefore, it provokes both tumor killing and hepatocyte damage, making it a challenging virus to test the hypothesis. Predosing alone with replication-defective Ad before i.v. injection of Ad-EGFPLuc increased transgene expression in the liver 40-fold at day 3 after the injection, but also increased liver damage. Warfarin treatment alone or with predosing to block blood factor–mediated hepatocyte infection markedly reduced liver transduction and liver damage. When Ad and these interventions were tested in the context of treating aggressive Hep3B tumor model, predosing and warfarin showed significant effects. Whereas single i.v. injection of Ad-EGFPLuc did not show significant antitumor efficacy, combination of Ad-EGFPLuc with predosing and warfarin significantly reduced tumor growth kinetics and prolonged survival of the treated mice.

These data suggest that predosing and inhibition of blood factor binding may have utility to improve virus delivery to distant tumors after systemic virus therapy. The original hypothesis was that predosing would release more virus from Kupffer cells and that preventing their uptake in hepatocytes with warfarin would increase the amount that could be delivered to tumors. Consistent with this, depletion of Kupffer cells has previously been shown to increase the half-life of i.v. injected Ad (8). When virus distribution was tested with replication-defective Ad-EGFPLuc-RD, only modest effects were observed on virus distribution to the liver and spleen. This is consistent with previous work that has shown that either predosing or warfarin alone have only weak effects on reducing total viral genome delivery to the liver (14, 23). One explanation for this is that not all virus reservoirs have been identified. For example, other components of mononuclear phagocyte system, such as hepatic sinusoidal endothelial cells and spleen macrophages (19), may represent other virus reservoirs that may be revealed as we block uptake into Kupffer cells and hepatocytes.

While we did not observe substantial changes in the amounts of virus in nontumor sites, both real time PCR and luciferase activity at 24 hours after injection showed that warfarin or predosing increased virus delivery to the tumors after i.v. injection. These data suggest that detargeting liver hepatocytes and Kupffer cells can enhance tumor targeting. This is consistent with the observation that depletion of peripheral macrophages with clodronate liposomes increased intratumoral titers of herpes simplex virus and improved antiglioma activity of the virus (46). Whereas the single agents increased virus titers in tumors, the combination of predosing and warfarin did not. This may reflect the small number of animals tested or the complexity of virus distribution by replication-competent versus replication-defective Ad in the mice. In particular, testing the distribution of replication-defective virus at an early time point of 24 hours cannot capture the effects that combined predosing and warfarin may have on the movement of progeny virions that are produced after this time point in the liver or the tumor. Nevertheless, the fact that predosing and warfarin mediate increased tumor kill and survival indicate the merits of combining these detargeting strategies.

In our tests, we used Ad itself for predosing as this has known abilities to deplete Kupffer cells. Other predosing approaches that have been tested for Ad include the use of gadolinium chloride or clodronate liposomes. Whereas both are effective in animals, neither is approved for use in humans and are therefore an impediment to translating this approach into the clinic. Ad itself may be the simplest predosing agent because one might be able to deliver the same virus in two bolus injections: the first to deplete Kupffer cells and the second for distribution to tumors. Alternatively, there are a number of contrast agents that are taken up by Kupffer cells that are in use in humans for magnetic resonance imaging and ultrasound imaging that may be able to block Kupffer cell uptake. If these prove able to block Kupffer cell uptake of Ad, they may provide an alternate clinically relevant method to apply Kupffer cell detargeting approaches for systemic therapy with oncolytic Ad.

In this study, we show for the first time that warfarin pretreatment reduces hepatotoxicity of i.v. injected oncolytic Ad in normal and Kupffer cell depleted mice. As warfarin is currently in rampant use in humans, this intervention is clinically relevant and may also be useful in mitigating liver toxicity during oncolytic therapy or perhaps even during Ad-mediated hepatitis after transplantation. Given that warfarin markedly reduced liver toxicity by fully replication-competent virus, we expect that detargeting other genetically targeted oncolytic Ads (9) from hepatocytes with warfarin or other approaches will also decrease their toxicity and provide an even wider therapeutic window for these viruses. It will be interesting to test if Ad vectors bearing capsid modifications that block blood factor binding (47, 48) can mimic the warfarin effect on tumor killing. Likewise, it will be interesting to test if cell-targeted Ads bearing capsid targeting modifications (49, 50) can mediate better tumor killing when hepatocyte infection is inhibited and when the animals are depleted of Kupffer cells.

In conclusion, we show that depletion of Kupffer cells allows for increased anticancer activity of a single i.v. injection of oncolytic Ad. Combination of this treatment with anticoagulant therapy increased antitumor efficacy and decreased Ad-mediated hepatotoxicity leading to an improved therapeutic window of the treatment.

Disclosure of Potential Conflicts of Interest

K. Doronin: coinventor on patent owned by Saint Louis University for E3-11.6K (ADP) overexpression.
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References
Macrophage Depletion Combined with Anticoagulant Therapy Increases Therapeutic Window of Systemic Treatment with Oncolytic Adenovirus

Elena V. Shashkova, Konstantin Doronin, Julien S. Senac, et al.


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