Biodistribution and kinetics of the novel selective oncolytic adenovirus M1 after systemic administration

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Abstract
Oncolytic adenoviruses represent a promising novel therapeutic option for the treatment of cancer. Despite their demonstrated safety in human clinical trials, the fundamental properties of oncolytic adenovirus biodistribution, spread, viral persistence, and replication in vivo have not been well characterized. The aim of this study was to evaluate the kinetics of viral distribution, spread, replication, and antitumoral efficacy after i.v. administration of a novel oncolytic mutant M1. This mutant consists of the E1A CR2-deleted Adv5 with a fragment of antisense polo-like kinase 1 (plk1) cDNA inserted into the deleted 6.7K/gp19K region, which combines oncolytic properties with efficient plk1 silencing, as described in our previous reports. In the present study, we established a new human orthotopic gastric carcinoma with a high frequency metastasis mouse model and showed that M1 spread not only in local primary tumors but also in disseminated metastases. M1 could effectively replicate in tumor cells leading to “oncolysis” and was able to eliminate expression of the targeted gene plk1 in human orthotropic gastric carcinoma model mice. Therefore, i.v. administration of M1 could prolong the survival time of tumor-bearing mice. [Mol Cancer Ther 2008;7(6):1624–32]

Introduction
Oncolytic adenoviruses, with the favorable feature of multiplying preferentially in malignant cells, represent a promising new platform for the treatment of cancer (1, 2). Despite the rapid placement of systematic administration of oncolytic adenoviruses in human clinical trials and its demonstrated safety, direct intratumoral injection, which severely limits the potential of oncolytic adenoviruses in vivo, is now still the major administration route of adenovirus therapy in clinical use (3–7). Systematic administration of therapeutic adenoviral agents, on the other hand, is not generally accepted because several fundamental issues remain to be further evaluated. (a) Can the oncolytic adenovirus reach, spread, and effectively replicate in the local primary tumor and disseminated metastases after systematic administration? (b) What is the kinetics of viral biodistribution in vivo? (c) Could the oncolytic adenovirus display the anticipated antitumoral efficacy when used as single agent? Efforts have been taken to develop tools for monitoring the performances of conditionally replicative adenoviruses in vivo (8–10). For example, extracting DNA from peripheral blood and running PCR has been widely used to detect viremia, which indirectly reflects adenovirus replication in vivo; however, this approach fails to determine the distribution of adenoviruses in tissues (11). Others have exploited the reporter gene expression technique to precisely detect adenoviral replication in vivo; however, reporter genes can only provide indirect information with respect to virus replication and localization based on transgene expression, which hardly reflects the real situation of the oncolytic adenovirus in vivo. Therefore, the aim of this study was to use a new in situ histology method, which can be directly extracted from the viral mass, to measure the real level of virus accrualment from the initial administration and to determine the biodistribution, replication, and antitumoral efficacy after i.v. administration of an oncolytic mutant.

Previously, we constructed a novel oncolytic adenovirus named M1 (12). M1 was derived from Adv5/dE1A. Adv5/dE1A contained a 27-bp deletion in the E1A CR2 region necessary for Rb protein binding. This modification enables Adv5/dE1A to replicate efficiently in and lyse cells with a defective G1-S checkpoint, which is seen in almost all tumor cells as a result of their loss of function of the Rb pathway. Use of Adv5/dE1A as a vector for delivery of antisense polo-like kinase 1 (plk1) cDNA conferred M1 with the combined functions of oncolysis and plk1 targeting. M1 has been shown to combine oncolysis with efficient plk1 silencing in our previous reports. In those studies, to our surprise, systematic administration of M1 plus cisplatin...
induced complete tumor regression in 80% of orthotopic hepatic carcinoma model mice that were otherwise resistant to cisplatin and disseminated metastases. The potent antitumoral efficacy in orthotopic hepatic carcinoma model mice may relate to the native tropism of adenovirus to the liver. Whether systemic administration of M1 could effectively induce tumor regression in other orthotopic carcinoma models and prevent metastases needs to be further clarified.

In the present study, we established a human orthotopic gastric carcinoma with high frequency metastasis in a mouse model. We showed that M1 spread not only in the local primary tumor but also in the disseminated metastasis. M1 effectively replicated in tumor cells leading to "oncolysis" and eliminated targeted plk1 gene expression in human orthotopic gastric carcinoma model mice, therefore prolonging the survival time of tumor-bearing mice.

Materials and Methods

Adenoviruses

M1 is an E1A CR2-deleted Adv5 with a fragment of antisense plk1 cDNA inserted into the deleted 6.7K/gp19K region (12). The replication-defective adenovirus mutant Adv-TK, containing a herpes simplex virus-thymidine kinase gene under control of a Rous sarcoma virus long terminal repeat promoter in the region of the excised E1 adenoviral genes, was constructed in this laboratory and used as a vehicle control. Adenoviruses were propagated in 293 cells (American Type Culture Collection), harvested 48 h after infection, and purified by the standard protocol of cesium chloride gradient centrifugation (13). The purified adenovirus was stored in PBS containing 10% glycerol at -80°C.

Mouse Tumor Model Studies

To ensure the uniformity of study, female athymic BALB/c mice were obtained from the Animal Experimental Center of Slaccas. Mice were used between ages 4 and 2 weeks, 4 weeks, and 6 weeks) after infusion of adenoviruses, mice were sacrificed, and the primary tumor, the liver, lung, lymph nodes in the peritoneal cavity, and other organs were harvested for virus quantification, histology, immunohistochemistry, in situ hybridization, and Western blotting. Findings were compared with model mice treated with Adv-TK and mock (PBS).

Virus Quantification

At the indicated time points (30 min, 3 days, 1 weeks, 2 weeks, 4 weeks, and 6 weeks) after infusion of adenoviruses, mice were sacrificed, and the primary tumor, the liver, lung, lymph nodes in the peritoneal cavity, and other organs were harvested for endogenous plk1 protein levels (n = 2 for each group). To address whether an early initiation of treatment could enhance the antitumoral efficacy of M1, two additional experiments were done by beginning the i.v. infusion of M1, Adv-TK, or PBS at 10 or 14 days after tumor implantation, respectively.

To further analyze the kinetics of viral distribution and spread after i.v. injection of virus in this orthotopic human gastric carcinoma model, mice were injected with 1 × 1012 vector particles/kg by tail vein daily for 5 consecutive days beginning at 10 days after implantation, and animals were sacrificed at the indicated time points. The primary tumor, as well as the liver, lung, lymph nodes in the peritoneal cavity, and other organs, were harvested for virus quantification, histology, immunohistochemistry, in situ hybridization, and Western blotting. Findings were compared with model mice treated with Adv-TK and mock (PBS).

Histology and Immunohistochemistry

Formalin-fixed tissues were processed, embedded in paraffin, sectioned at 5 μm, and stained with H&E. Immunohistochemical analyses were carried out to detect adenoviruses in situ using an anti-adenovirus mouse monoclonal antibody (MAB8052; Chemicon) according to the recommendation of the manufacturer. Briefly, slides were deparaffinized in xylene and then hydrated through graded alcohols. Endogenous peroxidase in the sections was blocked with hydrogen peroxide (0.03%). Mouse serum was added to minimize the background. A 1:200 dilution of the adenovirus antibody was then applied to the sections and incubated overnight at 4°C and the sections were then incubated with a biotinylated anti-mouse secondary antibody. Horseradish peroxidase was applied,
and diaminobenzidine/hydrogen peroxidase was used as the chromogen substrate. All slides were counterstained with hematoxylin. Negative controls were similarly processed by omitting the primary antibody. The intensity of staining was rated according to the following scale: 3, most intense immunostaining; 2, immunostaining between 1 and 3; 1, light immunostaining; 0, no staining. In each sample, the percentage of cells expressing adenovirus protein was determined. To obtain a numerical assessment of the expression of protein, we calculated the multiple of intensity of expression by the percentage of positive cells. This was termed the expression score. Thus, the expression score provided a semiquantitative assessment of the expression of adenovirus protein in a given sample. Two observers rated the samples blindly; we used the average of their scores as the final score.

**In situ Hybridization**

Slides were prehybridized for 30 min at 37°C. Hybridization was carried out overnight at 42°C with 1 µg/mL biotinylated viral fiber oligonucleotide probe complementary to the fiber coding region (5'-GGAACGCCCTTAATTGGACAGCACAGGTGCCATTACAG-3'). Negative controls were similarly processed by omitting the probe. An alkaline phosphatase–conjugated anti-biotin antibody (Boehringer Mannheim) was added to bind to the hybridized probe. The slides were then incubated with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate for 30 min and counterstained once with nuclear fast red. The evaluation of the hybridization signal was similar to that of immunohistochemistry.

**Western Blot**

Preparation of protein samples and Western blotting were done as described previously (17). Antibodies to plk1 and β-actin were purchased from Santa Cruz Biotechnology.

**Statistical Analysis**

The cumulative probability of survival was determined by the Kaplan-Meier method and the significance of differences with the log-rank test. Data are presented as mean ± SD of at least triplicate experiments, and a two-tailed unpaired t test was used to compare the statistical significance of the differences in data from the two groups. All P values were two-sided. SPSS version 11.5 software was used for statistical analysis.

**Results**

**Orthotopic Human Gastric Carcinoma Mice Model Exhibited Extensive Growth of Local and Metastatic Tumors**

We first established the orthotopic human gastric carcinoma mice model. Orthotopic implantation of the human gastric carcinoma cell line MKN-45 reached a 100% take rate for local tumor growth. Local tumor growth was rapid, and tumor volume reached 1,837 ± 804 mm³ at 6 weeks (Table 1). There was no evidence of invasive and metastatic tumor growth 2 weeks after tumor implantation. However, the frequency of local invasive growth and metastasis to local lymph nodes and liver increased significantly with time. Four weeks after tumor implantation, 6 of 8 mice had local invasive growth (Table 1). At this time point, liver metastases were detected in 4 of 8 model mice and lymph node metastases were detected in 3 of 8 mice (Table 1; Fig. 1). With the growth of local and metastatic tumors, the model mice began to die after 4 weeks. Surviving model mice showed signs of advanced clinical stages of gastric carcinoma characterized by extensive growth of local and metastatic tumors. In subsequent experiments, the model mice were employed to study the biodistribution and therapeutic outcome of M1 after systemic administration.

**Adenovirus M1 Prolongs the Survival Time of Tumor-Bearing Mice**

Previously, we have shown that M1 in combination with cisplatin showed potent antitumoral efficacy in orthotopic hepatic carcinoma model mice (12). The therapeutic outcome of i.v. administration of M1 as a single agent in metastatic tumor models is yet to be determined. Initially, we examined the antitumoral efficacy of M1 in treatment of late-stage orthotopic human gastric carcinoma mice. M1 was i.v. infused 4 weeks after orthotopic implantation of the tumor. The survival of mice was not prolonged by treatment with M1 when compared with Adv-TK or PBS control (P > 0.05; Fig. 2A). It is possible that the tumor volume at 4 weeks was so big that adenoviruses could not produce an effect, so the treatment was instead attempted 2 weeks after tumor implantation (~5 mm in diameter; Fig. 2B). Whereas all of the mice treated by PBS or Adv-TK died within 55 days following orthotopic implantation, the mice treated by M1 survived significantly longer (P < 0.05). When infusion of M1 was done 10 days after orthotopic implantation, mice survival increased significantly following treatment with M1 compared with Adv-TK or PBS (P < 0.01; Fig. 2C). However, complete tumor remission was not achieved and metastatic tumors developed in all M1-treated mice by the end of the study.

**Active In vivo Viral Replication Was Detected in Local and Metastatic Tumors after Systemic Administration of M1**

To determine whether systemic administration of M1 gives rise to efficient infection and viral replication in local and metastatic tumors, orthotopic human gastric carcinoma mice were injected by tail vein with a dose of 1 × 10¹² viral particles/kg 10 days after tumor implantation. Four weeks after the initiation of tumor implantation, local and metastatic (liver and lymph nodes) tumors were collected surgically for immunohistochemistry and in situ hybridization assays. Viral particles were readily detected in the local and metastatic (liver and lymph nodes) tumors (Fig. 3A). The i.v. route of administration of adenovirus mutants could thus give rise to efficient infection in vivo. In this experiment, Adv-TK was chosen as a control for replication-defective adenovirus mutants. In contrast to Adv-TK-treated model mice, tumor tissues from M1-treated model mice were detected with many more viral particles, which diffused throughout the entire sections examined (Fig. 3A and B). To examine in vivo replication of...
M1 in local and metastatic tumors, in situ hybridization for transcripts of viral fiber gene, an indicator of active viral replication, was done. Active viral replication was detected preferentially in local and metastatic tumor sites after i.v. injection of M1 but not Adv-TK (Fig. 3A and B). These findings showed the feasibility of using i.v. administration of M1 to target primary and metastatic tumors and showed that M1 preferentially replicated in local and metastatic tumors but not in normal tissues, allowing M1 to target metastatic diseases.

Dynamic Biodistribution of M1 after Systemic Administration in a Human Gastric Carcinoma Mice Model

To compare the viral distribution after systemic administration of M1 or Adv-TK, the levels of M1 or Adv-TK in serum and local and metastatic (liver and pelvic wall) tumors were determined at various time points after virus infusion. Local and metastatic tumors were collected at various time points and lysed to release viral particles for titer assay. The TCID$_{50}$ method was used to titer M1 or Adv-TK. The virus mutants were rapidly cleared away from the circulation, and no virus was present after 3 days (Fig. 4A). M1 and Adv-TK were readily detected in local or metastatic tumors 30 min after i.v. infusion, and the titers of virus ranged from $10^4$ to $10^6$ pfu/g tumors (Fig. 4B and C). The titers of M1 from local and metastatic tumors increased robustly after undergoing a reduction in the first 2 weeks; an average titer of $\geq 10^5$ pfu/g was detected in malignant tissues after M1 treatment, whereas the amount of Adv-TK

<table>
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<tr>
<th>Time after implantation (wk)</th>
<th>Survival rate</th>
<th>Primary tumor volume (mm$^3$)</th>
<th>Metastasis</th>
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<tr>
<td></td>
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<td>Local invasive growth</td>
</tr>
<tr>
<td>2</td>
<td>22/22*</td>
<td>76 ± 14</td>
<td>0/7 $^1$</td>
</tr>
<tr>
<td>4</td>
<td>15/15</td>
<td>978 ± 384</td>
<td>6/8</td>
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<td>6</td>
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<td>1,837 ± 804</td>
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$^1$Number of survival mice at the indicated time point/total number of mice.
$^2$Number of mice in which primary tumor growth or metastases were observed/number of mice evaluable.
gradiually decreased until almost no virus was detected at 6 weeks (Fig. 4B-D). Again, the data support the feasibility of i.v. administration of M1 in targeting primary and metastatic tumors and show that M1 preferentially replicates in local and metastatic tumors.

Selective Replication of M1 in Tumor Cells Led to Reduction of plk1 Protein Levels and Tumor Tissue Necrosis

Because active viral replication was evident 4 weeks after M1 infusion, we examined local tumors for evidence of oncolysis and levels of plk1 protein. As shown in Fig. 5A, a circle-like projection of viable tumor tissue was closely surrounded by virus-infected cells, and a significant number of virus-infected cells were seen in adjacent necrotic tissue. This suggests that the virus may spread through the tumor cells and produce an oncolytic effect and then infect adjacent viable tumor cells. Consequent depletion of tumor-associated plk1 protein was seen in the mouse model treated by i.v. injection of M1 but not Adv-TK or PBS (Fig. 5B). These findings showed the desired actions of M1: that oncolysis and plk1 targeting were actually taking place in vivo following systemic administration. The oncolysis and inhibition of plk1 protein would thus either kill metastatic tumor cells or sensitize them to other antitumoral agents and facilitate more complete eradication of tumor.

Discussion

Oncolytic adenoviral vectors represent a rapidly expanding novel option for cancer therapy. One of the first oncolytic viruses to enter the clinical trial, ONYX-015, provided evidence for both the safety and the antitumoral potential of this approach (3–5, 18–21). However, clinical application has been thwarted by poor viral distribution within the tumor and high immunogenicity, both of which limit the potential utility of oncolytic adenovirus in vivo. This has made direct intratumoral injection to be the most common administration route in clinical use and severely affects antitumoral therapeutic efficacy, which is clearly exemplified by clinical trials with oncolytic adenoviruses. To our knowledge, the present study is the first report to show that systemic administration of M1, spread and replicated not only in the local primary tumor but also in the disseminated metastases, eliminated the targeted gene plk1 expression in model mice and therefore prolonged the survival time of tumor-bearing mice.

Although current methods of chemotherapy, radiotherapy, and surgical resection effectively eradicate local primary tumors, they are less effective in controlling metastatic disease. Therefore, metastasis represents the most lethal stage of cancer progression. In a previous study, we have shown that the oncolytic virus M1 in combination with cisplatin could control both the local primary tumor and the disseminated metastases in an orthotopic human hepatic carcinoma model. Considering that most adenoviruses will accumulate in liver when given i.v., we have here established a new human orthotopic gastric carcinoma model to evaluate the intrinsic antimetastatic potency of M1 after i.v. injection. In contrast to previous studies (22–24), the human orthotopic gastric carcinoma models developed rapidly growing and highly invasive metastases in 100% of mice, causing the mice to begin to die at 4 to 6 weeks. This metastatic model had a variety of clinical behaviors that occur in human patients, including (a) local invasive growth, (b) abdominal metastasis, (c) lymph node metastasis, and (d) liver metastasis, which were very rare in other animal models of xenograft tumors. The data we obtained suggest that the orthotopic human gastric carcinoma model is a potential metastatic model in which variables including not only viral biodistribution, spread, and replication but also antitumoral metastasis can be experimentally modified and optimized. However, there are some defects in this model, such as the observation that
athymic mice are immune-incompetent and insensitive to Adv5. Well-designed clinical trials will be needed to determine the clinical efficacy and safety of M1.

Another important finding of this study is that systemic administration of M1 as a single agent could not prolong the survival of mice after i.v. administration at late time points. The outcome of viral therapy is dependent on the balance between several host and viral variables, including the initial loading of tumor, the viral replication, and antiviral immune response (25). Established tumors are more difficult to eradicate because of infiltrating macrophages and fibroblasts, resulting in connective tissue formation, the ingrowth of blood capillaries, and the tumor matrix, all of which impose blocks to the spread of viruses (26). One of the possible strategies overcoming these barriers is early administration of oncolytic adenovirus

Figure 3. Distribution and replication of M1 in local and metastatic tumors. Animals were injected i.v. with M1 or Adv-TK at a dose of $1 \times 10^{12}$ viral particles/kg daily for 5 consecutive days at 10 d after implantation. The local and metastatic (liver and lymph nodes) tumors were harvested at 4 wk and subjected to immunohistochemistry and in situ hybridization analysis. A, viral distribution was determined by immunohistochemistry staining using an anti-adenovirus mouse monoclonal antibody. Viral replication was detected by in situ hybridization with biotinylated viral fiber oligonucleotide probe complimentary to the fiber coding region. Cells containing viral particles were stained brown (black arrows) and those containing replicative virions were stained dark blue (red arrows). Original magnification, $\times 200$. B, horizontal bar chart, values of expression score of immunohistochemistry or in situ hybridization in local and metastatic tumors. *, $P < 0.05$. 

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Figure 4. Kinetics of biodistribution after systemic administration of M1. Viral (M1 or Adv-TK) injection was done at a dose of $1 \times 10^{12}$ viral particles/kg daily for 5 consecutive days at 10 d after implantation, and the mice were sacrificed at the indicated time points. Infectious virus particles per milliliter of serum, per gram of primary tumor, per gram of liver, and per gram of metastasis were quantified by titration onto 293 cells. Four model mice were used per time point, and each dot represents one data point. No virus was detected in samples represented by symbols below the dotted line. A, virus particles per milliliter of serum of the group treated with M1 were not higher than that of groups treated with Adv-TK at all time points ($P > 0.05$). B, level of M1 in primary tumor was higher than that of Adv-TK at 4 wk ($t = 3.206, P = 0.018$) and 6 wk ($t = 2.783, P = 0.032$). C, level of M1 in liver was higher than that of Adv-TK at 4 wk ($t = 2.826, P = 0.030$) and 6 wk ($t = 2.463, P = 0.049$). D, level of M1 in metastatic tumor was higher than that of Adv-TK at 4 wk ($t = 2.584, P = 0.042$) and 6 wk ($t = 2.718, P = 0.035$).
Once tumor is detected or after surgical resection. In addition, the combined use of conventional treatments such as chemotherapy and radiotherapy with oncolytic adenoviruses may improve oncolysis and the spread of the viruses into the tumor mass and may help to overcome structural barriers (27–29). The molecular mechanisms underlying the synergy are currently unknown but involve interactions between viral genes and stress-activated host cellular factors leading to enhancement of the tumor cell-killing effects of viruses (30–32). We have shown previously that cisplatin-refractory tumor xenograft models responded to i.v. administration of M1 plus cisplatin with reduced incidence of metastasis and substantially improved survival. The synergy is more important to the human patients, because the immune system of human beings may interfere with viral function via neutralizing antibodies or macrophage-mediated phagocytosis.

Overall, despite the limitations of immune-incompetent models, this study suggests that systemic administration of oncolytic adenovirus M1, combining oncolytic properties with the benefits of targeting plk1, allows it to effectively spread and replicate in local and distant metastasis, ultimately eradicating the primary and metastatic tumors. This would be a significant advance and provide the impetus to design a rational clinical trial for gene therapy in a future clinical setting.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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