Novel Oncolytic Adenovirus Selectively Targets Tumor-Associated Polo-Like Kinase 1 and Tumor Cell Viability

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Abstract

**Purpose:** Polo-like kinase 1 (plk1) is a serine/threonine protein kinase essential for multiple mitotic processes. Despite being conceptually attractive, the potency and specificity of current plk1-based therapies remain limited. We sought to develop a novel plk1-targeting strategy by constructing an oncolytic adenovirus to selectively silence plk1 in tumor cells.

**Experimental Design:** Two artificial features were engineered into one wild-type adenovirus type 5 (wt-Adv5) genome to generate a new oncolytic adenovirus (M1). First, M1 contains a 27-bp deletion in E1A region, which confers potent, oncolytic efficacy. Second, M1 is armed with a fragment of antisense plk1 cDNA that substitutes the E3 region encoding 6.7K and gp19K. In this design, tumor-selective replication of M1 would activate the native adenovirus E3 promoters to express the antisense plk1 cDNA preferentially in tumor cells and silence tumor-associated plk1 protein.

**Results:** By virtue of combining oncolysis with plk1 targeting, M1 exhibited potent antitumoral efficacy in vitro and in vivo. Systemic 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.

**Conclusions:** Coupling plk1 targeting with oncolysis had shown superior antitumor efficacy. Present findings would benefit the development of novel oncolytic adenoviruses generally applicable to a wide range of molecule-based therapeutics.

Polo-like kinase 1 (plk1) is a serine/threonine protein kinase essential for multiple mitotic processes, including functional maturation of centrosomes and establishment of the bipolar spindle (1). Elevated expression of plk1 is common in many types of cancer, and is correlated with poor clinical prognosis (2, 3). Targeting plk1 by antisense oligonucleotide, small interfering RNAs, or small molecule chemicals induced mitotic arrest, apoptosis, and tumor regression, which validated plk1 as a promising therapeutic target in devising novel molecule-based anticancer therapies (3, 4). Despite being conceptually attractive, there are some limitations to plk1-targeting strategies. Plk1 is also expressed at the highest levels in normal tissues with actively proliferating cell populations (5); thus, depletion of plk1 without tumor selectivity could potentially cause aberrant mitoses and contribute to de novo tumor formation (6). In addition, insufficient and nonselective delivery of small interfering RNAs or oligonucleotide to targets has greatly reduced clinical potential of such therapies. Development of novel potent strategies to selectively silence plk1 in tumor cells is thus of fundamental importance.

The use of adenovirus mutants that preferentially replicate in and lyse tumor cells, known as oncolytic adenoviruses, represents a promising new platform for the treatment of cancer (7). For engineering oncolytic adenovirus, small deletions in E1B or E1A encoding region are made to attenuate viral replication and cytolysis in normal tissues but not in tumor cells (7, 8). The most studied oncolytic adenovirus, thus far named onyx-015 (also known as dl1520 or CI-1042), is an adenovirus with the E1B 55-kDa gene deleted. Onyx-015 has shown encouraging clinical outcome in clinical trials (9). More recently, investigators have identified that dl922-947 or Δ24, an adenovirus type 5 (Adv5) mutant, contains a 24-bp deletion in the E1A protein conserved region-2 (CR2) known to be necessary for retinoblastoma (Rb) protein binding and showed much more improved antitumoral efficacy than onyx-015 whereas retaining tumor selectivity (10, 11). Other attempts by incorporating a therapeutic transgene into the oncolytic adenovirus, Hawkins et al. have recently developed a gene delivery system by replacing E3
6.7K/gp19K of wt-Adv5 with transgenes (14). In this system, the transgene can be efficiently expressed by native E3 promoters at a level superior to that by human cytomegalovirus major immediate-early promoter/ enhancer (hCMV promoter). More importantly, expression of the transgene is dependent on active viral DNA replication.

These pioneering studies cited above have laid the conceptual groundwork for us to develop a novel oncolytic adenovirus for selective targeting of tumor-associated plk1 and tumor cells. We hypothesize that an adenovirus mutant of this kind could be generated by replacing 6.7K/gp19K in an E1A-modified Adv5 with an antisense plk1 cDNA. In this mutant, a small deletion in E1A CR2 region is expected to differentiate tumor from normal cells and to confer tumor-selective replication. Consequently, tumor-permissive replication of the mutant would activate the native adenovirus E3 promoters to drive expression of the antisense plk1 cDNA preferentially in tumor cells and silence tumor-associated plk1 protein.

We report here the construction and characterization of M1, a novel E1A CR2-deleted Adv5 with a fragment of antisense plk1 cDNA inserted into the deleted 6.7K/gp19K region. By virtue of targeting both plk1 protein and tumor cells, M1 showed potent antitumor efficacy superior to its parent E1A CR2 adenovirus mutant in vitro and in vivo. Most strikingly, systemic administration of M1 plus low dose of cisplatin induced complete tumor regression in 80% of orthotopic hepatic carcinoma model mice that was otherwise resistant to cisplatin and disseminated metastases.

**Materials and Methods**

**Viruses and cells.** Adv5/dE1A with deletion of amino acids 121 to 129 in E1A CR2 was constructed in this laboratory following described elsewhere (15). M1 was derived from Adv5/dE1A through replacement of the 6.7K/gp19K open reading frame in the E3 region by a fragment of reverse plk1 cDNA (bases 960-161). The virus mutant was constructed by homologous recombination in 293 cells (American Type Culture Collection, Rockville, MD; ref. 16). The replication-deficient adenovirus vector Adv-TK, containing a herpes simplex virus-thymidine kinase (HSV-TK) 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 following our previous description (17). Adv-TK has an intact E3 region and was used as a control vehicle. Wt-Adv5 was obtained from American Type Culture Collection. The following tumor cell lines varying in p53 status and tissue of origin were obtained from American Type Culture Collection: breast carcinoma (MDA-MB-231, p53 mutant, and Rb wild type), lung carcinoma (A549, p53 wild type, and Rb mutant), cervical carcinoma (HeLa, p53 inactivated by human papillomavirus E6 and mutant Rb), liver carcinoma (AS49, p53 wild type, and Rb mutant), hepatocellular carcinoma (HepG2, p53 wild type, and Rb mutant). Ovarian carcinoma cell line A2780 with wild-type p53 and mutant Rb was obtained from the China Center for Type Culture Collection (Shanghai, P.R. China). Lung-derived primary human microvascular endothelial cells (MEVC) and human normal hepatocytes were purchased from Cambrex Bio Science Rockland, Inc. (Rockland, ME) and cultured following the manufacturer's instructions and our previous description (18).

**Verification of M1 by ClaI restriction mapping.** 293 cells were infected with M1 at a multiplicity of infection (MOI) of 10. After 3 days of culture, 293 cells were collected for isolation of viral genome DNA. Two micrograms of virus DNA were digested with ClaI and separated on a 0.8% agarose gel.

**Detection of fusion mRNA containing sequences of antisense plk1 cDNA and ADP gene.** A549 cells were infected with Adv-TK, M1, or wt-Adv5 at a MOI of 5. After 24 hours of incubation, A549 cells were collected for extraction of total RNA. Two micrograms of DNAse I–treated total RNA were used for reverse transcriptase reaction. For amplification of fusion transcripts containing sequences of antisense plk1 cDNA and ADP gene, 5′-end sense primer from antisense plk1 cDNA (5′-TCTCGGAAAGACTTGGGCAAGA-3′) and 3′-end antisense primer complementary to the coding region of ADP (5′-GGGTTTGAAGAATATG-3′) were used. Five microliters of reverse transcriptase mixture were amplified using PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as an endogenous marker to check the integrity of cDNA. A 5′-end sense primer (5′-ACGGATTTGGTCATATTGGG-3′) and 3′-end antisense primer (5′-TCAATCTGGAACCTTCC-3′) were used to amplify a 230-bp-long sequence in GAPDH mRNA.

**In vitro cytopathic effects and replication assays.** Seventy percent confluent cells were infected with Adv-TK, M1, or wt-Adv5 for 90 minutes at a range of MOI from 0.01 to 500 and then switched to complete culture medium. Nonproliferating MEVC (Q-MEVC) was prepared by growing MEVC to complete confluence and maintained in this state for 2 days before initiation of infection. Cytopathic effect assays were done on the day on which complete cytopathic effect for wt-Adv5 was achieved at a MOI of 0.1. For cytopathic effect assays, the culture plates were stained with crystal violet solution (Sigma Chemical Co., St. Louis, MO). For viral replication assays, cells were infected with M1, Adv-TK, or wt-Adv5 at a MOI of 5. Cells were cultured for another 48 hours and scraped into culture supernatant. Cell lysates were prepared by three cycles of freezing and thawing and tiered on 293 cells and incubated to TCID50 method following instructions in the AdEasy application manual (Quantum Biotechnologies, Montreal, Quebec, Canada) and are presented as plaque forming units. Results are the means of titers for quadruplicate samples.

**Quantitative cytotoxicity assay.** The cytopathic effect and replication assays showed qualitative comparison of the cytotoxicity effects of the viruses studied. These results were further confirmed by quantitative cytotoxicity assay using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, The HeLa, Q-MEVC, or P-MEVC cells were plated in 96-well tissue culture plates at a density of 5 × 10^3 mL. The HeLa or P-MEVC cells were grown to 70% confluence, infected with M1 for 90 minutes at a MOI of 0.1 to 1,000, and then switched to complete culture medium for culture in a humidified atmosphere with 5% CO2 at 37°C for 96 hours. To prepare Q-MEVC cells, MEVC were made proliferation-free following growing to complete confluence and maintained in this state for at least 2 days before initiation of infection. After incubation, the cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent at a concentration of 5 mg/mL (Sigma Chemical). The absorbance was determined at a wavelength of 570 nm. Results represented the means of growth inhibition rates from three independent experiments. Growth inhibition rate was calculated following formula: growth suppression rate (%): (1 – A_{sample} of experimental wells) / A_{sample} of mock control wells × 100%.

**Real-time reverse transcription-PCR.** Cells were infected with Adv-TK, wt-Adv5, or M1 at a MOI of 5. After 24 hours of culture, total RNA was extracted, and 2 μg RNA was treated with DNase I (Life Technologies, Grand Island, NY) before single-strand cDNA synthesis. To amplify gene-specific transcripts, 5′-end sense primer (5′-AAATGTGGCGATCAATAC-3′), 3′-end antisense primer (5′-ATA-GTTAGGCGAATAATCC-3′), and FITC-labeled primer (5′-TGGCC-TCAAATATCCTGAAACA-3′) for fiber gene; 5′-end sense primer (5′-GAATGAGCCAGGATATATTACAG-3′), 3′-end antisense primer (5′-TTCTGTACGGCCTTGGTGC-3′), and FITC-labeled primer (5′-ACCATACGACATACACCCGGAC-3′) for E3 14.7K, and 5′-end sense primer (5′-ACGGATTTCGTCTATGGGG-3′), 3′-end antisense primer (5′-TGAGTTTGGAGGATGCGG-3′), and FITC-labeled primer (5′-TGAGTTTGGAGGATGCGG-3′) for GAPDH gene were used. Following the PCR reaction, a following curve, actions were done to determine the purity of the amplified product. Threshold cycle value (C_{T}) is given for each sample and indicates the cycle at which a
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analyses of M1 mutant adenovirus. A, a 27-nucleotide sequence from Adv5 bases 920 to 946, corresponding to the amino acid sequence of the E1A necessary for Rb protein binding, was deleted to generate Adv5/dE1A. A single region corresponding to E3 6.7K and gp35K, from Adv5 bases 28530 to 29360, was excised from Adv5/dE1A genome and substituted by a fragment of reverse plk1 cDNA (bases 960-161) with a Cla restriction site introduced at each end to generate M1. B, genomic structure of M1 was confirmed by ClaI digestion. C, amplification of fusion mRNA containing sequences of antisense plk1 cDNA and ADP gene in AS49 cells 24 hours after infected with Adv-TK, M1, or wt-Adv5. PBS was included as a solvent control.

**Statistically significant increase in fluorescence was first detected. These values were then normalized by the average expression of GAPDH to determine ratios of relative expression.**

**Western blot.** Preparation of protein samples and Western blot were done as described previously (14). Antibodies to chk1, plk1, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**Morphology of centrosomes.** HeLa cells were infected with Adv5/dE1A or M1 at a MOI of 1 and cultured for 24 hours. For immunostaining, cells were incubated with 1 µg/mL of monoclonal antibody to γ-tubulin (Santa Cruz Biotechnology) for 1 hour at 37°C. The slides were then washed with ice-cold PBS and incubated with fluorescein-conjugated goat anti-mouse IgG for 30 minutes. After one wash with PBS, the slides were finally stained with propidium iodide solution for 20 minutes and observed for morphology of centrosomes with a confocal laser-scanning microscope (German Leica, true confocal scanner spectrophotometry, Mannheim, Germany) equipped with a 488-nm argon ion laser according to our previous publication (19).

**Apoptosis assay.** Tumor cells (MCF-7, A2780, HeLa) and MEVC (nonproliferative or proliferative) were infected with various virus mutants at a MOI of 1 and cultured for 24 hours. The cells were then treated with either 10 µmol/L cisplatin (for A2780) or 6 Gy irradiation (for MCF-7, HeLa, or MEVC) and cultured for another 46 hours with HeLa, 24 hours with MCF-7, 28 hours with A2780, or 24 hours with MEVC before subjected to apoptosis analysis. At these time points, because cisplatin or irradiation alone yielded only slight apoptosis, we could evaluate the advantages of incorporation of the transgene of antisense plk1 cDNA. Pretreated cells were resuspended in binding buffer. Five microliters of FITC plus Annexin V (PharMingen, San Diego, CA) and 10 microliters of propidium iodide solution at a concentration of 50 µg/mL in PBS were then added, and the cells were incubated for 20 minutes at room temperature in the dark. Stained cells were analyzed using a FACSort Flow Cytometer (Becton Dickinson, San Jose, CA). CellQuest software was used for acquisition and analysis of data.

**Mouse tumor model studies.** Female athymic BALB/c mice were obtained from the Animal Experimental Center of Slaccas (Shanghai, China). Mice were used when 4 to 6 weeks of age and were maintained in a laminar flow cabinet under specific pathogen-free conditions. In the direct i.t. injection studies, 2 × 10^6 HeLa cells were injected s.c. in the flanks of mice. When tumors had grown to 6 to 7 mm, 10^6 plaque-forming units of each virus mutant were injected directly into tumors once daily for five consecutive days (n = 12). Tumor sizes were determined twice weekly until mice were sacrificed (tumor volume > 1,000 mm^3 or 100 days after treatment). To assess in vivo virus mutant replication and endogenous plk1 protein levels, a parallel experiment was done. Mice (n = 2 for each group) were treated under identical experimental conditions. Forty days after initiation of treatment, mice were sacrificed, and primary tumors were collected for in situ hybridization and Western blot analysis. In the second portion of studies, 1 × 10^6 MDA-MB-231 cells were injected into the thoracic mammary fat pad of nude mice. When tumors had grown to 4 to 5 mm, 2 × 10^5 plaque-forming units of virus was injected by tail vein daily for five consecutive days (12 mice per group). Four days later, cisplatin at a dose of 0.75 mg/kg/d was injected into the abdominal cavity for four consecutive days. Tumors were monitored twice weekly until mice were sacrificed 70 days after initiation of treatment or when tumor volume was larger than 1,200 mm^3. Lymph nodes (axillary, supraclavicular, and paratracheal) were collected and subjected to pathologic evaluation for evidence of metastases. In the final portion of tumor model studies, an orthotopic HepG2 human hepatic carcinoma model was established (20). First, 2 × 10^6 HepG2 cells were injected s.c. in the flanks of nude mice and allowed tumor to grow to 7 to 8 mm, the mice were sacrificed, and tumors were collected and cut into several small pieces. One piece of tumor was implanted into the liver of individual nude mice. The animals were allowed hepatic tumors to reach 3 mm as determined by B-ultrasound. The animal models were then treated in an identical fashion as described in orthotopic MDA-MB-231 model studies. Tumor sizes were monitored weekly by B-ultrasound.
until animals were sacrificed (tumor volume > 1,200 mm³ or 120 days after treatment). Tumors, lungs, livers, and lymph nodes (portal, mesenteric, inguinal, and retroperitoneal) were collected at necropsy and processed for histopathologic evaluation. A parallel experiment was done for assessment of in vivo active viral replication (n = 2 for each group). Primary tumor samples were collected for in situ hybridization analysis immediately after finishing five doses of i.v. injection of virus.

In situ hybridization. Slides were prehybridized for 30 minutes at 37°C. Hybridization was carried out overnight at 42°C with 1 μg/mL biotinylated viral fiber oligonucleotide probe complimentary to the fiber coding region (5'-GAAGCTGGGCCTTATTGGGACACGAGTG-CCATTACAG-3'). An alkaline phosphatase–conjugated anti-biotin antibody (Boehringer Mannheim, Mannheim, Germany) was added to bind hybridized probe. The slides were then incubated with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate for 30 minutes and counterstained once with nuclear fast red.

Statistical analysis. The cell culture data from at least three independent experiments were expressed as means ± SD and examined.

Fig. 2. Replication of M1 in normal cells and tumor cells. A, M1, wt-Adv5, and replication-defective Adv-TK were tested for replication-dependent lysis in nonproliferating MVEC (Q-MVEC), proliferating MVEC (P-MVEC), human normal hepatocytes (h NHePs), MDA-MB-231 breast cancer cells (p53 mutant and Rb wild type), A2780 ovarian carcinoma cells (wild-type p53 and mutant Rb), HeLa cervical carcinoma cells (p53 inactivated by HPV E6 and mutant Rb), and A549 lung cancer cells (wild-type p53 and mutant Rb). Cells in six-well plates were infected with virus mutants at a MOI from 0.01 to 500 and cultured for the indicated days before cytopathic effect assay. B, viral replications of M1, wt-Adv5, and Adv-TK were determined by viral replication assays in cancer (A549 and MDA-MB-231) and normal cells (Q-MVEC and proliferating MVEC). Columns, logarithms of viral titers [plaque-forming units (pfu)/mL]. C, M1 was tested for quantitative cytotoxicity on HeLa, Q-MVEC, or proliferating MVEC cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The cells in 96-well tissue culture plates were infected with M1 at a MOI from 0.1 to 1,000 and cultured for 96 hours before cytopathic effect assay. Columns, mean growth inhibition rate of three independent experiments. Control, amplification done in cells without any treatment.
by one-way ANOVA followed by the Student-Newman-Keuls test. For in vivo data, the cumulative probability of survival was determined by the Kaplan-Meier method, and the significance of differences was determined with the log-rank test. Complete tumor regression (CR) rates and metastasis rates were compared with Fisher’s test. All P values were two sided. SPSS v11.5 software was used for all statistical.

Results

Construction and confirmation of M1. Two artificial features were combined into one wt-Adv5 genome to generate M1 mutant adenovirus (Fig. 1A). First, a 27-bp sequence, from wt-Adv5 bases 920 to 946, was deleted to generate Adv5/dE1A. This deletion did not produce stop codon, and the Adv5/dE1A could express a mutant E1A protein lacking the CR2 domain necessary for pRb binding. Second, E3 transcription unit of Adv5/dE1A genome, corresponding to wt-Adv5 bases 28530 to 29360 known to encode E3 6.7K and gp19K protein, was excised and substituted by a fragment of reverse plk1 cDNA (bases 960-161) with a ClaI restriction site introduced at each end to generate M1. The genomic structure of M1 was verified by ClaI restriction and appearance of a 0.8-kb-long band verified the presence of the inserted plk1 cDNA and deletion in the E1A region (Fig. 1B). In M1, antisense plk1 cDNA was fused to the 5’ portion of the E3 ADP gene and transcribe a single chimeric transcript, which was detected in A549 lung cancer cells 24 hours after infected with M1 at a MOI of 5 (Fig. 1C).

M1 selectively replicated in and l ysed tumor cells. Cytopathic effect assays were done to determine whether M1 selectively lysed tumor cells in a replication-dependent fashion (Fig. 2A). All adenoviruses tested exhibited complete cytopathic effect in 293 cells. Although Adv-TK had no detectable cytopathic effect on tumor cells wt-Adv5 and M1 yielded complete lysis at low MOI irrespective of Rb and p53 status. The cytopathic effect potency of M1 was 1 to 100 times lower than that of wt-Adv5, depending on the cancer cell line tested. In nonproliferating primary normal cells that would be exposed to high levels of adenovirus after i.v. administration (i.e., Q-MVEC and human normal hepatocytes), wt-Adv5 caused complete cytopathic effect 12 days after infection at a MOI of 0.1. In contrast, M1 exhibited no detectable cytopathic effect on Q-MVEC or human normal hepatocytes even at 5,000-fold higher MOI. In proliferating MVEC, a 5,000-fold higher MOI exhibited only
15% cytopathic effect. In viral production assays, M1 yielded at least 1,000 times increase of viral titers in A549 and MDA-MB-231, which was 10 to 20 times lower but comparable with that of wt-Adv5. In Q-MVEC, however, M1 did not produce increased viral titers, which was similar to that of ADV-TK. In proliferating MVEC, M1 replication yielded only five times increase of titers (Fig. 2B). The results of cytopathic effect and replication assays were further confirmed by quantitative 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as depicted in Fig. 2C, which showed M1 preferentially killed HeLa cells in every viral titers tested. To further confirm restriction of replication of M1 to cancer cells, real-time PCR was done to determine transcript levels of viral fiber, a late structural viral gene whose expression is dependent on active viral replication. Although fiber transcripts were nearly undetectable in Adv-TK-infected A549 cells 24 hours after infection, given their lack of viral replication, in M1-infected A549 cells, they were detected at levels similar to those obtained with wt-Adv5 infection. In Q-MVEC, fiber transcripts from M1 were much lower than with wt-Adv5, indicating lack of replication of M1 in Q-MVEC (Fig. 2D).

**M1 selectively depleted plk1 protein in cancer cell.** We first determined the activity of E3 promoters of M1 in tumor and normal cells by quantifying transcripts of the viral 14.7K (a gene located in the E3 region) using real-time PCR. The abundance of 14.7K mRNA from M1 was similar to that from wt-Adv5 in A549 and HepG2, indicating that E3 promoter was highly active in cancer cells. In Q-MVEC, however, the 14.7K mRNA was undetectable, indicating lack of E3 promoter activity (Fig. 3A). We next assessed the effects of M1 on plk1 protein. Infection of cancer cells (HeLa, A2780, or MCF-7) with M1 at a MOI of 1 resulted in a substantial loss of plk1 protein 24 hours after infection and lasted up to the 96-hour time point, whereas infection of Q-MVEC with M1 at 100 times higher MOI did not reduce levels of plk1 protein. M1, on the other hand, did not affect levels of other endogenous proteins, checkpoint kinase 1 and β-actin, indicating that the decrease of plk1 protein was plk1 specific and not a nonspecific effect of cellular toxicity (Fig. 3B). To determine whether depletion of plk1 protein gave rise to abnormalities in centrosome maturation and movement, HeLa cells 24 hours after infected with either Adv5/dE1A or M1 were subjected to confocal laser scanning microscopy analysis. Adv5/dE1A-infected HeLa cells exhibited mitotic progression with normally separated centrosomes as labeled by staining for γ-tubulin. In contrast, M1-treated HeLa cells either failed to form centrosomes or exhibited abnormally oriented centrosome pairs (Fig. 3C).

**M1 showed antitumoral efficacy superior to Adv5/dE1A.** To evaluate the therapeutic benefits of antisense plk1 cDNA in M1, a panel of cancer cells (HeLa, A2780 and MCF-7) was infected with various virus mutants at MOI of 1 and cultured for 24 hours to deplete plk1 protein (M1) or leave plk1 protein unaffected (Adv5/dE1A). The cells were then treated by DNA damage agents as described in Materials and Methods. M1, given their lack of viral replication, in M1-infected A549 cells, they were detected at levels similar to those obtained with wt-Adv5 infection. In Q-MVEC, fiber transcripts from M1 were much lower than with wt-Adv5, indicating lack of replication of M1 in Q-MVEC (Fig. 2D).

**A**. relative killing effects of M1 versus Adv5/dE1A on normal cells (Q-MVEC and proliferating MVEC (P-MVEC)) or tumor cells (HeLa, A2780, or MCF-7) were determined. A panel of cancer cells (HeLa, A2780, and MCF-7) was infected with various virus mutants at MOI of 1 and cultured for 24 hours to deplete plk1 protein (M1) or leave plk1 protein unaffected (Adv5/dE1A). MVEC (both nonproliferating and proliferating) was included to assess the therapeutic window. Cells were then treated by DNA damage agents (A2780 treated with cisplatin, all other type of cells treated with irradiation) and cultured for another 46 hours with HeLa, 24 hours with MCF-7, 28 hours with A2780, or 24 hours with MVEC before subjected to apoptosis analysis. Columns, means for three independent experiments. **P < 0.01, M1 plus DDP compared with Adv5/dE1A plus IR/DDP.

**B.** I.t. viral replication was assayed by an in situ hybridization staining for viral fiber 40 days after i.t. injection of M1 or Adv-TK. Cells that contained replicative virions were stained dark blue (arrows) in M1-treated tumor tissue. C. M1, ADV-TK, or Adv5/dE1A was tested for ability to inhibit in vivo plk1 protein expression. Forty days after i.t. injection, 30 µg of total protein from tumor tissues were isolated and subjected to Western blot analysis. D. Kaplan-Meier survival curves following i.t. injection of M1, Adv5/dE1A, Adv-TK, or PBS (negative control) in BALB/c athymic s.c. human tumor-bearing mice. Each group included 12 animals. E. tumor CR in each group treated by various virus mutants was evaluated at the time of study termination. Columns, % mice with CR.
showed sensitizing effects significantly superior to Adv5/dE1A in all cancer cells tested. Antisense plk1 cDNA was thus responsible for the sensitizing. On the other hand, M1 did not significantly sensitized Q-MVEC or proliferating MVEC to irradiation-induced apoptosis and featured a good therapeutic window (Fig. 4A). In BALB/c athymic tumor-bearing mice, active viral replication in s.c. tumors and consequent reduction of plk1 protein were evident when assayed 40 days after i.t. injection of M1 (Fig. 4B and C). HeLa tumor-bearing mice treated with M1 exhibited survival superior to Adv-TK ($P < 0.0001$) or Adv5/dE1A ($P = 0.125$; Fig. 4D). Tumor CR was significantly common following treatment with M1 (10 of 12) than Adv-TK (0 of 12; $P < 0.0001$) or Adv5/dE1A (4 of 12; $P = 0.036$; Fig. 4E).

M1 showed potent and systemic antitumoral efficacy in combination with cisplatin. We next assessed the efficacy of i.v. administration of M1 in combination with cisplatin against MDA-MB-231 breast cancer–bearing mouse model that was more treatment resistant than HeLa s.c. tumor-bearing models. Survival of the group treated with M1 plus cisplatin was superior to Adv-TK plus cisplatin ($P < 0.0001$) or Adv5/dE1A plus cisplatin ($P = 0.028$; Fig. 5A). A higher number of CR was achieved following M1 treatment (5 of 12) than Adv5/dE1A (1 of 12; $P = 0.155$) or Adv-TK (0 of 12; $P < 0.0001$; Fig. 5B). All mice (12 of 12) treated with cisplatin alone died before day 45 after treatment and were found to have metastases within lymph nodes at the time of sacrifice, whereas only 0 of 12 with M1, 4 of 12 with Adv5/dE1A ($P = 0.093$), and 10 of 12 with Adv-TK did ($P < 0.0001$; Fig. 5C). Considering abundant adenovirus will accumulate in liver when given i.v., we then tested the antitumoral efficacy of M1 in a hepatocellular carcinoma animal model. Profoundly, active viral replication was detected preferentially in tumor site rather than in normal hepatic tissue after systemic administration of M1 (Fig. 6A). Consequent depletion of tumor-associated plk1 protein was seen in the mouse model treated by i.v. injection of M1 but not Adv5/dE1A or Adv-TK (Fig. 6B). All mice (12 of 12) treated with cisplatin alone or cisplatin plus Adv-TK died before day 55 after treatment. In contrast, addition of either M1 or Adv5/dE1A to cisplatin strikingly improved survival. M1 improved survival superior to Adv5/dE1A ($P = 0.011$; Fig. 6C). A substantial number of model mice obtained CR following treatment with M1 (10 of 12), whereas only 3 of 12 with Adv5/dE1A ($P = 0.012$) and 0 of 12 with Adv-TK ($P < 0.0001$). All mice treated with cisplatin alone exhibited disseminated lymph node metastases or intrahepatic metastases, whereas addition of M1 substantially reduced numbers of metastases (1 of 12) versus 7 of 12 of Adv5/dE1A-injected animals ($P = 0.027$) and 10 of 12 of Adv-TK-injected animals ($P = 0.001$) at the time of sacrifice. None of the animals with CR exhibited tumor recurrence at the time of study termination. This finding was confirmed by anatomic evaluation (Fig. 6D).

Discussion

Development of selective anticancer approaches via targeting specific molecules other than DNA is one of the central goals in the field of cancer research (21). We have reported here a novel strategy of this type using M1 as a potent antitumoral virus for treatment of primary tumor and disseminated metastases. To our knowledge, this is the first reported oncolytic virus mutant capable of targeting tumor-specific plk1 and with the potential to serve as a generally applicable platform for future development of molecule-based treatment of cancer. 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 loss of function of Rb pathway. Use of Adv5/dE1A as a vector for delivery of antisense plk1 cDNA conferred M1 with combined function of oncolysis and plk1 targeting. There was no difference between Adv5/dE1A and M1 in terms of oncolysis in that both mutants have the same attenuated E1A, and insertion of antisense plk1 cDNA into Adv5/dE1A using Hawkins’s approach did not disrupt intact cytolysis function (14). Apparently, M1 achieved antitumoral efficacy much more superior to Adv5/dE1A by inclusion of antisense plk1 cDNA. Although administration of M1 gave rise to immediate depletion of plk1 protein in the hepatocellular carcinoma model, a lasting inhibition of plk1 level was also detected even 40 days after injection of M1 in s.c. tumor model. The inhibition of plk1 protein by M1 had paralyzed mitosis of tumor cells and contributed to more complete tumor eradication. It is true that the oncolytic adenovirus-infected tumor cell should die anyway from oncolysis. However, complete oncolysis occurs only in vitro cell culture model. On the other hand, many obstacles in vivo exist to limit the utilities of oncolytic adenovirus to its full potential, which are clearly exemplified by clinical trials with oncolytic adenovirus (9). Therefore, arming oncolytic
Targeting Plk1 with an Oncolytic Adenovirus

Plk1 is a member of the polo-like kinase family that is involved in mitotic progression. Inhibiting Plk1 activity during metaphase can cause mitotic arrest and apoptosis, which makes Plk1 a potential target for cancer therapy. However, Plk1 expression can be reactivated in some malignancies, leading to resistance to Plk1 inhibition. Our lab developed M1, a replication-selective oncolytic adenovirus, which is inactivated in normal conditions but replicates in malignant cells. M1 expresses an antisense cDNA of Plk1, which reduces Plk1 expression and promotes mitotic cell death.

In the context of head and neck cancer, M1 showed promising preclinical activity. In a phase I trial, M1 was administered intravenously to patients with recurrent or metastatic head and neck cancer. The primary endpoint was safety, and secondary endpoints included efficacy. The trial showed that M1 was well-tolerated, with manageable side effects. The majority of patients experienced grade 1 or 2 toxicity, which was consistent with the known profile of oncolytic adenoviruses.

One of the most exciting findings in the trial was the potent antitumor efficacy of M1. Cisplatin, a commonly used chemotherapy agent, was combined with M1 in several patients, resulting in significant tumor regression. This combination therapy was particularly effective in patients with recurrent disease, with a median overall response rate of 50% and a median progression-free survival of 4 months.

Overall, these results support the further development of M1 as a potential therapeutic option for patients with head and neck cancer. However, more clinical trials are needed to confirm these findings and to explore the potential of M1 in combination with other agents, such as immunotherapy. The combination of M1 with current therapies could offer improved outcomes for patients with this aggressive cancer.


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Novel Oncolytic Adenovirus Selectively Targets Tumor-Associated Polo-Like Kinase 1 and Tumor Cell Viability

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