Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses

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Abstract

Natural and genetically modified oncolytic viruses have been systematically tested as anticancer therapeutics. Among this group, conditionally replicative adenoviruses have been developed for a broad range of tumors with a rapid transition to clinical settings. Unfortunately, clinical trials have shown limited antitumor efficacy partly due to insufficient viral delivery to tumor sites. We investigated the possibility of using mesenchymal progenitor cells (MPC) as virus carriers based on the documented tumor-homing abilities of this cell population. We confirmed preferential tumor homing of MPCs in an animal model of ovarian carcinoma and evaluated the capacity of MPCs to be loaded with oncolytic adenoviruses. We showed that MPCs were efficiently infected with an adenovirus genetically modified for coxsackie and adenovirus receptor–independent infection (Ad5/3), which replicated in the cell carriers. MPCs loaded with Ad5/3 caused total cell killing when cocultured with a cancer cell line. In an animal model of ovarian cancer, MPC-based delivery of the Ad5/3 increased the survival of tumor-bearing mice compared with direct viral injection. Further, tumor imaging confirmed a decrease in tumor burden in animals treated with oncolytic virus delivered by MPC carriers compared with the direct injection of the adenovirus. These data show that MPCs can serve as intermediate carriers for replicative adenoviruses and suggest that the natural homing properties of specific cell types can be used for targeted delivery of these virions. [Mol Cancer Ther 2006;5(3):755–66]

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Introduction

Replication-competent oncolytic viruses have emerged in recent years as a promising novel class of therapeutics for the treatment of human cancer. Among this group, oncolytic adenoviruses have proven to be particularly suitable to adaptation as virotherapy agents. Consequently, conditionally replicative adenoviral agents (CRAd) have been developed for a broad range of tumor targets with a rapid transition from preclinical evaluations to clinical trials (1).

Early-phase clinical trials have validated the safety profile of CRAds in the context of cancer therapy; however, there has only been limited evidence of antitumor efficacy. In this regard, clinical studies have clarified the barriers limiting the efficacy of CRAd interventions, including inefficient virus delivery to the tumor site and poor transduction of the tumor cells (2). High viral doses must be applied to overcome these obstacles, which can result in toxicity. Thus, a means by which adenoviruses can be effectively and specifically delivered to tumors is needed.

Several approaches to improving overall virus delivery to tumors have been evaluated. Several of these approaches involve capsid proteins that are genetically engineered to alter viral tropism and redirect viral infection to tumor cells (3–5). Other strategies seek to mitigate the nonspecific viral sequestration at nontumor sites (6–12), because adenovirus is preferentially taken up by cells in the liver and other organs (13–15). Exploration of alternative CRAd delivery schemes may lead to the development of methods with improved clinical outcomes.

One strategy to enhance viral delivery makes use of vehicles that have endogenous tumor-targeting activity and can thereby chaperone viral delivery *in vivo*. In this regard, specific autologous cell types have been considered as potential vehicles for viral delivery to the tumor site, where replication and amplification should increase the local viral dose (16). The utility of cells as vehicles for toxic genes, antiangiogenic molecules, and immunostimulatory genes has been shown in several studies (17–19). Although the application of cell vehicles for virotherapy agents has also been proposed, only a few practical attempts using cells as viral carriers have been made to date (20, 21).

In our previous studies, we investigated the properties of mesenchymal progenitor cells (MPC) as cell carriers of anticancer therapeutics (22). These studies established that MPCs possess key functional vector attributes for cancer gene therapy. The recently reported tumor-homing properties of this cell population add further weight to the value of this cell type as potential vehicles (23, 24). Thus, we hypothesized that the lower viral replication rates and tumor-homing properties of MPCs could be exploited for effective tumor delivery of CRAd agents. The lower viral replication rates should delay virus production until the

MPC-based carriers migrate to tumor sites, thereby avoiding nonspecific sequestration, neutralization, and the endothelial barriers of targeted virus delivery. In the present study, we tested the suitability of MPCs to serve as a carrier system for the delivery of replication-competent adenoviruses to ovarian tumors *in vivo*. Our studies showed that MPC cell vehicles represent a viable means to enhance the benefits of CRAd agents and may offer a rational approach that could be easily translated to the clinical setting.

Materials and Methods

Reagents

Fibroblast antigen antibody (Ab-1) clone AS02 (Oncogene Research Products/Novagen, Madison, WI) was used for detection of MPCs in human ovarian cancer xenografts. The secondary antibody was rat anti-mouse IgG1 (PharMingen, San Diego, CA). CFDA-SE fluorescent dye was from Molecular Probes (Eugene, OR).

Cell Lines

The human ovarian carcinoma cell line SKOV3ip1 was obtained from Dr. Janet Price (University of Texas M. D. Anderson Center, Houston, TX). Firefly luciferase-expressing ovarian carcinoma call line SKOV3luc was kindly provided by Dr. Negrin (Stanford Medical School, Stanford, CA). Cells were maintained in DMEM/F-12 containing 10% fetal bovine serum (HyClone, Logan UT) and 2 mmol/L glutamine at 37°C in a humidified atmosphere of 5% CO_2. A549 and HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as recommended.

Isolation and Culture of MPCs

Primary human MPCs were obtained from leftover materials (screen filters with bone marrow cells remaining) from several individuals undergoing bone marrow harvest for allogeneic transplantation at the University of Alabama at Birmingham Stem Cell Facility under an approved institutional review board protocol. MPCs were isolated and cultured as described previously (22). Cells were expanded by consecutive subcultivations in α-MEM with 10% fetal bovine serum at densities of 5,000 to 6,000 cells/cm^2 and used for experiments at passages 2 to 8.

Recombinant Adenoviruses

Replication-incompetent recombinant adenoviral vectors having wild-type and genetically modified Ad5 fibers were used for experiments to determine their transduction efficiency on MPCs. All adenoviral vectors expressed firefly luciferase in E1 region under cytomegalovirus promoter and differ only in the fiber gene. Ad5luc has wild-type Ad5 fiber, Ad5/3luc has a chimeric fiber where the knob of Ad5 fiber is replaced by the Ad3 fiber knob (25), Ad5RGDluc has a fiber protein with an integrin-binding motif (CDCRGDCFC) inserted in the HI loop (26), and Ad5/3RGDluc has the same RGD motif inserted in the HI loop of the Ad3 knob in Ad5/3 chimeric fiber. All viruses were constructed at University of Alabama at Birmingham Gene Therapy Center.

Replication-competent viruses used in the study were Adwt, Ad5luc3, and AdΔ24wt (27) with unmodified fiber and Ad5/3 (28) and Ad5/3luc3 (25) with chimeric fiber having the knob of Ad5 fiber replaced by the knob of Ad3 fiber. Ad5luc3 and Ad5/3luc3 had a luciferase gene inserted in E3 region. Ad5RGD (28) and AdΔ24RGD (29) had fiber protein with a RGD motif in HI loop of Ad5 fiber. Viruses labeled as Δ24 bear a 24-bp deletion in E1A, resulting in a mutant E1A protein unable to bind the Rb protein, which normally allows S-phase entry, needed for virus replication (30). The rest of the viruses had unmodified E1 region.

MPC Homing to Preestablished Ovarian Tumors

I.p. tumors were established in mice by i.p. injection of 5×10^6 SKOV3ip1 cells. After 14–20 days of tumor development, mice received labeled or unlabeled MPCs i.p. at 1×10^6 cells per injection.

To detect MPCs by luciferase expression, MPCs were transduced with Ad5RGDluc at 1,000 viral particles (vp)/cell (MPC-luc) 1 day before the injection into mice with established tumor xenografts. Mice received one or two (7 days apart) injections of MPC-luc. Two days after last injection, mice were sacrificed; tumor nodules, spleen, liver, and part of the intestine were collected. Luciferase activity was measured in tissue homogenates using luciferase assay system (Promega, Madison, WI) according to the protocol. Protein concentration was measured in tissue homogenates using DC Protein Assay (Bio-Rad, Hercules, CA) according to the protocol.

To detect MPCs by fluorescence, cells were labeled with fluorescent dye CFDA-SE. The dye was added to the cells attached to the plastic at 1 mmol/L/2×10^5 cells in PBS, incubated 15 minutes, and washed away. Cells were trypsinized and used for injections. Two days after injection, tumor nodules, liver, and spleen were collected and frozen in OCT. Fluorescent MPCs were detected on fresh cryosections (6–8 μm) of tumor samples using a fluorescent microscope.

Immunostaining for unlabeled MPCs with was done with AS02 AB, specifically reacting with human MPCs. Tumor cryosections were fixed in acetone followed by several blocking steps. The endogenous peroxidase activity was blocked by 3% H_2O_2 in distilled water for 10 minutes at room temperature. Nonspecific binding was blocked by subsequent incubation with Fc-receptor block and background buster for 15 minutes (Innovex Bioscience, Pinole, CA). Primary mouse anti-human antibody Ab-1 (clone AS02) was applied in 1:20 dilution and incubated in a humid chamber overnight at 4°C. After washing, secondary antibody rat anti-mouse IgG1 in a dilution of 1:500 was applied for 1 hour at room temperature. Positive reaction was visualized with 3,3′-diaminobenzidine staining followed by hematoxylin counterstain.

In vitro Adenoviral-Mediated Gene Transfer

Recombinant replication-incompetent adenoviral vectors were used to transduce cells as described previously (22). In brief, cells were plated 24 hours before infection at 5×10^4 per well in a 24-well plate. The transduction was

done with the virus diluted in a small volume of appropriate cell-specific medium containing 2% fetal bovine serum for 2 hours at 37°C. Then, medium was replaced with complete medium containing 10% fetal bovine serum. Twenty-four hours after plating, SKOV3ip1 cells and MPCs were infected with modified adenoviral vectors at multiplicities of infection (MOI) of 100 vp/cell. Luciferase expression was determined in cell lysates after 48 hours of incubation in complete medium using luciferase assay system (Promega) according to the protocol. The luciferase activities were measured in a Lumat LB 9507 luminometer (Lumat, Wallac, Inc., Gaithersburg, MD) in relative light units and normalized by the protein concentration in cell lysates (DC Protein Assay kit). All of the experiments were done in triplicate.

Cytotoxicity Assay

For determination of virus-mediated cytotoxicity, cells were plated in 24-well plates at 1×10^5 per well and infected with adenoviruses at indicated titers or mock infected. Adenoviral-permissive A549 cells were used as a positive control for adenoviral replication and virus-induced cytopathic effect. Plates were incubated at 37°C for 7 days. To visualize cell killing, cells were fixed and stained with 1% crystal violet in 4% formaldehyde for 20 minutes followed by washing with tap water to remove excess dye. Images of plates were captured with a with a Kodak DC260 digital camera (Kodak, Rochester, NY).

Quantitative PCR Analysis of Adenoviral DNA

MPCs and HeLa cells were plated on six-well plates at a density of 2×10^5 per well 24 hours before infection. Cells were infected with replication-competent Ad5luc3 and Ad5/3luc3 viruses at MOI of 1 vp/cell. The infected cells and virus-containing supernatants were harvested 1, 3, 5, 7, and 9 days after infection and used for viral and total cell DNA isolation. Total DNA from cells was purified using the QIAamp DNA Blood kit (Qiagen, Valencia, CA) according to the kit instruction. Total cell DNA was used for quantitative PCR with E1 primers to determine copy numbers of viral DNA and with actin primers to determine number of actin DNA copies. To isolate only encapsidated viral DNA from cultural medium and destroy naked viral DNA present in the supernatant, DNase1 treatment was done. Then, EDTA, SDS, and proteinase K were added (final concentration 20 mmol/L, 0.5%, 0.2 mg/mL correspondingly) and samples were incubated at 56°C for 1 hour followed by phenol/chloroform extraction and ethanol precipitation. The purified viral DNA was dissolved in TE buffer. Real-time PCR (LightCycler, Roche, Indianapolis, IN) analysis was used for quantitative evaluation of adenoviral DNA copy number. Oligonucleotides corresponding to the sense strand of adenoviral E1 region (5′-AACCAGTTGCCGTGAGAGTTG-3′; 1,433-1,453), the antisense strand of E1 region (5′-CTCGTTAAGCAAGTCCTC-GATACA-3′; 1,500-1,476), and TaqMan fluorigenic probe (5′-CACAGCCTGGCGACGCCCA-3′; 1,473-1,455) were synthesized and used as primers and probe for real-time PCR analysis. To correct for differences in total DNA concentration for each sample, adenoviral E1A copy numbers were normalized by actin DNA copy number. Primer sequences to detect transcripts of actin DNA were as follows: sense strand 5′-CAGCAGATGTGGATCAGCAAG-3′, antisense strand 5′-CTAGAAGCATTTGCGGTGGAC-3′, and TaqMan probe 5′-AGGAGTATGACGAGTCCGGCCCCTC-3′.

Loading MPC Carriers with Ad5/3 (MPC-Ad5/3)

MPC cultures at 60% to 80% confluence were infected with Ad5/3 at MOI of 2,000 vp/cell. The next day, MPC-Ad5/3 were trypsinized, counted, and used for mixed culture experiment or for *in vivo* injections. Samples of the MPC-Ad5/3 used for injections were saved to quantitatively determine viral load of cell carriers.

Analysis of Cytolytic Effect of MPC-Ad5/3 in Mixed Cultures *In vitro*

One day before establishing mixed culture, MPCs were infected with replication-competent Ad5/3luc3 or Ad5luc as a nonreplicative control at MOI of 2,000 vp/cell as described above. The next day, infected MPCs were detached, mixed with SKOV3ip1 in ratios ranging from 5% to 50%, plated in 96-well plates at 2×10^4 per well, and cocultured. Viable cells were stained with crystal violet on days 7 and 14 after plating.

Therapeutic Ovarian Cancer Model

Female CB17 severe combined immunodeficient mice (Charles River, Boston, MA) were obtained at 6 to 8 weeks of age and quarantined for 2 weeks. Mice were kept under pathogen-free conditions according to the American Association for Accreditation of Laboratory Animal Care guidelines. Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of University of Alabama at Birmingham. On day 0, mice were injected i.p. with 5×10^7 SKOV3ip1 cells. On days 4, 8, and 12, mice were injected i.p. with either PBS ($n = 15$), MPCs at 1×10^6 per injection ($n = 15$), MPC-Ad5/3 at 1×10^6 per injection ($n = 15$), or Ad5/3 at 1×10^{10} vp/injection ($n = 15$). On day 21, five randomly selected animals from each group were sacrificed and examined for i.p. tumors. All visible tumor nodules were collected, combined, and weighed to estimate tumor burden. Data are mean ± SD. Statistical differences among groups were assessed with a two-tailed Student's t test. $P < 0.05$ was considered significant.

The rest of mice ($n = 10$) were followed daily to record survival times. Mice were killed when there was any evidence of pain or distress. Survival data were plotted on a Kaplan-Meier curve, and the MPC-Ad5/3 group was compared with the other groups using the log-rank[2] test (GraphPad Prism Software version 4, San Diego, CA).

In imaging experiments, mice were injected i.p. with SKOV3luc on day 0 followed by the injection on days 4, 8, and 12 of PBS only ($n = 5$), MPC-Ad5/3 at 1×10^6 per injection ($n = 5$), or Ad5/3 at 1×10^{10} vp/injection ($n = 5$) in 0.5 mL PBS. Three animals from each group were imaged for bioluminescence on days 3, 10, 16, 22, and 28 after establishing tumors and followed to record survival times.

Imaging and Quantification of Bioluminescence Data

An *in vivo* optical imaging was done with a custom-built optical imaging system with a liquid nitrogen–cooled 1 KB digital CCD camera (Princeton Instruments VersArray:

Roper Scientific, Trenton, NJ). Mice were anesthetized with 2% isoflurane before i.p. injection of D-luciferin. D-Luciferin potassium salt, the substrate for firefly luciferase, was purchased from Molecular Imaging Products (Ann Arbor, MI). Each mouse received injection of 30 μg D-luciferin diluted in 100 μL PBS. Mice (three animals per group) were placed in the supine position within the imaging chamber with continuous isoflurane sedation. Whole-body luminescent images were obtained during the 5- to 10-minute interval after injection of the substrate. I.p. injected luciferin is relatively stable in the interval of 10 to 30 minutes after injection (31). Luminescence images and bright-field images were acquired with an exposure time of 60 and 0.02 seconds, respectively, using WmView/32 software (Roper Scientific) without a filter at f/16. Index color image overlays were done in Photoshop 7. 0 (Adobe, Seattle, WA). The range of acquisition signal was kept constant at all imaging time points. The gray-scale photographic images and bioluminescence color images were superimposed using the Adobe Photoshop 7.0 software.

Results

MPCs Preferentially Home to Ovarian Tumors

To determine if MPCs preferentially home to human ovarian xenografts, MPCs labeled with the luciferase (MPC-luc) or fluorescent dye CFDA-SE were injected into the peritoneal cavity of severe combined immunodeficient CB17 mice carrying preestablished i.p. tumors. For labeling of MPCs with luciferase, we used replication-deficient adenoviral vectors encoding the luciferase as reporter gene. Luciferase activities higher than background therefore reflected the presence of labeled cells in tumors and other organs after the MPC-luc transplant. Two separate experiments were carried out using either single (Fig. 1A, *top*) or double (Fig. 1A, *bottom*) MPC-luc injections. Most of the tumor xenografts collected from animals that received MPC-luc showed a luciferase activity that was 1 to 2 orders of magnitude higher (mice 1–4, *top* and *bottom*) than background tumor luciferase activity (mice 6 and 7), validating an increased content of MPC-luc in tumors. A few mice had detectable levels of luciferase in the spleen and liver; however, it was generally 10 times lower than the levels observed in tumors. Interestingly, we did not detect any luciferase activity in the organs of mice that failed to develop tumors after SKOV3ip1 injection (mice 5, *top* and *bottom*). This finding suggests that MPCs did not have any homing preference in mice without tumors, in corroboration with previously reported data on MPC homing in unconditioned mice (32). Thus, the cells likely disseminated nonspecifically and could not be detected by the method used.

In the experiment where the MPCs were labeled with fluorescent dye, fluorescent cells were detected in tumor samples, located in the tumor parenchyma, or attached to the tumor nodules (Fig. 1B). Specific staining of the tumor xenograft sections for MPCs (Fig. 1C) with the AS02 antibody also confirmed the tumor-homing properties of the MPC carriers. In summary, we have shown that MPCs are capable of preferentially localizing to metastatic ovarian tumors after i.p. injection. Next, we wanted to exploit this property of cells to deliver anticancer therapeutics.

MPCs as Intermediate Adenovirus Carriers

MPC Transduction with Retargeted Adenoviral Vectors. MPCs must be highly permissive to adenoviral infection to be useful virus delivery vehicles. Previous studies have documented low level of MPC transduction with conventional adenoviral vectors due to low expression of the adenoviral attachment receptor coxsackie and adenovirus receptor (22, 33). However, adenoviruses with modified cellular integrin tropism, such as AdRGD, are able to overcome coxsackie and adenovirus receptor deficiency and achieve efficient infection of MPCs (22). To select the virus suitable for our strategy, we evaluated the transduction efficiencies of several adenoviruses genetically modified with coxsackie and adenovirus receptor–independent viral entry that were previously designed and characterized by our group. We specifically tested adenoviral vectors having the Ad5 knob replaced with the knob of Ad3 (Ad5/3luc), vectors with the RGD-4C peptide incorporated in the HI loop of the Ad5 fiber knob (Ad5RGDluc), or vectors with an Ad3 fiber knob (Ad5/3RGDluc). These structural modifications confer an expanded tropism to the adenoviruses via binding to either cellular integrins, the Ad3 receptor, or both.

Figure 2A presented the transduction patterns of aforementioned adenoviruses on a panel of primary MPC isolates obtained from different donors (MPC1–MPC5). A certain degree of variability in expansion and differentiation potentials as well as surface antigen expression profiles has been widely noted for primary MPC cultures (34). These factors may also result in variable expression of receptors involved in viral infection. For our cell-based strategy, we wanted to select an adenovirus, which combines efficient loading of carrier cells with maximal killing potency of the tumor cell targets. Therefore, ovarian cancer cell line SKOV3ip1 was tested in parallel with the MPC cultures. Despite the variability, the RGD-containing virus (Ad5RGDluc) had consistently higher transduction levels than Ad5 having wild-type fiber (Ad5luc) in all MPC isolates tested. Ad5/3luc and Ad5/3RGDluc showed variable rates of MPC transduction in different MPC samples: from the highest (MPC1) to the lowest (MPC5) compared with Ad5luc and Ad5RGDluc. This might indicate a variable pattern of expression of Ad3 receptors on different primary MPC cultures as well as a possible changing receptor expression pattern with MPC culturing. It is likely that cellular integrins, which are the entry receptors for RGD-modified virus, are more consistently expressed on MPCs than Ad3 receptors. At the same time, in the ovarian cancer cell line, Ad5/3luc showed the highest efficiency of gene transfer, in line with previous data from our group (35–37). The primary culture MPC2 was used for subsequent *in vitro* and *in vivo* experiments.

Next, we tested the level of cytopathic effect the tropism-modified replication-competent adenoviruses exerted on MPC carriers and on target ovarian tumor cells (Fig. 2B).

Replication-competent variants of viruses having Ad5 fiber (Adwt, AdwtΔ24), RGD-containing fiber (AdRGD, AdΔ24RGD), or chimeric 5/3 fiber (Ad5/3luc3, Ad5/3) were used for these experiments. Cells plated in 24-well plates were infected with the designated viruses at MOIs increasing from 0.1 to 1,000 vp/cell. A549 cells were included as a reference cell line highly sensitive to viral oncolysis. Results were read by crystal violet staining intensity on day 7, at which point the reference cell line A549 exhibited complete lysis at each viral dose applied. In agreement with data on transduction efficiency of replication-deficient adenoviral vectors, MPCs were most sensitive to the RGD-containing viruses AdΔ24RGD and Ad5RGD, both of which achieved cell lysis at MOI as low as 1 vp/cell. Adwt and Ad5/3 viruses had attenuated cytopathic effect on MPCs. SKOV3ip1 cells showed a different pattern of oncolysis with the array of viruses tested compared with MPC cultures. The most prominent oncolysis of SKOV3ip1 cells was attributed to Ad5/3 viruses, whereas AdRGD and Adwt had minimal and moderate lytic effects, respectively.

In summary, the Ad5/3 virus exhibited sufficient MPC infectivity, with limited cytopathic effect, although it was

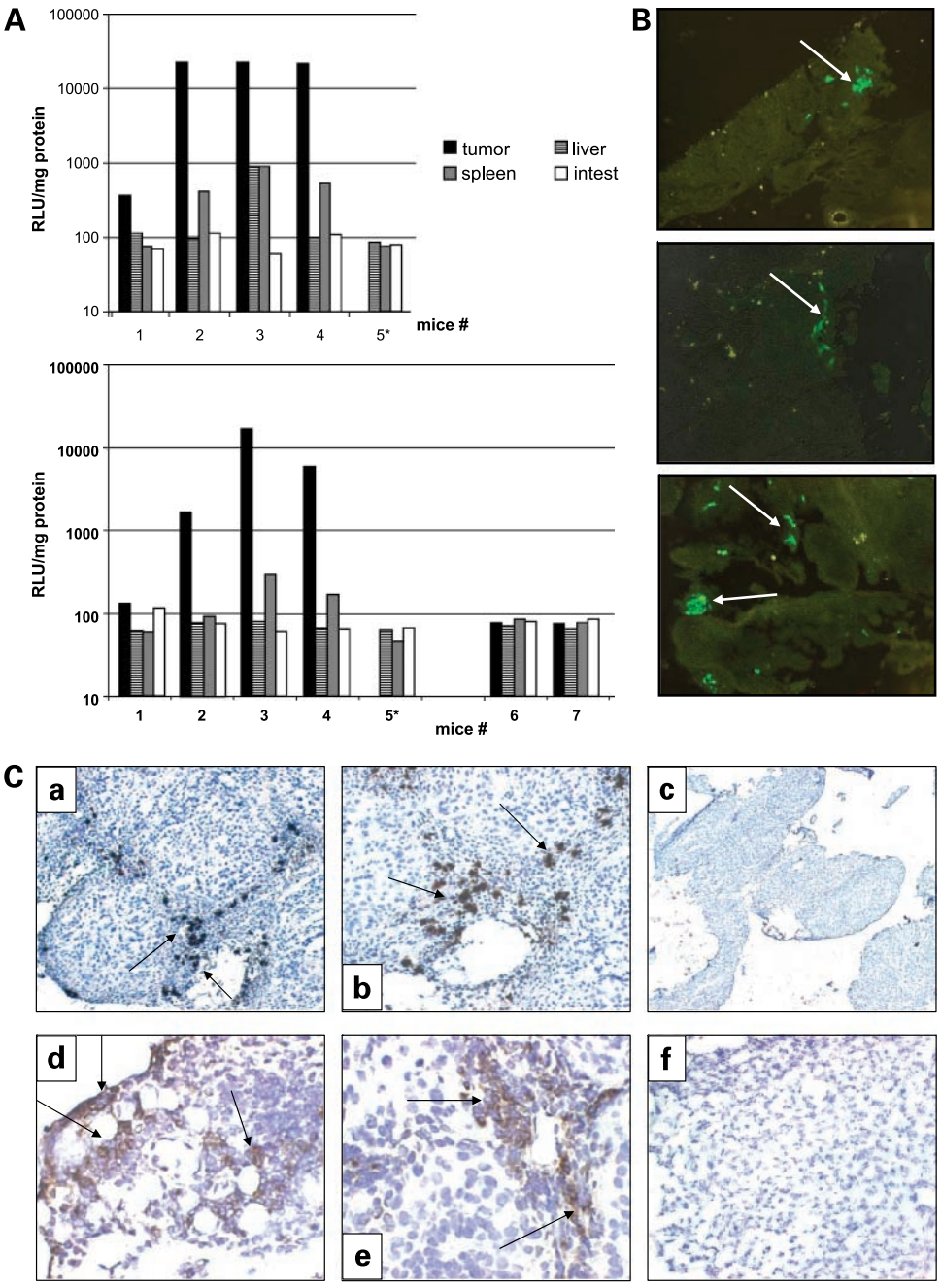

Figure 1. MPCs preferentially home to preestablished ovarian tumors. **A,** MPCs were labeled with luciferase by transduction with Ad5RGDluc (MPC-luc). Mice with preestablished i.p. tumors received one (*top*) or two (*bottom*) injections of MPC-luc. Two control mice (6, 7) did not receive MPC injections. *, mice did not develop tumor in experiment and were used as tumor-free control. Two days after last injection, mice were sacrificed; tumor nodules, spleen, liver, and part of intestine were collected. Luciferase activity was measured in tissue homogenates and presented as relative light units (*RLU*)/mg protein. **B,** MPCs were labeled with fluorescent dye CFDA-SE and injected i.p. in mice with ovarian tumor xenografts. Two days after MPC injection, mice were sacrificed and tumors were excised and frozen in OCT. Microphotographs are representing cryosections of tumor samples. *Arrows,* fluorescent MPCs located in tumor parenchyma or attached to the tumor nodules. **C,** detection of MPCs by immunohistochemistry. Tumor cryosections were stained with AS02 AB and counterstained with hematoxylin. Microphotographs were taken at lower power (**a**–**c**) and higher power (**d**–**f**) and show the distribution of MPCs in tumor nodules. Dark brown–stained MPCs (*arrows*) were detected both at the tumor periphery and tumor parenchyma. Ovarian tumor xenografts taken from animals without MPC injections were used as negative controls (**c** and **f**).

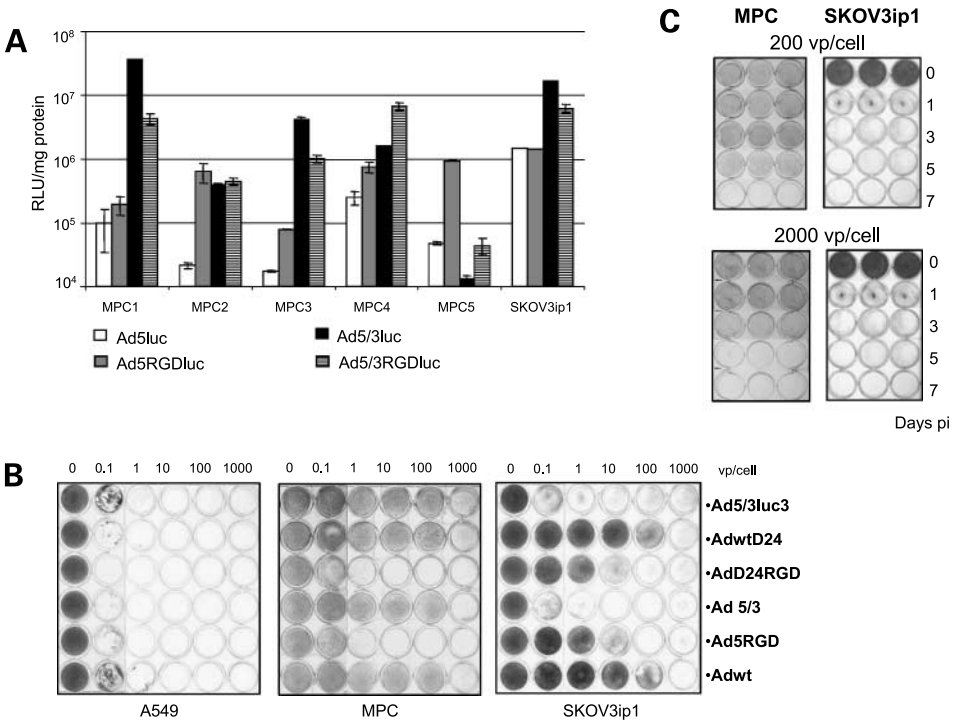

Figure 2. Loading MPC carriers with adenovirus. Retargeted adenoviruses increase efficiency of MPC infection. **A,** transduction efficiency of retargeted adenoviral vectors. Several primary MPC cultures and SKOV3ip1 cells were transduced with Ad5luc, Ad5RGDluc, Ad5/3luc, and Ad5/3RGDluc at MOI of 100 vp/cell. Luciferase activity was determined 48 h posttransduction and plotted in relative light units (*RLU*)/mg protein. *Columns,* average of four replicates; *bars,* SD. **B,** cytopathic effect of genetically modified replication-competent adenoviruses on carrier and target cells. MPCs, SKOV3ip1, and A459 cells were infected with replication-competent adenoviruses having either Ad5 wild-type fiber or genetically modified fibers Ad5RGD and Ad5/3 at MOI ranging from 0.1 to 1,000 vp/cell. Seven days after infection, cells were fixed and stained with crystal violet to visualize remaining viable cells. **C,** speed of development of cytopathic effect of Ad5/3 on MPC carriers and SKOV3ip1 target cells. MPCs and SKOV3ip1 cells were infected with replication-competent adenovirus Ad5/3luc3 at MOI of 200 and 2,000 vp/cell. At days 0, 1, 3, 5, and 7, cells were fixed and stained with crystal violet to visualize remaining viable cells.

substantially more cytopathic for ovarian tumor cells. Thus, the optimal proportion of infection and cell killing for our MPC-vehicle SKOV target strategy was achieved using the Ad5/3 virus. Based on these data, we selected the replication-competent Ad5/3 virus for our subsequent *in vitro* and *in vivo* experiments. Cells carrying this replication-competent virus were designated as MPC-Ad5/3.

We also tested the rate of cytopathic effect developed in MPC carriers and target SKOV3ip1 cells. This was done by comparing the cell viability over time for cultures infected with the same MOI (200 and 2,000 vp/cell). Oncolytic effect of Ad5/3 on SKOV3ip1 cell cultures developed much faster at both MOIs tested and was apparent 1 day after infection (Fig. 2C). On the contrary, MPCs did not show significant lytic changes until 3 days after infection even at the highest MOI tested. Thus, MPC carriers infected with Ad5/3 remained viable several days after infection, which would provide an opportunity for MPC manipulation *ex vivo* and a time window for loading, introduction, and homing of the MPC carriers.

MPCs Can Support Adenoviral Replication. One of the advantages of using cells as intermediate viral carriers is amplification of the initial viral load. The accumulation of viral DNA during infection is one of the indicators of adenoviral replication. Therefore, we quantitatively measured the viral DNA content in infected MPCs. Several primary MPC cultures were infected with the Ad5/3luc3 and Ad5luc3 replication-competent adenoviruses at a MOI of 1 plaque-forming unit/cell, and the development of infection was followed for 11 days. Real-time PCR was used to measure the amount of viral DNA in infected cells and the medium over time (Fig. 3). The replication of Ad5 in infected MPC cultures was 10 to 100 times lower than that of HeLa cells, a reference cell line supporting a high level of adenoviral replication (Fig. 3A). More dramatic differences were observed between the two cultures when the viral DNA content was determined in the medium (Fig. 3A, *top*); the MPC culture showed 1,000 times lower viral DNA content compared with HeLa cells. Ad5/3 showed enhanced replication of viral DNA versus Ad5 in MPCs (Fig. 3B) as judged by viral DNA content in infected cells and culture medium. These data suggest that adenovirus can successfully replicate in MPC carriers and the level of replication depends on the efficiency by which the MPCs were initially infected.

Virus-Loaded MPCs Exert Oncolytic Effect on Tumor Cells in Mixed Culture

Next, we tested whether MPC-Ad5/3 could exert oncolytic effect on target cells *in vitro*. We estimated the efficacy of the tumor cell killing caused by virus-loaded cell carriers. To this end, the oncolytic effect of target tumor cells cocultured with virus-loaded MPCs (MPC-Ad5/3) was compared with that produced by direct infection of SKOV3ip1 with A5/3luc3. MPCs were infected with Ad5/3luc3 at a MOI of 2,000 vp/cell 1 day before establishing mixed cultures. The next day, virus-loaded MPCs were plated with target SKOV3ip1 cells in ratios ranging from 5% to 50%. After 7 days, we observed cell lysis in the wells having only 5% of MPC-Ad5/3 (Fig. 4A). Direct infection of SKOV3ip1 cells with replication-competent adenovirus Ad5/3luc required a MOI of at least 10 vp/cell for similar cytopathic effects to be observed 7 and 14 days after infection (Fig. 4B). To exclude toxic effects of adenoviral infection itself, which may contribute to cell lysis, tumor cells were mixed at the same ratios with carriers infected with nonreplicative virus and were subjected to direct infection with nonreplicative virus. Nonreplicative virus did not cause cell lysis when applied directly or carried by cells. Thus, coculturing of SKOV3ip cells with MPC-Ad5/3 at low ratio resulted in the killing of tumor cells.

Therapeutic Effect of MPC-Based Delivery of Oncolytic Viruses *In vivo*

We hypothesized that administration of virus-loaded MPCs would allow selective delivery of virus to disseminated ovarian tumor nodules and would improve the therapeutic effect over virus alone. To test the MPC-based delivery *in vivo*, we generated orthotopic xenografts of human ovarian cancer in severe combined immunodeficient mice by injecting SKOV3ip1 cells. Four days after administration of tumor cells, mice received three doses of Ad5/3 alone (group T + Ad; $n = 15$) or MPC-Ad5/3 (group T + MPC/Ad; $n = 15$). Two control groups received either PBS (group T; $n = 15$) or uninfected MPCs (group T + MPC; $n = 15$). MPCs loaded with virus were given 1 day after *in vitro* infection. Five randomly selected mice from each group were sacrificed at day 21 and all visible tumor nodules were excised and weighed to estimate the tumor burden in each cohort. The average tumor weight for untreated PBS (groups 1) and uninfected MPCs (group 2) was 184 ± 39 and 228 ± 92 mg, respectively (Fig. 5A). The average tumor weight in animals that received direct Ad5/3 injections was 186 ± 75 mg, similar to control animals treated with PBS. However, in animals that received MPC-Ad5/3, the tumor growth was delayed and the average tumor burden at day 21 was 52 ± 7 mg, which was significantly less than the three others groups ($P = 0.006$). The remaining mice in each group were followed to determine survival rates (Fig. 5B). Based on log-rank tests, statistically significant differences in median survival time were calculated between control and treated groups ($P < 0.0001$). The median survival time was 34 days for the PBS-treated controls (T) and 44 days for the uninfected MPC transplanted controls (T + MPC). However, in groups treated with oncolytic virus (T + Ad5/3) or oncolytic virus-infected MPCs (T + MPC-Ad5/3), the median survival time was extended to 59 and 69 days, respectively. Administration of MPC-Ad5/3 increased the mean survival time compared with direct administration of Ad5/3; however, it only presented a trend and was not statistically significant. Assuming the survival advantage can be caused by higher amounts of virus introduced by cell carriers compared with direct viral injection, we

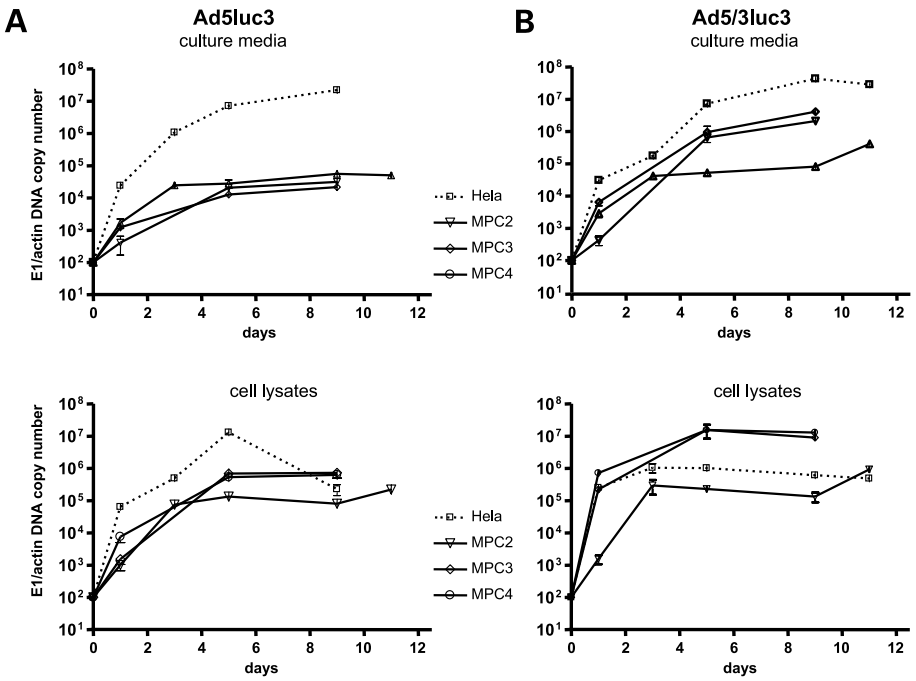

Figure 3. Amplification of viral load in MPC carriers. Increased viral burst of Ad5/3luc3. Several primary MPC cultures and HeLa cells were infected with Ad5luc3 (**A**) or Ad5/3luc3 (**B**) at MOI of 1 plaque-forming unit/cell. Infected cells and culture medium were collected at days 1, 3, 5, 7, and 11. Three wells for the same cell culture were used for each time point. Total DNA from cells and viral encapsidated DNA from culture medium were isolated. Amount of viral DNA corresponding to amount of virus produced was estimated by quantitative PCR. *Points,* mean ratios of viral DNA (E1) to human actin DNA copy number of triplicates; *bars,* SD.

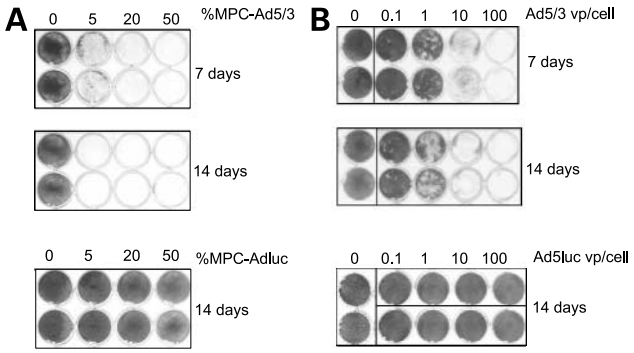

Figure 4. MPC-Ad5/3 exert oncolytic effect on ovarian SKOV3ip1 cells in mixed culture. **A,** coculture of infected MPCs and SKOV3ip cells mixed in different ratios. One day before establishing mixed culture, MPCs were infected with replication-competent Ad5/3luc3 or Ad5GFP as nonreplicative control at MOI of 2,000 vp/cell. The next day, infected MPCs were detached, mixed with SKOV3ip1 in ratios ranging from 5% to 50%, and plated. Viable cells were stained with crystal violet on days 7 and 14 after plating. **B,** direct oncolytic effect of Ad5/3luc3 on SKOV3ip1 cells was estimated in parallel by infection with Ad5/3luc3 at MOI of 1.0 to 100 vp/cell. Infection with nonreplicative virus Ad5GFP at the same MOI was used as control for nonreplicative adenoviral toxicity. Viable cells were stained with crystal violet on days 7 and 14 after infection.

quantitatively estimated the viral load in all samples of MPC-Ad5/3 before *in vivo* injection. The total viral load in MPC-Ad5/3, determined by quantitative PCR in viral genome copy numbers, corresponded to 0.7×10^9 to 2.3×10^9 vp, which was less than the viral dose used for direct injection (1×10^{10} vp). Therefore, the therapeutic advantage of adenoviruses delivered by MPCs was attributed to other factors, most likely to selective tumor delivery or ongoing amplification of virus in the cell carriers.

To visualize the potential therapeutic effects of our treatments, we took advantage of a SKOV3luc cell line that stably expresses luciferase, which allows noninvasive estimates of tumor burden *in vivo*. When introduced i.p., these cells develop tumor xenografts similar to SKOV3ip1 in terms of localization, metastasis, and growth rate. Xenografts were induced in mice (three groups; $n = 5$) by injection with 5×10^6 SKOV3luc cells, and the same treatment schedule with Ad5/3 or MPC-Ad5/3 was used as described above. Mice were imaged after i.p. injections of D-luciferin on days 3, 10, 16, 22, and 28 (Fig. 6A). Low bioluminescent signals of SKOV3luc were detected as early as 3 days after i.p. injection, which greatly increased by day 10. At the last imaging time point (28 days), two mice from the tumor only group had bioluminescent signals that exceeded the camera's limit of detection indicative of bulky tumor growth in the peritoneal space. One mouse from this group did not develop tumors and thus represented a negative control for luciferase imaging. Lower light intensities were detected in the groups that received direct Ad5/3 injections or Ad5/3-MPC compared with control groups, indicating a slower rate of tumor development in the groups exposed to the oncolytic adenovirus. Animals in the imaging groups were also followed to determine the length of survival. The animals in the treated group eventually succumbed to the tumors and died but at later time points than the untreated mice. The average survival time increased from 32 days for the untreated cohort to 42 days in the cohort injected with straight virus (T + Ad) and 60 days in the cohort transplanted with virus-infected MPCs (T + MPC/Ad; Fig. 6B). The rate of tumor development correlated with the tumor mass detected by image intensity. The median survival rates for each cohort in this experiment also correlated with the data obtained in the previous animal experiment. Moreover, statistically significant differences between T + Ad and T + MPC/Ad groups were calculated using log-rank test ($P = 0.006$).

In summary, MPCs loaded with replication-competent adenoviruses were able to exert an oncolytic effect on tumor cells *in vitro* and *in vivo*. Delivery of the virus by cell carriers with endogenous tumor-targeting properties resulted in a therapeutic benefit compared with viral injections alone.

Discussion

Although the benefits of cells as viral carriers have been recognized, only a few practical attempts of using

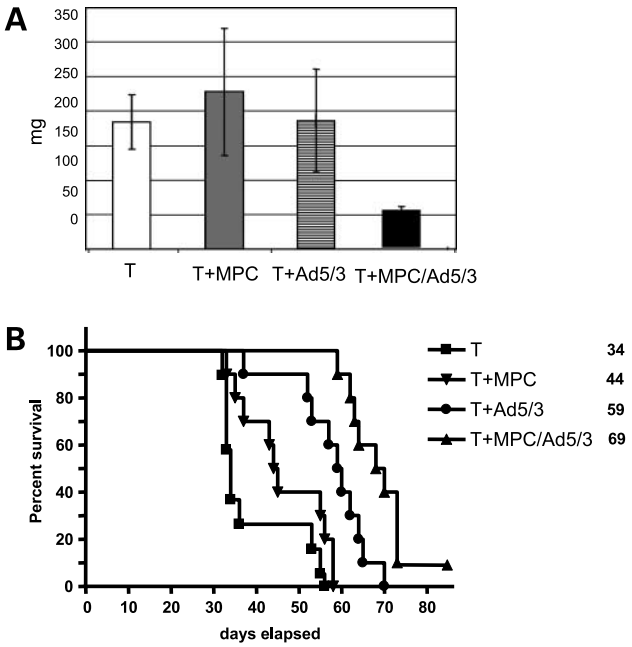

Figure 5. Therapeutic effect of oncolytic adenoviruses delivered by MPC carriers. **A,** average tumor burden in experimental groups. Ovarian tumor xenografts were established in peritoneum cavity of CB17 severe combined immunodeficient mice by i.p. injection of SKOV3ip1 cells at 5×10^6 per animal. On days 4, 8, and 12, animals received three i.p. injections of MPCs preloaded with Ad5/3 (T + MPC/Ad; $n = 15$) at 1×10^6 cell/injection or Ad5/3 (T + Ad; $n = 15$) at 1×10^{10} vp/injection. Control groups received PBS (T; $n = 15$) or uninfected MPCs (T + MPC; $n = 15$). Five randomly selected animals from each group were sacrificed on day 21 to estimate tumor development. All visible tumor nodules were collected, combined, and weighed. **B,** analysis of survival. Mice from the same experimental groups ($n = 10$ for each group) were monitored for survival. The percentage of surviving animals is plotted against the number of days after tumor cell introduction. Number adjacent to the group label is the median survival time.

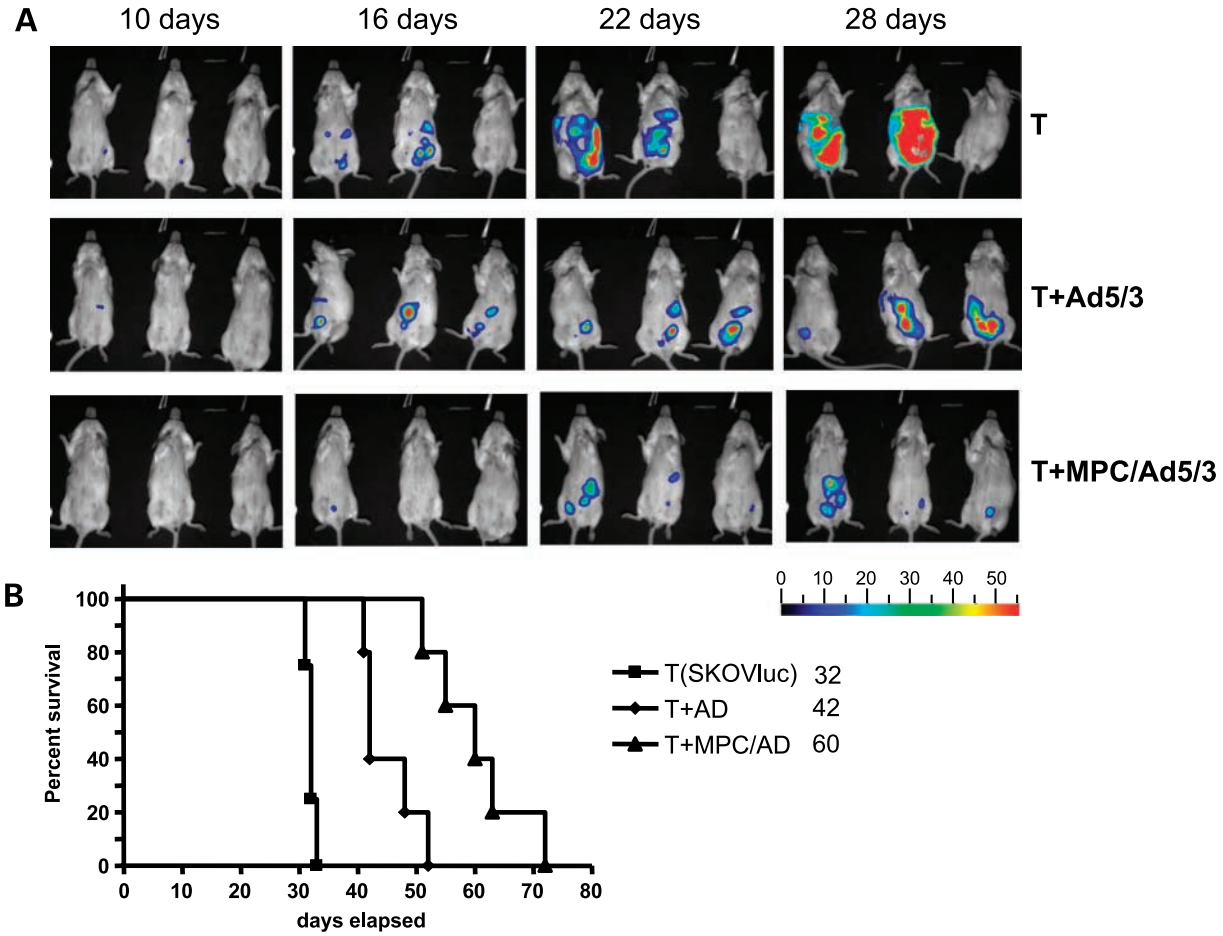

Figure 6. Noninvasive assessment of tumor burden in animals treated with oncolytic adenoviruses. **A,** ovarian tumor xenografts were established in peritoneum cavity of CB17 severe combined immunodeficient mice by i.p. injection of SKOV3luc cells at 5×10^6 per animal. On days 4, 8, and 12, animals received three injections of MPCs preloaded with Ad5/3 (T + MPC/Ad; $n = 5$) at 1×10^6 cells/injection or direct injections of Ad5/3 (T + Ad; $n = 5$) at 1×10^{10} vp/injection. Control groups received PBS only (T; $n = 5$). Three animals from each group were imaged weekly for bioluminescence starting on day 3. Location and magnitude of light captured by optical CCD imaging after i.p. injection of D-luciferin on days 10, 16, 22, and 28. A pseudocolor image representing light intensity in a scale from 0 to 55 relative light units (black, least intense and red most intense, corresponding to ≥55 relative light units) and black and white images were superimposed. **B,** survival of animals in imaging groups. The percentage of surviving animals is plotted against the number of days after tumor cell introduction. Number adjacent to the group label is the median survival time.

cells as viral vehicles have been reported to date. The use of cells for tumor delivery of retrovirus (20) and parvovirus (21) has previously been proposed. We reasoned that delivery of replication-competent adenoviruses would also benefit by exploiting intermediate cell carriers.

The cell type used for delivery of the virus is a critical determinant in the efficacy of the treatment. For instance, application of murine fibroblasts as retroviral producers for glioma treatment resulted in minimal viral spread low therapeutic benefit in these protocols (38, 39). The importance of the carrier cell type was shown in subsequent studies by Namba et al. (19, 40, 41), in which different cell carriers were tested for glioma treatment, with neural progenitor cells showing the best therapeutic efficacy (40). Tumor cells themselves are often chosen as carriers for ''suicide'' genes or viruses (19, 41). T cells have also been tested as carriers of retroviruses and adenoviruses (20, 42, 43). Recently, MPCs, for which tumor-homing capacity has been shown, have been considered promising cell populations for cancer applications (44–46).

The present study addresses the utility of MPCs as intermediate carriers for replication-competent adenoviruses, which has not been described hitherto. The choice of MPCs as cellular vehicles was based on their tumor-homing capacity (46, 47). The ability of MPCs to incorporate into tumors as stromal elements may allow these cells to release virus from the inside of the tumor. In addition, the fact that these cells can be obtained through noninvasive techniques and are readily expanded *ex vivo* suggests that autologous carrier cells could be used. Thus, we reasoned that MPCs represents an ideal population for virus carrier cells.

The extent of the tumor-homing ability of injected MPCs has not been widely investigated. Tumor localization and proliferation of injected MPCs has only been confirmed in animal models of lung metastases (23) and glioma (45, 46). These properties are thought to be attributed to intrinsic properties of MPCs in the presence of certain external signals released by local tumor environment (45). It is possible that different tumor types give out similar cues to attract MPCs. However, the specificity of MPCs may be influenced by the experimental conditions or a particular tumor type. In this regard, ''homing'' of MSCs in lung metastasis models may be enhanced after systemic injection due to the mechanical trapping of these cells in the capillary system. Intraperitoneal infusion of MPCs to target i.p.-based tumors presents a different experimental setting. Thus, we initially tested the ability of MPCs to preferentially localize in the vicinity or within growing ovarian tumors after i.p. injection. The accessibility of tumor nodules in the peritoneal ovarian cancer model also gave an opportunity to introduce cell carriers locally, avoiding the cell trapping effect of systemic injections before tumor homing. We used MPCs labeled with luciferase to show that MPCs preferentially localize in growing tumor nodules but not in the organs of abdominal cavity. Nevertheless, a low level of MPC dissemination into other organs may not be completely ruled out due to sensitivity of detection with this method.

In our study, we only investigated the homing effect of MPCs (2–4 days after introduction). We did not follow the remote fate of injected cells, because our strategy was to exploit only immediate or initial routing of cells to tumors. In this regard, delivery of oncolytic viruses does not rely on long-term survival and proliferation of cell carriers. It is anticipated that cell carriers would reach tumors to establish cell-to-cell contact with tumor cells and, after performing the delivery mission, would eventually be destroyed by viral replication.

Amplification of the initial adenoviral load in the MPC carriers is an additional attractive potential of cell-based viral delivery. Kinetics of viral DNA replication in MPCs showed that adenoviruses can be efficiently amplified in the cell carriers to produce a second generation of the virus. The level of amplification of Ad5/3 in MPCs almost reached that of the adenoviral-permissive HeLa cell line. Thus, MPCs could also function as viral factories, allowing the total amount of virus needed for therapeutic effects to be decreased compared with direct viral injection.

One of the practical issues for consideration in a cell-mediated virus delivery strategy is how to limit lytic viral activity in the carrier cells until the cells reach their tumor targets. We found that MPCs behave as semipermissive cells for adenoviruses, nevertheless allowing adenoviral replication and thus increasing the initial viral dose. We preselected the virus Ad5/3 as optimal for our strategy by screening a virus panel and picking a virus with limited cytopathic effect in MPCs and maximum cytopathic effect in the tumor cell line. It has been shown that wild-type adenoviral replicates and kills highly proliferating cells more efficiently and therefore only exerts an attenuated effect on quiescent cells (48). This could explain the lower cytopathic effect rate of adenoviruses on MPCs compared with tumor cells.

The issue of preventing or attenuating the cytolytic effect of viruses on cell carriers may be realized by various means. For example, in the study of Herrlinger et al. (49). Herpes simplex virus-1 vector replication was temporarily arrested in cell carriers by treating the cells with mimosine, allowing time for cell migration and virus delivery. Controlled replication of retroviruses in T-cell carriers was proposed in the study of Crittenden et al. (42) and Yotnda et al. (43). The cytotoxicity of adenoviruses for cell carriers can also be regulated by the introduction of regulatory elements that enable viral replication only under specific conditions. The most straightforward regulatory mechanism to control adenoviral replication would be to use the Tet-on/Tet-off systems (50). Another promising way is to use tumor-specific promoters, which have attenuated activity in noncancerous cell carriers but show high transcriptional activity in tumor cells. Importantly, in clinical settings, these selectively oncolytic viruses would represent safer variants for virotherapy. Multiple variants of CRAds have been constructed and tested to date, which would apparently benefit from cell-mediated tumor targeting and delivery. In our experiments, we used replication-competent virus Ad5/3, which in essence represents the human Ad5 wild-type virus with a fiber modified for targeting purposes. However, on a mouse model, the selective replication of Ad5/3 only in tumor cells is expected, because human adenoviruses do not replicate in mouse cells. Thus, using this virus for our proof-of-principle study created a situation similar to using CRAds in humans, which selectively replicates in tumors versus normal tissues.

Only a limited number of studies have been carried out to compare the therapeutic effects caused by direct administration of viruses and virus delivery via cell-based carriers (21, 51). In the study of Raykov et al. (21) and Coucos et al. (51), the authors reported that an *in vivo* oncolytic effect of attenuated herpes simplex virus-1716 on ovarian cells was enhanced by the utilization of a carrier cell line delivering herpes simplex virus-1. We show here that MPC-based viral delivery promoted the oncolytic effect of adenoviral treatment and increased the median survival of tumor-bearing animals, thus validating the feasibility of using MPCs as carriers for replication-competent adenoviruses and the advantage of this strategy over those using direct viral injections. Although complete tumor regression was not achieved in both treatment groups, the cell-based delivery was superior in terms of delayed tumor development. The dose of virus injected with cell carriers was measured on the time of introduction and corresponded to 0.7×10^9 to 2.3×10^9 vp, which was lower than the total dose of virus injected directly at 1×10^{10} vp/mouse. Therefore, the superior therapeutic effect in animals receiving MPC-Ad5/3 cannot be attributed to a higher dose of initially introduced adenoviruses. This effect

may result either from the amplification of viral load in cell carriers or targeted virus delivery and release of the virus in the vicinity of tumor cells at multiple foci. Both of these factors need to be confirmed and require further investigation. However, regardless of the mechanism, the rationale for using MPCs as adenoviral vehicles is valid.

The viral dose selected for our *in vivo* experiment (1×10^{10} vp) was in a high range comparable with viral doses (1×10^{8}–1×10^{10} vp) used in several CRAd protocols used for treatment in animal models of ovarian cancer (35–37). These studies documented that direct i.p. introduction of wild-type adenovirus lead to liver toxicity, which was the primary cause of lethality, rather than tumor development. In our experiment, we did not observe this extent of toxic effects because the animals in the Ad5/3-treated groups survived longer than those without treatment. At the time of death, animals in the adenovirus-treated and MPC-Ad5/3-treated groups had prominent tumors, indicating that deaths were related to carcinomatosis. Imaging experiments also revealed development of tumors in both experimental groups. However, the direct toxic effect of the virus cannot be completely ruled out and potentially could be the reason for moderate prolongation of survival in animals with treatment.

Virus-armed cells present a complex biotherapeutic system that has the attractive aspect of exploiting the intrinsic abilities of both biological agents. In our study, we initially aimed to optimize the cell-based virus carrier system from the perspective of choosing an appropriate virus for the cell carrier. To this end, we showed that viruses with modified tropism provide superior cargo for MPC-based carriers. However, the proposed delivery platform has sufficient flexibility and great potential for improving both components of this system through genetically engineered properties.

In summary, in the current work, we have evaluated the potential utility of MPCs as cellular vectors to chaperone delivery of oncolytic adenoviruses to tumors. We confirmed that MPCs have an intrinsic capacity to home to growing i.p. tumors. We tested the ability of MPCs to carry out productive adenoviral infection and showed that selection of the appropriate virus as the cell cargo results in an increased viral production by the cell carriers. We showed the oncolytic effects of adenoviruses produced by MPCs on the ovarian cancer cell line (SKOV3ip1) in mixed culture *in vitro*. Applying our strategy for the treatment of peritoneally disseminated ovarian carcinoma in a murine model, MPCs armed with replication-competent adenovirus resulted in augmented efficiency of MPC-Ad5/3 therapy. These findings are consistent with the concept that MPCs possess key properties that validate their use as cellular vehicles for virotherapy.

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Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses

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