Tumor and Vascular Targeting of a Novel Oncolytic Measles Virus Retargeted against the Urokinase Receptor

Yuqi Jing, Calli Tong, Jin Zhang, Takaumi Nakamura, Ianko Iankov, Stephen J. Russell, and Jaime R. Merchan

Division of Hematology-Oncology, University of Miami Miller School of Medicine and Sylvester Comprehensive Cancer Center, Miami, Florida and Department of Molecular Medicine, Mayo Clinic, Rochester, Minnesota

Abstract

Oncolytic measles virus (MV) induces cell fusion and cytotoxicity in a CD46-dependent manner. Development of fully retargeted oncolytic MVs would improve tumor selectivity. The urokinase-type plasminogen activator receptor (uPAR) is a tumor and stromal target overexpressed in multiple malignancies. MV-H glycoproteins fully retargeted to either human or murine uPAR were engineered and their fusogenic activity was determined. Recombinant human (MV-h-uPA) and murine (MV-m-uPA) uPAR-retargeted MVs expressing enhanced green fluorescent protein (eGFP) were rescued and characterized. Viral expression of chimeric MV-H was shown by reverse transcription-PCR and Western blot. In vitro viral replication was comparable to MV-GFP control. The receptor and species specificity of MV-uPAs was shown in human and murine cells with different levels of uPAR expression. Removal of the NH2-terminal fragment ligand from MV-uPA by factor Xa treatment ablated the MV-uPA functional activity. Cytotoxicity was shown in uPAR-expressing human and murine cells. MV-h-uPA efficiently infected human endothelial cells and capillary tubes in vitro. I.v. administration of MV-h-uPA delayed tumor growth and prolonged survival in the MDA-MB-231 breast cancer xenograft model. Viral tumor targeting was confirmed by immunohistochemistry. MV-m-uPA transduced murine mammary tumors (4T1) in vivo after intratumor administration. MV-m-uPA targeted murine tumor vasculature after systemic administration, as shown by dual (CD31 and MV-N) staining of tumor capillaries in the MDA-MB-231 model. In conclusion, MV-uPA is a novel oncolytic MV able to replicate in murine cells and target tumor vasculature in a uPAR-dependent manner.

Introduction

Oncolytic virotherapy is an innovative biological strategy that holds great promise for the treatment of cancer. Because oncolytic viruses could, in principle, be genetically engineered to specifically target, replicate in, and ultimately kill tumor cells, they may offer advantages over conventional treatments (1, 2). The Edmonston vaccine strain of measles virus (MV-Edm; ref. 3) is a novel oncolytic virus currently being evaluated in phase I clinical trials in ovarian cancer, multiple myeloma, and glioblastoma multiforme.3 MV-Edm exerts its cytopathic effects by formation of multinuclear cell aggregates (i.e., syncytia) resulting from fusion of infected cells (1). Cell fusion is mediated by the MV-H glycoprotein, which binds to the endogenous MV-Edm cell surface receptor CD46, and signals to MV-F to trigger cell fusion. As fusion progresses, surrounding nontransfected cells are recruited into expanding syncytia, generating a significant local bystander effect (4, 5).

Although MV-induced cytopathic effects seem to preferentially affect tumor cells, normal cells could also be affected (6–8), limiting the therapeutic potential of these agents. A desirable target for an oncolytic virus should be biologically relevant, overexpressed by tumors and tumor stromal cells, to potentially amplify the antitumor effects of the virus. Development of oncolytic viruses against murine tumor targets would allow the testing of retargeted oncolytic viruses in syngeneic cancer models to characterize and predict virus-tumor-host interactions that may be relevant for human clinical studies.

The plasminogen activator (PA) system consists of a family of proteases [urokinase (uPA), tissue plasminogen activator, and plasmin], receptors, and inhibitors and is involved in the regulation of coagulation, angiogenesis, and tumor growth (9–12). The importance of the PA system in breast and other human malignancies is well established (13–15). Binding of uPA with its receptor (uPAR) initiates a proteolytic cascade that results in the conversion of plasminogen to plasmin, extracellular matrix degradation, and activation of matrix metalloproteinases (10). Functionally, uPA can be divided into three independent regions: an NH2-terminal epidermal growth factor (EGF)-like domain, a kringle domain, and a COOH-terminal catalytic domain (16). The first two domains compose the NH2-terminal fragment (ATF). The receptor-binding module resides in the EGF-like domain, in residues 21 to 32 (17).

The urokinase receptor (uPAR) is a three-domain (D1, D2, and D3) glycosyl phosphatidylinositol–anchored protein with a high affinity (1 nmol/L) for uPA, pro-uPA, and the ATF (18). The molecular role of uPAR in cancer progression is well characterized. In addition to its participation in extracellular matrix degradation, uPAR elicits a number of nonproteolytic cellular responses involved in tumor progression and angiogenesis, such as cell migration, adhesion, differentiation, and proliferation (19–22).

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Requests for reprints: Jaime R. Merchan, Division of Hematology-Oncology, University of Miami Miller School of Medicine, 1475 Northwest 12th Avenue, Suite 3300, Miami, FL 33136. Phone: 305-243-1287; Fax: 305-243-9828; E-mail: jmerchan@med.miami.edu.

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uPAR is overexpressed in breast tumor cells as well as in tumor stroma, and its presence has been associated with an aggressive tumor phenotype and adverse prognosis (21, 23–26). Moreover, preclinical studies have shown that targeting the uPAR by monoclonal antibodies or antisense oligonucleotides is a promising tumor-selective strategy for the treatment of uPAR-overexpressing tumors (27–29).

In the present study, we report on the generation and characterization of fully retargeted oncolytic MVs against the human and murine uPAR, as well as the assessment of their tumor and endothelial targeting abilities in vitro and in vivo.

Materials and Methods

Cell culture. Vero (African green monkey kidney), NIH-3T3 (immortalized mouse fibroblasts), CHO (Chinese hamster ovary), 786-O (human renal carcinoma), MC38 (murine colon carcinoma), CT-26 (murine colon cancer), Hep3B (human hepatoma cell lines), and MDA-MB-468 (human breast carcinoma) cells were purchased from the American Type Culture Collection (ATCC) and maintained in DMEM containing 10% fetal bovine serum (FBS). HT1080 (human fibrosarcoma cells) and MDA-MB-231 (human breast cancer) cells were obtained from ATCC and maintained in RPMI 1640 with 10% FBS. 4T1 (murine mammary carcinoma) cells (a gift from Dr. Carlos Arteaga, Vanderbilt University, Nashville, TN), NIH3T3/RAS cells (a gift from Dr. Y. Ikeda, Mayo Clinic, Rochester, MN), and Vero-aHis cells (30) were maintained in DMEM with 10% FBS. The rescue helper cell line 293-3-46 (30) was grown in DMEM with 10% FBS and 1.2 mg/mL G418 (Invitrogen). Coronary artery smooth muscle cells, human mammary epithelial cells, human umbilical vein endothelial cells (HUVEC), and human dermal fibroblasts were purchased from Lonza and maintained in growth medium as recommended by the vendor. All cells were grown at 37°C and 5% CO₂.

Generation of stable uPAR-overexpressing and knockdown cell lines. Total RNA was isolated from 4T1 cells using the RNeasy Mini Kit.
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(Qiagen). The cDNA encoding murine uPAR was obtained by reverse transcription-PCR (RT-PCR) as a BamHI/NotI fragment using the following primers: forward, 5'-TTTGATCGATCATGAGAAGCTCACAG; reverse, 5'-TTTGGGCGGCTCAGAGGGACGGACAG-3'. The purified PCR product was cloned into the BamHI/NotI site of the lentivector viral pH-5-SIN-CSGWd1NotI (a gift of Dr. Ikeda) and the cDNA sequence was verified. Lentiviral packaging was done as previously reported (30). CHO cells were infected with lentivirus, and clones expressing high levels of murine uPAR were sorted by fluorescence-activated cell sorting (FACS). Stable uPAR knockdown 4T1 cells were generated using vectors containing microRNA-based shRNA targeting mouse uPAR and nonselecting control vectors (Open Biosystems). Vectors were delivered into 4T1 cells using Arrest-In transfection reagent (Open Biosystems) following the manufacturer's recommendations. The levels of expression of uPAR were analyzed by real-time PCR (Applied Biosystems) and flow cytometry.

**Flow cytometry.** Cells were harvested by cell dissociation buffer (Invitrogen) and the surface expression of relevant receptors was detected using anti- uPAR FITC-conjugated antibody (R&D systems) and anti-human CD46 FITC-conjugated antibody (Abcam). Washed cells were analyzed on a Becton-Dickinson FACScan Plus cytometer and analyzed using CellQuest software (Becton-Dickinson). Data were displayed as flow cytometric histograms of >5,000 events. Relative changes in cell surface uPAR or CD46 expression levels were determined by quantitative assessment of fluorescence shifts (from flow cytometry data) using WinMDI 2.9 software (J. Trotter, Scripps Research Institute) and expressed as fold changes of the mean fluorescence intensity.

**Construction of chimeric MV-H Glycoproteins retargeted against the uPAR.** Total RNA was isolated from 786-O cells. The cDNA encoding human uPA was obtained by RT-PCR using the following primers: forward, 5'-TTTTGCTAGATCATGAGAAGCTCACAG; reverse, 5'-TTTTGGGCGGCTCAGAGGGACGGACAG-3'. The cDNA was cloned into the Nhel/NotI site of pcDNA3.1(+) (Invitrogen). The ATF of human uPA was amplified as a SfiI/NotI fragment by PCR from pcDNA3.1(+)-uPA using the following primers: forward, 5'-GGCCGACGCCGCAATGAACTCTCATCA-3'; reverse, 5'-TGGCGGGCCTTTCTTCTGCGGACTGATG-3'. ATF of mouse uPA was amplified by PCR from pcDNA3.1(+)-muPA (31) using the following primers: forward, 5'-GGCCGACGCCGCAATGAACTCTCATCA-3'; reverse, 5'-TGGCGGGCCTTTCTTCTGCGGACTGATG-3'. Human and murine uPA-ATFs were cloned into the SfiI and NotI cloning sites of pTNH6AALS, respectively, and verified by DNA sequencing. The constructs containing the chimeric MV-H glycoproteins were named pTNH6AALS-b-ATF (targeting human uPAR) and pTNH6AALS-m-ATF (targeting murine uPAR), respectively.

**Cotransfections and cell fusion assay.** Cells (2 x 10^5 per well) were seeded in each well of six-well plates. After overnight incubation, cells were cotransfected with 0.5 μg of pCGF, which encodes the full-length infectious clone of MV-Edm, resulting in the expression of the E protein, and 1 μg of the following: 1 μg of the cDNA encoding human uPA (29), 1 μg of the cDNA encoding murine uPA (31), or the expression vector pCMV-pAG (28). At 48 h after transfection, the syncytia were scored by fluorescence microscopy (Nikon). Experiments were done in triplicate.

**In vivo experiments to test the oncolytic activity of retargeted MVs.** Animal studies were approved by the Institutional Animal Care and Use Committee of University of Miami and the Mayo Clinic. Human breast cancer xenografts were established by inoculating MD-MBA-231 cells (2 x 10^6 in 50 μL PBS) using a 27-gauge needle into the fifth mammary fat pads of 9- to 10-wk-old female immunodeficient [nonobese diabetic (NOD)/severe combined immunodeficient (SCID)] mice (The Jackson Laboratory). When tumors reached a diameter of 0.4 to 0.5 cm, animals were randomized into two groups [(n = 10 per group) and received seven i.v. injections of either MV-H-uPA at 1 x 10^8 TCID50 in 50 μL Opti-MEM (treatment group) or equal volumes of Opti-MEM alone (mock therapy group) on days 0, 2, 4, 7, 9, 12, and 14. Tumor-bearing animals were followed until they reached sacrifice criteria (tumor burden reached 10% of body weight, tumor ulceration occurred, or mice became moribund). Tumor size was measured every 3rd day and the volume (product of 0.52 x length x width^2) was recorded.

**In vivo experiments for detection of measles.** For assessment of tumor targeting after i.v. viral administration, human breast cancer xenografts were inoculated as described above and the tumors reached 0.6 cm in diameter, mice received two i.v. injections (3 μg apart) of recombinant MVs at 1.5 x 10^6 TCID50 in 50 μL Opti-MEM. Seventy-two hours after the last treatment, tumors were resected and frozen for further studies. For assessment of in vivo viral transduction in a murine mammary cancer model, 2 x 10^6 4T1 cells in PBS were implanted s.c. into the fifth mammary fat pads of 9- to 10-wk-old female SCID (n = 3) and immunocompetent (n = 3) BALB/c mice (Harlan Sprague-Dawley). When tumors have reached 0.5 cm in diameter, mice were given two intratumoral injections of MV-m-uPA at a dose of 1 x 10^8 TCID50. Forty-eight hours after the last treatment, mice were sacrificed and tumors were resected. Enhanced green fluorescent protein (eGFP) expression of fresh tumor sections was analyzed by a Zeiss LSM 410 laser scanning confocal microscope (Carl Zeiss).

**Immunohistochemistry studies.** Frozen tumor sections were used for immunostaining for measles N protein and CD31. Reagents were obtained from Vector Laboratories unless otherwise specified. Crystallized sections were fixed in cold acetone for 10 min and endogenous peroxidase activity was quenched with 0.3% H2O2 for 10 min. The slides were incubated with biotinylated mouse anti-MV-nucleoprotein antibody (Chemicon International) for 30 min at 37°C. The slides were developed with VECTASTAIN ABC-alkaline phosphatase (AP) kit and an AP substrate kit. For dual staining (MV-N and CD31) of tumor capillaries, slides were prepared as above and incubated with biotinylated antibodies to murine CD31 (BD Biosciences Pharmingen). Positive staining was visualized with a VECTASTAIN ABC (HRP) kit and 3',3'-diaminobenzidine substrate. The slides were then treated with an avidin blocking kit, washed in PBS, and incubated with rabbit HRP second antibody (Millipore) and applied, and the peroxidase activity was revealed with the enhanced chemiluminescence system (Amersham Bioscience).

**Matrigel tube formation assay and infection with viral particles.** The Matrigel tube formation assay was done as previously described (33, 34). When HUVEC tubes were formed (16 h after HUVEC plating into Matrigel coated wells), they were infected with either MV-GFP control or MV-uPA diluted in Opti-MEM (MOI 1) for 2 h. After 2 h, medium containing the virus was removed and endothelial growth medium (EGM-2) was added. Infection efficiency was measured as mean fluorescence intensity in arbitrary units by NIS-Elements image analysis software (Nikon). Experiments were done in triplicate.

**Assessment of in vitro cytotoxic effects.** Cells were plated in six-well plates at a density of 10^5 per well. Twenty-four hours after seeding, the cells were infected at a MOI of 1 in 1 mL of Opti-MEM for 2 h at 37°C. The same number of uninfected cells in six-well plates was used as controls. At 48, 72, and 96 h after infection, the number of viable cells (determined by trypan blue exclusion) in each well was counted using Vi-Cell cell viability analyzer (Beckman Coulter). The percentage of surviving cells was calculated by dividing the number of viable cells in the infected well by the number of viable cells in the uninfected well corresponding to the same time point, as previously reported (35, 36). Infection was confirmed by fluorescent microscopy (Nikon) at the corresponding time points.

**Tumors were infected at a MOI of 1 in 1 mL of Opti-MEM for 2 h at 37°C.** The same number of uninfected cells in six-well plates was used as controls. At 48, 72, and 96 h after infection, the number of viable cells (determined by trypan blue exclusion) in each well was counted using Vi-Cell cell viability analyzer (Beckman Coulter). The percentage of surviving cells was calculated by dividing the number of viable cells in the infected well by the number of viable cells in the uninfected well corresponding to the same time point, as previously reported (35, 36). Infection was confirmed by fluorescent microscopy (Nikon) at the corresponding time points.
Biotinylated mouse anti-MV-nucleoprotein antibody for 30 min at 37°C. After washing in PBS, the slides were developed with VECTASTAIN ABC-AP kit and an AP substrate kit.

**Statistical analysis.** *In vivo* data are presented as mean ± SD. Results from *in vivo* studies are shown as mean ± SD. Statistical analysis among groups was done by ANOVA followed by Tukey-Kramer, Fisher’s, or Wilcoxon rank sum test, and *P* < 0.05 was considered to be statistically significant. Overall survival was analyzed by the Kaplan-Meier method and differences were analyzed by the log-rank test. All statistical tests were two-sided.

**Results**

**Functional activity of chimeric MV-H glycoproteins retargeted against uPAR.** Human and murine uPAR–retargeted MV-H glycoproteins were generated by displaying ATF of human and murine uPA, respectively, at the COOH terminus of pTNHₐALS (Fig. 1A). Functional activity of the chimeric MV-H glycoproteins was assessed by cotransfection assays. Human and murine pTNHₐALS-ATF glycoproteins contain a (coagulation) factor X(a) cleavage linker (IEGR; ref. 32) before the NotI restriction site (Fig. 1A). An aliquot of MV-GFP or MV-h-uPA viral particles was pretreated with 20 μg/mL of activated factor X(a) (New England Biolabs) or PBS (mock treatment) for 30 min at room temperature, in sterile conditions, before infection of MDA-MB-231 cells. Western blot analysis shows successful factor X(a)-induced cleavage of the linker and detachment of the uPA-ATF from the H glycoprotein (Western blot, far right lane). Untreated MV-GFP and MV-h-uPA (MOI, 0.5) induced cell fusion in MDA-MB-231 (bottom; 1, MV-GFP; 2, MV-h-uPA). Factor X(a) treatment of MV-GFP did not affect cell fusion (3). Factor X(a) treatment of MV-h-uPA prevented fusion and syncytia formation in MDA-MB-231 cells (4). CHO cells stably overexpressing murine uPAR (2) underwent fusion and syncytia formation after infection with MV-m-uPA, compared with wild-type CHO cells (1). 3, 4T1 cells express murine uPAR and undergo fusion after infection with MV-m-uPA (MOI, 1). uPAR expression in this cell line was knocked down by a retroviral vector encoding microRNA-based shRNA against mouse uPAR. uPAR expression was significantly decreased as determined by real-time PCR (data not shown) and by flow cytometry (86% decrease, as assessed by FACS analysis of uPAR). uPAR knockdown (4) of 4T1 cells reduces the ability of MV-m-uPA to induce cell fusion. Bar, 100 μm.

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differences between human and murine uPAR and ATF, where human urokinase (ATF) does not bind to murine uPAR and vice versa (37). Expression of the chimeric MH-V glycoproteins (of larger size than unmodified MV-H) was shown at the RNA level by RT-PCR of Vero-aHis cells exposed to the recombinant viruses (Fig. 1C-I) and at the protein level by Western blot of MV-H in naked viral particles (Fig. 1C-II). In vitro viral propagation was assessed by the previously described one-step growth curve (38). The retargeted viruses propagated efficiently in Vero-aHis cells in a His-dependent manner compared with (unmodified) MGFP control virus.

Receptor and species specificity of MV-uPA. To assess uPAR species specificity, human and murine cancer cells overexpressing uPAR were infected with human or murine retargeted oncolytic MVs. MDA-MB-231 cells were sensitive to MGFP- and MV-h-uPA–induced cell fusion but resistant to MV-m-uPA (Fig. 2A). MC-38 cells were resistant to unmodified MV-GFP and MV-h-uPA (Fig. 2B). MV-m-uPA, however, efficiently infected MC-38 cells and induced cell fusion (Fig. 2B).

The receptor specificity of MV-uPA was assessed by the following experiments: First, the ATF ligand was removed from the retargeted virus by factor X(a) treatment of MV-h-uPA. Western blot analysis showed successful cleavage of the linker and separation of the ATF fragment of uPA from MV-H glycoprotein (Fig. 2C, Western blot, second versus fourth lane). Factor X(a)–treated MV-h-uPA virus, but not mock-treated virus, lost its ability to induce cell fusion in MDA-MB-231 cells (Fig. 2C, compare 4 with 2), confirming that MV-h-uPA–induced cell fusion is dependent on binding of MV-H-ATF to uPAR. Factor X(a) treatment of MGFP did not affect cell fusion (Fig. 2C, compare 1 with 3).

Next, CHO cells and CHO-muPAR cells were exposed to MV-m-uPA (Fig. 2D). MV-m-uPA infection of CHO cells, which do not express mouse uPAR (they express hamster uPAR), resulted in modest cell fusion (Fig. 2D-I); CHO-muPAR cells, on the other hand, underwent cell fusion and syncytia formation on infection with MV-m-uPA (Fig. 2D-II). MGFP and MV-h-uPA did not induce significant cell fusion in CHO cells (data not shown).

Finally, 4T1 cells were exposed to MV-m-uPA and syncytia were observed (Fig. 2D-2). 4T1-muPAR knockdown cells became less susceptible to infection with MV-m-uPA (Fig. 2D-4). The above data clearly show that human and murine MV-uPA enter cells and induce cell fusion/syncytia formation in a uPAR-dependent and species-specific manner.

uPAR-dependent in vitro endothelial cell and capillary infection. The importance of uPAR during angiogenesis is well established, with roles in migration, invasion, and cell signaling (16, 39–41). uPAR expression was up-regulated in HUVEC cells stimulated with 2% serum and angiogenic growth factors, as compared with HUVECs maintained in basal conditions (Fig. 3B). Levels of CD46 (MV-Edm natural receptor) did not significantly change after HUVEC exposure with EGM-2 (Fig. 3A). Increased uPAR expression after stimulation of HUVEC monolayers was associated with more efficient infection by MV-h-uPA (mean fluorescent intensity, 312,826 ± 25,379; Fig. 3C-2) compared with the unmodified MGFP control (mean fluorescent intensity, 102,558 ± 21,567; P < 0.001; Fig. 3C-1). Next, the ability of MV-h-uPA to infect capillary tubes was assessed. HUVEC capillary tubes were exposed to MV-h-uPA. Compared with MGFP, MV-h-uPA was associated with more efficient capillary infection, as evidenced by more frequent fluorescent capillary tubes (higher mean fluorescence intensity, 53,040 ± 8,356) induced by the retargeted versus the control virus (mean fluorescence intensity, 11,497 ± 2,393; P < 0.001; Fig. 3C-3, 4 and 6 versus 3 and 5).

In vitro cytopathic effects. Human and murine cancer cell lines were exposed to the recombinant viruses, and cell viability was determined at different time points by trypan blue exclusion (35, 36). In human cancer cells (MDA-MB-231 and 786-O), MV-h-uPA

Figure 3. uPAR-dependent in vitro endothelial cell infection. HUVECs were grown in full endothelial growth medium (EGM-2; stimulated) or in EBM-2 medium with 1% FBS (unstimulated). Stimulation of HUVEC monolayers with 2% serum and growth factors (endothelial growth medium, EGM-2) was associated with up-regulation of uPAR (B), but not CD46 (A), compared with unstimulated HUVECs (endothelial basal medium and 1% FBS). Changes in HUVEC expression of CD46 and uPAR were determined by FACS analysis, and displayed as fold increase of mean fluorescence index (MFI) before and after stimulation. HUVEC monolayers were infected with viruses at a MOI of 0.5. C, in stimulated HUVECs, MV-h-uPA induced cell fusion more efficiently than MGFP (2, versus 1). Bar, 100 μm. HUVECs (grown in EGM-2) were plated on Matrigel and tubes were allowed to form (16 h). Once tubes were formed, they were infected with either MGFP (3 and 5) or MV-h-uPA (4 and 6) at a MOI of 1. Pictures of the areas of abundant tubes mostly in the center of wells were taken at 72 h after infection. MV-h-uPA was associated with more efficient capillary infection compared with the unmodified virus control (4 and 6 versus 3 and 5). Experiments were done in triplicate. Bar, 500 μm (3 and 4); 50 μm (5 and 6).
and MV-GFP induced significant (P < 0.001, Tukey-Kramer test) cytotoxicity at 48, 72, and 96 hours, compared with MV-m-uPA (Fig. 4A and B). The cytopathic effects of MV-h-uPA were more prominent than those of MV-GFP (72-hour cytotoxicity: 84% versus 74% in MDA-MB-231 cells and 79% versus 70% in 786-O cells, respectively). MV-m-uPA, on the other hand, induced significant cytotoxicity (P < 0.001, Tukey-Kramer test) in the murine tumorigenic cell lines 4T1 (72-hour cytotoxicity: 64%; Fig. 4C) and 3T3 Ras (72-hour cytotoxicity: 57%; Fig. 4D), compared with MV-h-uPA (cytotoxicity: 7% and 16% in 4T1 and 3T3 Ras, respectively). Murine cell lines were resistant to MV-GFP and MV-h-uPA because they do not express human CD46 or human uPAR. **, P < 0.001, MV-m-uPA versus MV-GFP and MV-h-uPA at 72 h.

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Figure 4. *In vitro* cytopathic effects of MV-uPA. Human and murine tumorigenic cells were infected with different viruses at a MOI of 1 and viability was determined at different time points (48, 72, and 96 h) by trypan blue exclusion and presented as percentage of uninfected cells. Human cancer cell lines MDA-MB-231 (A) and 786-O (B) underwent significant cytotoxicity when treated with MV-h-uPA at 48, 72, and 96 h. *, P < 0.001, MV-GFP, MV-h-uPA versus MV-m-uPA at 72 h. C and D, MV-m-uPA induced significant cytotoxicity in the murine tumorigenic cell lines 4T1 (C) and 3T3-Ras (D). Murine cell lines were resistant to MV-GFP and MV-h-uPA because they do not express human CD46 or human uPAR. **, P < 0.001, MV-m-uPA versus MV-GFP and MV-h-uPA at 72 h.

**In vivo antitumor effects and in vivo tumor targeting.** To determine whether the *in vitro* cytopathic effects are relevant on human breast cancer *in vivo*, recombinant oncolytic viruses were administered systemically into (MDA-MB-231) tumor-bearing mice. MV-h-uPA treatment was associated with significant antitumor effects compared with mock treatment (Fig. 5A) Tumor growth in MV-h-uPA–treated mice was inhibited by 76% at day 39 (P = 0.0009, Wilcoxon rank sum test). Significant prolongation of survival was observed in mice treated with MV-h-uPA compared with mock [80-day survival rate in the control group: 0% versus 70% in the MV-h-uPA–treated group; median survival in control group: 63 days (95% confidence interval, 42–70 days), versus median survival not reached in the MV-h-uPA–treated group by the end of the study: 100 days; P = 0.0039, log-rank test; Fig. 5B]. Systemic administration of MV-h-uPA was not associated with acute or subacute toxicity in tumor-bearing mice during or after i.v. administration.

Tumor targeting of the recombinant viruses after systemic administration of the viral agent was assessed in additional groups of tumor-bearing mice. They were treated with two i.v. injections of recombinant MVs, and tumors were resected for immunohistochemistry studies for MV-N protein. As shown in Fig. 5C, viral protein was detected in the tumors treated with i.v. MV-h-uPA, but not in the mock-treated tumors, confirming the *in vivo* tumor targeting abilities of MV-h-uPA after systemic administration.

**In vivo murine tumor transduction and tumor vascular targeting of MV-m-uPA.** *In vitro* viral replication of MV-m-uPA was shown in Fig. 1D, in Vero-oHis (monkey) cells, in a His-dependent manner. To show replication of MV-m-uPA in a murine uPAR–dependent manner, the one step replication curve was done in NIH-3T3 cells infected with MV-m-uPA. As seen in Fig. 6A and B, MV-m-uPA was able to replicate in the murine tumorigenic cells, showing murine uPAR–dependent viral replication in *in vitro*. Replication of MV-m-uPA in murine cells, however, was less efficient than its replication in Vero-oHis cells.

To evaluate *in vivo* viral transduction in a syngeneic model, BALB/C mice bearing 4T1 tumors were injected with MV-m-uPA intratumorally. Fresh tumors were sectioned and examined by laser confocal microscopy. Tumors treated with MV-m-uPA showed GFP-positive structures, consistent with intratumor syncytia (Fig. 6C-1 and C-2 and Supplementary Fig. S2C, SCID mice; Fig. 6C-3 and Supplementary Fig. S2B, immunocompetent mice). Tubular GFP-positive structures were identified, resembling infected tumor capillary structures (Fig. 6C-3). This data strongly suggests successful *in vivo* viral transduction in a murine uPAR–dependent manner. No signs of acute toxicity were observed in mice after intratumoral treatment with MV-m-uPA.

To assess the ability of MV-m-uPA to target tumor vasculature *in vivo*, mice bearing MDA-MB-231 tumors were injected systemically with MV-m-uPA, and double staining for MV-N
protein and CD31 in tumor capillaries was done on resected tumors. As shown in Fig. 6D-3 (and Supplementary Fig. S3C, F, and H), after systemic administration of MV-m-uPA in tumor-bearing mice, strong staining for MV-N protein (blue) was observed around tumor capillaries (CD31 in brown color). Tumor capillaries from mice treated with vehicle control or MV-GFP stained positive for CD31 but did not stain for MV-N (Fig. 6D-I and Supplementary Fig. S3A, D, and G for vehicle control; Fig. 6D-2 and Supplementary Fig. S3B, E, and H for MV-GFP). Because MV-m-uPA does not efficiently bind to MDA-MB-231 cells (which express human uPAR; Fig. 2A), the above findings show the ability of the murine version of MV-uPA to target murine tumor capillaries in vivo in a uPAR-dependent manner, after systemic administration. Importantly, we did not observe any acute toxicity in tumor-bearing mice treated with two i.v. injections of the murine uPAR–retargeted oncolytic virus (MV-m-uPA), suggesting the safety and specificity of the murine retargeted oncolytic virus after systemic administration.

**Discussion**

In this report, we successfully rescued and characterized a novel Edmonston vaccine strain oncolytic MV (MV-Edm) fully retargeted against the uPAR, a highly relevant tumor and tumor stromal target (42, 43). uPAR-retargeted MVs were generated by displaying the uPAR binding ATF of either human (MV-h-uPA) or murine (MV-m-uPA) uPA into the COOH terminus of a mutant MV-H glycoprotein (H_{AALS}; ref. 32) that lacks the ability to attach to its endogenous receptors. We showed that the human and murine uPAR–retargeted viral agents were able to attach to, replicate, and induce cell fusion and cytotoxicity in a receptor- and species-specific manner. MV-h-uPA significantly delayed tumor progression and improved survival in a breast cancer xenograft model. Finally, we presented evidence that MV-h-uPA can efficiently infect activated endothelial cells and that MV-m-uPA can transduce murine tumors and target tumor capillaries in vivo.

Clinical development of fully retargeted oncolytic MVs requires a comprehensive assessment of the pharmacokinetic and pharmacodynamic properties of the viral agents in appropriate preclinical models. This information will be important to predict potential safety or biodistribution issues before human testing. Previously reported oncolytic MVs redirected against tumor targets such as CD38, EGF receptor (EGFR), and EGFRvIII have shown potent antitumor effects (7, 32, 44). However, full development of these agents into phase I trials is hindered by the inability to test these viruses in syngeneic cancer models because the above agents cannot replicate in murine cells. Attempts have been made to generate targeted oncolytic MVs to be used in syngeneic cancer models. Ungerechts and colleagues (45) reported on the activity of MV-PNP-anti-carcinoembryonic antigen (CEA) against murine colon tumors expressing human CEA. However, because murine tissues do not express human CEA, studies to predict virus-host-tumor interactions and tissue biodistribution are not possible, and the in vivo utility of MV-PNP-anti-CEA is limited to murine tumors artificially expressing CEA.

MV-uPA is the first oncolytic MV retargeted against a tumor target (uPAR) that is relevant for and naturally expressed in human and murine tumors. Because MV-m-uPA can infect and transduce murine tumors in vitro and in vivo, it provides a valuable tool to assess important issues relevant to the safety and biodistribution of the viral agents in unmodified, immunocompetent murine tumors. As shown in Fig. 6D, after systemic administration of MV-m-uPA in tumor-bearing mice, strong staining for CD31 but did not stain for MV-N (Fig. 6D-I and Supplementary Fig. S3A, D, and G for vehicle control; Fig. 6D-2 and Supplementary Fig. S3B, E, and H for MV-GFP). Because MV-m-uPA does not efficiently bind to MDA-MB-231 cells (which express human uPAR; Fig. 2A), the above findings show the ability of the murine version of MV-uPA to target murine tumor capillaries in vivo in a uPAR-dependent manner, after systemic administration. Importantly, we did not observe any acute toxicity in tumor-bearing mice treated with two i.v. injections of the murine uPAR–retargeted oncolytic virus (MV-m-uPA), suggesting the safety and specificity of the murine retargeted oncolytic virus after systemic administration.

**Discussion**

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**Figure 5.** In vivo antitumor effects and tumor targeting. A, MDA-MB-231 xenografts were established orthotopically by implantation of MDA-MB-231 into the mammary fat pads of female NOD/SCID mice. When the tumors reached a mean diameter of 0.4 to 0.5 cm, the animals (10 per group) were treated with seven doses of MV at 1 × 10^9 TCID_{50} per dose. Mice in the mock therapy group were injected with equal volumes of Opti-MEM. A, systemic MV-h-uPA treatment was associated with a significant retardation in tumor growth compared with controls. *P < 0.0009, Wilcoxon rank sum test. B, Kaplan-Meier analysis of survival of tumor-bearing mice treated with vehicle control or MV-h-uPA. Mice were monitored until they reached sacrifice criteria (see Materials and Methods). There was a significant prolongation of survival in the MV-h-uPA treatment group compared with control. ***, P < 0.0039, log-rank test. C, in vivo experiments for detection of MV. In separate experiments, tumor-bearing mice were twice injected i.v. (n = 3 per group) with 2 × 10^9 TCID_{50} viruses. Tumors were harvested 3 d later and frozen tumor sections were used for immunostaining for measles N protein. Viral protein was detected in the tumors after i.v. administration of the virus. Bar, 100 μm.
models of cancer naturally expressing uPAR in a way that may more closely resemble the human cancer situation.

The tumor endothelium represents a barrier to the effective delivery of therapeutic agents into tumor cells (46). Because endothelial cells are easily accessible after i.v. administration of a therapeutic agent, oncolytic viruses that target tumorendothelium may have the advantage of circumventing this problem because they may bind to endothelial cells, replicate in them, and facilitate viral entry into tumor tissue (46, 47). In this report, we showed that human endothelial uPAR expression is increased on stimulation with serum and endothelial growth factors (Fig. 3B). This is concordant with previous reports suggesting that vascular endothelial growth factor or tumor cell–conditioned medium up-regulates endothelial uPAR expression (40, 48), and emphasizes the importance of uPAR in tumor angiogenesis (41, 48). Under those conditions, MV-h-uPA infects HUVEC monolayers and capillary tubes (in Matrigel) more efficiently than the unmodified MV-Edm. Importantly, systemic administration of MV-m-uPA successfully targeted and infected tumor vasculature, as evidenced by positive double staining (CD31 and MV-N) of tumor capillaries in MV-m-uPA–treated mice (Fig. 6D-3 and Supplementary Fig. S3C, F, and I), as compared with controls (Fig. 6D-1 and D-2 and Supplementary Fig. S3A, D, G and B, E, H). This provides first-line evidence of the ability of a murine retargeted oncolytic virus to reach the tumor vasculature compartment in vivo after i.v. administration.

A previous study reported on the activity of an oncolytic MV-Edm (called “MV-ERV”) displaying echistatin, a disintegrin that binds to αvβ3 (38). Antitumor effects and in vivo capillary infection were shown by local administration of MV-ERV into tumors or chick chorioallantoic membranes, respectively. MV-uPA differs from MV-ERV in that it induced antitumor effects (MV-h-uPA) and tumor vascular targeting (MV-m-uPA) in vivo after systemic administration (Fig. 6D-3). Moreover, i.v. administration of MV-m-uPA in tumor-bearing mice was safe, whereas safety of MV-ERV after systemic administration was not assessed.

Although we have achieved the important goal of rescuing and characterizing a murine retargeted oncolytic MV, which is safe and able to target tumor vasculature in vivo, a number of questions are raised in this study. MV-m-uPA replicates in murine cells in vitro (Fig. 6A and B); however, the efficiency of viral replication (viral
titers) in rodent cells is lower than its replication in nonmurine (e.g., Vero-α His) cells (Fig. 1D). This suggests the presence of intracellular restriction of mechanisms to replication and generation of progeny virus in rodent cells, which needs to be addressed in future studies. Second, uPAR is expressed in a number of normal tissues, potentially raising issues about the specificity and safety of targeting this receptor. Tumor selectivity of uPAR directed therapies has been shown by Rabbani and colleagues, who showed in rat models of cancer that antisense uPAR antibodies specifically target tumor tissues and are able to detect occult tumor metastases in vivo (29). Such tumor selectivity may be explained by the fact that uPAR expression is significantly higher in tumor and tumor stroma compared with normal tissues (42, 43, 49). Induction of uPAR expression by activated endothelium provides an additional advantage for MV-uPA by increasing its ability to bind to tumor capillaries. Finally, we found that intratumor or i.v. administration of MV-muPA was not associated with acute toxic effects in tumor-bearing mice.

In summary, this study shows successful rescue and characterization of a uPAR-retargeted MV, evidence of successful infection and replication in a receptor and species specific manner, and, for the first time, the ability of a murine retargeted virus to reach murine tumor vasculature safely and efficiently. MV-uPA is a highly promising retargeted oncolytic virus with the potential of becoming an important novel therapeutic agent against breast cancer and other uPAR-overexpressing malignancies. Studies aimed at overcoming barriers to viral replication in vitro and in vivo and characterizing the virus-host-tumor interactions in syngeneic, immunocompetent models of cancer are under way.

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

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References


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