The relationship between ligand–receptor affinity and antitumor potency of an oncolytic virus was investigated using a panel of six HER2/neu (HER2)-targeted measles viruses (MV) displaying single-chain antibodies (scFv) that bind to the same epitope on HER2, but with affinities ranging from $10^{-6}$ to $10^{-11}$ M. All viruses were able to infect SKOV3ip.1 human ovarian cancer cells in vitro, but only the high-affinity MV (Kd $\geq 10^{-8}$ M) induced cytopathic effects of syncytia formation in the cell monolayers. In contrast, all six viruses were therapeutically active in vivo against orthotopic human ovarian SKOV3ip.1 tumor xenografts in athymic mice compared with saline-treated controls. The oncolytic activities of MV displaying the high-affinity scFv (Kd $= 10^{-9}, 10^{-10}, 10^{-11}$ M) were not significantly superior to MV displaying scFv with Kd of $10^{-8}$ M or less. Results from this study suggest that increasing the receptor affinity of the attachment protein of an oncolytic MV has minimal impact on its in vivo efficacy against a tumor that expresses the targeted receptor.

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INTRODUCTION

Replication competent and tumor selective viruses are being developed as oncolytic agents for cancer therapy. Several phase II trials are in progress and a recently completed phase III melanoma trial with intralesional injection of Talimogene laherparepvec (Oncovec, herpes simplex virus) showed promising survival data. Ideally, the oncolytic virus should infect and spread efficiently in cancer cells but minimally in normal cells. With some viruses, this can be achieved by transcription targeting through use of tissue-specific promoters or transductional targeting through specific receptor–ligand interactions to achieve tumor selective activity. Adenoviruses, which have poor infectivity in cancer cells due to downregulation of the coxsackie adenovirus receptor, have been engineered to display binding peptides to enhance their infectivity and spread on cancer cells. Viruses such as vesicular stomatitis virus that have a broad tropism can be redirected to bind to tumor-associated receptors by pseudotyping them with Sindbis or measles-envelope glycoproteins displaying single-chain antibodies (scFv) with specificity for tumor-associated receptors.

The attenuated Edmonston B strain of measles virus (MV) has potent oncolytic activity against various types of cancers. It selectively causes extensive syncytia formation and cell death in tumor cells and shows minimal cytopathic effects in normal cells. Fully retargeted MV that do not bind to their natural receptors, CD46 and SLAM (signaling lymphocyte activation molecule), but efficiently infect and fuse cells through alternative receptors have been generated. The virus tropism is now dependent on the displayed ligand at the C terminus of the MV hemagglutinin (H) attachment protein.

HER2/neu (HER2), also known as ErbB-2, is a member of the epidermal growth factor receptor family. Aberrant HER2 expression, generally attributed to gene amplification and receptor overexpression, has been described in various types of cancer, especially breast and ovarian cancers. Retargeted HER2-specific oncolytic viruses have been established from MV, herpes simplex virus, adenovirus serotype 5, simian adenovirus serotype 24 and vesicular stomatitis virus. Due to its importance as a cancer target, there has been intense interest in the development of HER2-targeted therapeutics. A panel of anti-human HER2-specific scFvs was generated by phage display and sequential mutagenesis. These scFvs target the same epitope but bind to HER2 with affinities (Kd) ranging from $10^{-6}$ to $10^{-11}$ M. We previously established a panel of six recombinant MV displaying this panel of HER2 scFvs. The scFv is displayed as a C-terminal extension on the measles H attachment protein that is ablated for binding to MV receptors, CD46 and SLAM (signaling lymphocyte molecule). As such, the HER2-targeted MV binds to and infects cells exclusively through the HER2/neu receptor. The panel of HER2 viruses selectively infected and caused cytopathic effect specifically in HER2-positive cells. In vitro, there was a threshold scFv affinity (Kd $= 10^{-8}$ M) required for the efficient scFv–receptor interaction below which viral infectivity and intercellular fusion were severely compromised. Viruses displaying scFv with affinities above $10^{-8}$ M did not induce larger syncytia in the HER2-positive cells in vitro. In contrast to the lower-affinity viruses, the higher-affinity viruses ($\geq 10^{-9}$ M) were able to induce syncytia formation in low-HER2-expressing cells.

The role of ligand–receptor affinity in oncolytic activity of MV has not been previously evaluated in vivo. Here, we used multicellular spheroids composed entirely of HER2-positive tumor cells and human tumor xenografts grown in athymic mice as models. Virus infectivity, size of infectious centers, intratumoral spread and antitumor activity in an orthotopic intraperitoneal (i.p.) model of ovarian cancer were evaluated.
MATERIALS AND METHODS

Cell lines and viruses

The human epithelial ovarian carcinoma cell line, SKOV3ip.1 (a kind gift from Ellen Vitetta, University of Texas Southwestern Medical Center) and SKOV3ip.1-Fluc cells, which stably express firefly luciferase, were maintained in alpha-MEM supplemented with 20% fetal bovine serum and 2 mM L-glutamine.16,17 Human rhabdomyosarcoma TE671 and Vero-xHis cells, which stably express a membrane-anchored scFv that recognizes a six-histidine peptide, were maintained in Dulbecco modified Eagles medium supplemented with 10% or 5% fetal bovine serum, respectively.18 The six fully retargeted HER2-specific MV (MV-xHER-6 to MV-xHER-11) were propagated and titered on Vero-xHis cells as previously described.21 These viruses were aborted for binding to their natural receptors, CD46 and SLAM, and encode an enhanced green fluorescent protein (GFP) reporter gene.10 A six-histidine peptide was inserted at the C terminus of H to enable virus rescue and propagation on Vero-xHis cells.

Generation and infection of multicellular tumor spheroids

Multicellular spheroids of tumor cells were prepared by using the liquid overlay technique.19,20 Spheroids were incubated with viruses (multiplicity of infection 0.5) at 37 °C. To quantitate the extent of virus infection, spheroids were fixed with 4% paraformaldehyde and nuclei were stained with 20 ng ml

-1 Hoechst 33342 (Molecular Probes, Eugene, OR, USA). Virus infection and viral spread (size of GFP foci) were assessed using a Zeiss LSM-510 confocal system on an Axioplan-2 microscope (Carl Zeiss, Dublin, CA, USA).

In vivo experiments

All procedures involving animals were approved by and performed according to the guidelines of the Mayo Foundation Institutional Animal Care and Use Committee. Female 4–5-week-old NCR athymic mice (Harlan-Sprague-Dawley, Indianapolis, IN, USA or Taconic Laboratories, Germantown, NY, USA) were injected i.p. with 2 × 10^6 SKOV3ip.1-Fluc cells in 250 μl Dulbecco phosphate-buffered saline. Five days later, when tumors were established in the omentum, mice (n = 10 per group) were treated i.p. with three doses, given every other day, of 2 × 10^6 TCID50 of each of the HER2-targeted viruses or saline as control. Mice were killed if they developed ascites, subcutaneous injection site tumors that were >10% of body weight, or if they lost >20% of body weight. All surviving mice were killed at the end of the experiment (day 90 after first virus treatment). Kaplan–Meier survival curves were plotted and compared by log-rank sum test. Tumor burden was monitored weekly by non-invasive bioluminescence imaging for firefly luciferase activity (Xenogen Corporation, Alameda, CA, USA).

Immunohistochemistry

Tumor samples frozen in OCT were cryosectioned (5 μm thick) and fixed in –20 °C pre-chilled acetone. Non-specific binding was blocked with 5% normal horse serum in 0.01% Triton X-100/phosphate-buffered saline. Sections were then incubated with biotinylated anti-measles nucleoprotein antibody (Chemicon, Temecula, CA, USA) and subsequently with Vectastain ABC-AP Kit (Vector Labs, Burlingame, CA, USA). Signals were developed using a blue substrate kit (Vector Labs) and nuclei were counterstained with Nuclear Fast Red (Vector Labs).

Statistical analysis

In the spheroids experiment, comparison of successful infections between all viruses was tested by χ^2 test. Differences in tumor burden were analyzed by two-way analysis of variance of the photon counts from the imaging data. Survival curves were represented using the Kaplan–Meier method. The log-rank test was used to examine the significance of differences in the survival between groups using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A P-value of <0.05 was considered to be significant.

RESULTS

Infection and spread of MV-xHer2 viruses in multicellular spheroids

We previously showed that a scFv Kd of 10^-8 M is required for mediating efficient virus infection (40–60% at multiplicity of infection 1) and syncytial formation (intercellular fusion) in SKOV3ip.1 cell monolayers.21 The low-affinity viruses did not induce syncytium and only low levels of infection (<10%) were observed. Here, we exposed spheroids composed entirely of tumor cells to media containing MV. A χ^2 or Fisher’s exact test was used to test the null hypothesis of equal proportion of successful infection between all viruses. Experiments were undertaken in three to four independent replicates, each time with 12–36 spheroids per virus. A spheroid with one or more GFP-positive foci (independent of size) is classified as ‘infected’. In Vero-xHis spheroids, all viruses had equally high successful infection rates (80.4%–100%), confirming that equivalent amounts of virus were used in the assay. In SKOV3ip.1 spheroids, the infection rates were: MV-xHER-9 (63.9%), MV-xHER-7 (68.8%), MV-xHER-10 (79.0%), MV-xHER-8 (83.9%), MV-xHER-11 (88.3%) and MV-xHER-6 (94.4%). There was no apparent correlation between scFv affinity and infection rates in SKOV3ip.1 spheroids. We also evaluated the size of infected areas. Spheroids of similar sizes with successful infection events were analyzed. The extent of virus infection throughout the spheroid, as reflected by GFP expression, was captured using confocal microscopy through the Z axis and analyzed using the NIH ImageJ program (Bethesda, MD, USA). There was no correlation between the size of infected area and scFv affinity (data not shown). As expected, receptor abundance plays a predominant role in determining the size of infected areas. In the low HER2 TE671 human rhabdomyosarcoma spheroids (4.3 × 10^5 molecules per cell), the size of infected area was significantly lower (about 10 times) than in SKOV3ip.1 spheroids (1.5 × 10^5 molecules per cell).

MV-xHER2 infection of tumors in vivo

Subcutaneous SKOV3ip.1 and TE671 flank tumors grown in athymic mice were injected directly with 10^6 TCID50 viruses. Three days or seven days post virus administration, tumors were harvested. The cut into half and observed MV+ were quantitated using a bioluminescent imaging system (Figure 2). Tumor burden was quantitated by measuring whole abdominal photon counts from the bioluminescent imaging data. Survival curves were represented using the Kaplan–Meier method. The log-rank test was used to examine the significance of differences in the survival between groups using GraphPad Prism (GraphPad Software, San Diego, CA, USA). A P-value of <0.05 was considered to be significant.

Antitumor activity of MV-xHER2 in an orthotopic model of ovarian cancer

The antitumor potential of MV-xHER2 was examined in athymic mice bearing disseminated SKOV3ip.1 tumors expressing the firefly luciferase gene (SKOV3ip.1-Fluc). Mice received three IP doses of MV (2 × 10^6 TCID50 per dose) or saline (n = 10 mice per group) every other day. All HER2-targeted viruses significantly inhibited tumor growth compared with saline-treated controls (Figure 2). Tumor burden was quantitated by measuring whole abdominal photon counts from the bioluminescent imaging studies (Figure 2a). By day 28 after the first treatment, 50% of mice in the saline-treated group had to be killed due to tumor burden. The cut into half and observed MV+ were quantitated using a bioluminescent imaging system. Median survival of saline-treated mice was 28.5 days. The median survival of MV-xHER-6-, MV-xHER-7-, MV-xHER-8-, MV-xHER-9-, MV-xHER-10-, MV-xHER-11-treated mice were 56.5, 51.5, 55, 54, 53 and 58 days, respectively. MV treatment significantly increased median survival by a factor of 1.98, 1.8, 1.93, 1.9, 1.86 and 2.04, respectively (P < 0.0001). Importantly, all viruses were therapeutically active and the higher-affinity viruses did not perform better than the lower-affinity viruses (P = 0.05). Tumors were harvested on day 4, 10 or 14 after the first treatment and immunohistochemical staining for measles N protein was performed on omental tumors. There was no apparent difference in the numbers or size of infectious foci.
between the high- and low-affinity viruses. None of the viruses were able to efficiently penetrate into the center of the omental tumors even at later time points (data not shown).

DISCUSSION

The panel of scFv displayed on the HER2-retargeted MV is composed of affinity mutants of the parental C6.5 scFv (Kd = 10^{-8} M) and all scFv bind to the same epitope on HER2 but with affinities ranging from 10^{-10} to 10^{-11} M.28,29 The virus hemagglutinin attachment protein is ablated for binding to two of the three MV cellular receptors; CD46 which is ubiquitously expressed on nucleated cells, and SLAM which is expressed on activated immune cells.34,35 These HER2 viruses are not ablated for the binding to nectin-4, the recently identified third receptor of MV.36 Nectin-4 is overexpressed on lung, ovarian and breast cancers and may enhance infectivity of the HER2-targeted viruses on the ovarian cancer cells.36 However, SKOV3ip.1 tumor cells do not express detectable levels of nectin-4 as determined by antibody staining and analysis by flow cytometry (K-WP, unpublished data). Thus, MV-αHER2 entry and infection of SKOV3ip.1 cells and tumors are mediated through scFv binding to HER2 receptor.

Our goal was to evaluate the importance of ligand–receptor affinity on the antitumor activity of oncolytic MVs. Results indicate that the antitumor activity of the low- and high-affinity viruses were comparable against i.p. SKOV3ip1. ovarian tumors. The ‘seemingly negative’ result that we report is the first study to demonstrate that receptor affinity of the attachment protein of an oncolytic virus has minimal impact on its in vivo efficacy against a tumor that expresses the targeted receptor. The study therefore challenges a fundamental assumption about the in vivo behavior of viruses, that is, high-affinity interactions would be desirable for therapeutic activity of an oncolytic virus. This finding has not yet been generalized to all virus families and currently, on the basis of the presented work, applies only to retargeted MV.

The HER2 receptor is overexpressed on 15–20% of breast cancers and its expression is correlated with more aggressive tumors.37 HER2 is overexpressed in 20 of 20 ovarian tumor cell lines derived from advanced stage III and stage IV tumors.38 The monoclonal antibody trastuzumab is currently the only approved adjuvant treatment specifically for patients with HER2-positive early-stage breast cancer.37 A limitation of trastuzumab monoclonal antibody therapy is that its activity is largely restricted to cancers with the highest level of HER2 overexpression or HER2 gene amplification. To test suitability for trastuzumab therapy, patient tumor samples are stained with an anti-HER2 antibody and
need to be graded as +3 or +4 for immunopositivity. However, there is a large population of breast or ovarian cancers that have low or moderate HER2 expression. We propose that these low-HER2-positive tumors can potentially benefit from oncolytic MV-targeted therapy using the high affinity MV, which efficiently infects low-HER2-expressing tumor cells.

The panel of HER2 scFv affinity mutants that was developed by Adams et al. has been very useful for investigations in understanding the role of receptor–ligand affinity in modulating the biodistribution, tumor penetration and the antitumor activity of various therapeutic agents. In nephrectomized SCID mice bearing SKOV3 tumors, Adams et al. showed scFv need to have sufficiently high affinity to achieve good tumor localization. As such, the $10^{-7}$ Kd scFv failed to accumulate in significant amounts in the tumors compared with the higher-affinity scFvs ($10^{-8}$ and $10^{-9}$ M). Accumulation in the tumor ceased to increase with affinity and was nearly the same for scFv with Kd of $10^{-9}$, $10^{-10}$ and $10^{-11}$ M. However, undesirable side effects could arise with high-affinity binders. Immunohistochemical analysis of well-vascularized tumors showed the highest affinity scFv limited to tumor space adjacent to the blood vessel while the low-affinity scFv diffused uniformly throughout the tumor interior.

In contrast to the studies above, we did not observe a significant difference in the in vivo performance of low- and high-affinity HER2-targeted MV after i.p. administration into mice with orthotopic ovarian disease. Immunohistochemical staining for MV-N protein indicate sites of virus infection in the tumors, but there were no significant areas of intercellular fusion or differences in size of infected areas by the low- and high-affinity viruses. In contrast to the in vitro study where extensive intercellular fusion was seen in infected cell monolayers, intercellular fusion may not be a major factor involved in oncolytic activity of these fusogenic MVs in the tumors. Importantly, the in vitro results were not predictive of the antitumor activity of the retargeted MV in vivo. Unlike scFv or antibodies, a virus has several hundred copies of the scFv displayed on the viral coat, thereby significantly increasing the avidity of the agent. Results from this study indicate that increasing the affinity of attachment protein–receptor interaction does not enhance virus delivery or therapeutic activity in vivo, and that future studies could focus on improving delivery of

Figure 2. In vivo antitumor activity of measles virus (MV)-HER2. Mice were implanted with SKOV3ip.1_Fluc cells. Five days later, mice were injected intraperitoneally with three doses of $2 \times 10^{6}$ TCID50 MV-HER-6 to MV-HER-11 or saline, given every other day. (a) Bioluminescence images showing tumor burden in treatment groups. (b) Quantitation of tumor burden from the bioluminescence imaging study; 10 mice per group. (c) Kaplan–Meier survival curves of mice in each treatment group compared with saline control group. (d) Statistical difference between survival curves of mice in respective treatment groups was compared. The P-values were calculated using the log-rank sum test.
therapeutic viruses by other strategies other than increasing affinity.

**CONFLICT OF INTEREST**

Dr SJR and Dr K-WP and Mayo Clinic have financial interests in the technology used in this research.

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