

ORIGINAL ARTICLE

An armed oncolytic measles vaccine virus eliminates human hepatoma cells independently of apoptosis

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Due to late diagnosis and a pronounced chemoresistance, most patients with hepatocellular carcinoma (HCC) have an overall poor prognosis. Measles vaccine viruses (MeV) have been shown to possess anti-tumor properties and their efficacy has been enhanced by arming with suicide genes. To test armed MeV for the treatment of HCC, we equipped it with the suicide gene Super-cytosine deaminase (SCD) and tested the efficacy in cell culture and in a mouse xenograft model of human HCC. Prodrug conversion was investigated in cell culture and quantified by high-performance liquid chromatography. We observed a strong oncolytic activity of MeV-SCD against human HCC *in vitro* and *in vivo*. The prodrug was efficiently converted in infected cells leading to a significant enhancement of the cytotoxic effect. Treatment of HCC xenografts with MeV caused long-term virus replication in tumor tissue. We show that the suicide gene therapy induces an apoptosis-like cell death but is not dependent on intact apoptosis pathways. These results demonstrate that MeV-based suicide gene therapy is a promising novel therapy regimen for HCC overcoming resistance towards conventional therapy. The independence from apoptosis raises hopes for the treatment of patients whose tumor cells exert defects in this cell death mechanism.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third most common cause of cancer deaths worldwide,¹ constituting a major obstacle especially in advanced stages.² Despite approval of the multikinase inhibitor sorafenib,³ median survival times have only improved marginally.² Oncolytic viruses are under investigation for cancer treatment. These tumor-specific viruses have progressed from preclinical to clinical studies.⁴ Safety has been shown in humans, and efficacy has come into the focus of research. Measles vaccine virus (MeV) is under investigation as an oncolytic virus and has been tested preclinically for the treatment of several tumor entities, including HCC.⁵ MeV is also undergoing clinical investigation against ovarian carcinoma, multiple myeloma and glioblastoma multiforme.^{6,7} MeV-induced cell death has been ascribed to apoptosis.^{8,9} However, as many cancer cells possess severe defects in this cell death program, it is of utmost importance for novel cancer therapies to be efficient also in tumor cells unable to execute apoptosis. Several methods to enhance the oncolytic effect are under investigation, including insertion of suicide genes. Here, a gene is inserted into the viral genome encoding an enzyme that converts a non-toxic prodrug into a toxic drug, leading to a localized chemotherapy with minimized systemic side effects.¹⁰ MeV has been armed with the prodrug convertase *Escherichia coli* purine nucleoside phosphorylase.^{11,12} As the corresponding prodrug fludarabine can cause severe cytotoxicity when administered systemically,¹³ we sought to develop a novel armed MeV expressing an optimized

suicide gene called Super-cytosine deaminase (SCD).¹⁴ SCD is a fusion protein of yeast cytosine deaminase (CD) and yeast uracil phosphoribosyltransferase (UPRT), converts the prodrug 5-fluorocytosine (5-FC) into the approved chemotherapeutic 5-fluorouracil (5-FU) and facilitates further conversion into 5-fluorouridine monophosphate (5-FUMP).¹⁵ 5-FUMP is converted by cellular enzymes into metabolites, which interfere with DNA repair, DNA and RNA synthesis, and with protein synthesis.^{16–18} Anti-tumor efficacy of adenovirus-encoded SCD has been demonstrated in hepatoma.¹⁴ A clinical study testing this suicide gene expressed by oncolytic vaccinia virus on HCC patients has been completed (clinical trial NCT00978107; sponsor: Transgene SA, France). Activity of an oncolytic MeV armed with a fusion of *E. coli* CD and UPRT against head and neck cancer has been demonstrated.¹⁹ However, as the bacterial CD has a 22-fold higher K_m value for 5-FC than the yeast enzyme,²⁰ yeast CD might be the suicide gene of choice. 5-FU is a highly diffusible substance and has a strong bystander effect on neighboring non-infected cells. To enhance this bystander effect, fusion of the intercellular transport protein VP22 from herpes simplex virus 1 to SCD (VP22SCD) was tested in our laboratory and was found to be superior to SCD.²¹

In the study presented here, we hypothesized that oncolytic MeV expressing SCD or VP22SCD exert enhanced anti-tumor effects in HCC compared with the virus alone in the context of an HCC cell line and in a mouse xenograft model of human HCC. Further, we examined whether this combinatorial approach is effective also in a tumor background lacking functional apoptosis pathway.

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RESULTS

Generation and characterization of armed MeV

We generated a MeV cDNA with 100% identity to the Schwarz vaccine strain and introduced an empty additional transcription unit (ATU) at genome position one (resulting in parental cDNA plasmid pc3MerV2 Id-ATU). Based on this cDNA, two new viruses were generated (Figure 1a), encoding the suicide gene SCD (MeV-SCD) or VP22SCD (MeV-VP22SCD). Expression levels of viral N protein and transgenes were confirmed by immunoblotting (Figure 1b). For VP22SCD, only a faint signal was detected, which might be due to a probable partial masking by VP22 of the epitope detected by the antibody. Both armed viruses were found to replicate with similar kinetics and to similar titers; replication of MeV-VP22SCD was slightly, but not significantly, delayed compared with MeV-SCD (Figure 1c). Further replication kinetics experiments clearly demonstrated no differences between the two new viruses MeV-SCD and MeV-VP22SCD and the parental virus (MeV-ATU) generated from parental cDNA plasmid pc3MerV2 Id-ATU, which 'only' contains an empty transcription unit (84 bp) but no transgene (data not shown). Thus, we could provide experimental evidence proving that the transgene expression of the new vectors, MeV-SCD and MeV-VP22SCD, did not inadvertently lead to viral attenuation or other changes to the viral kinetics in comparison to the parental virus encoding no transgene.

SCD-based suicide gene therapy is superior to MeV-mediated oncolysis alone

To determine the most efficient treatment schedule *in vitro*, Hep3B and HepG2 human hepatoma cells were infected with

MeV-SCD or MeV-VP22SCD and treated with 5-FC after different incubation periods. Late prodrug addition (2 days post infection (dpi)) killed cells more efficiently than treatment *ab initio* (data not shown). Thus, to investigate prodrug-enhanced cell killing, cells were treated with 5-FC at 2 dpi and the remaining cell mass was determined at 3–6 dpi (Figure 2). In both cell lines with and without additional prodrug treatment, the oncolytic effect of MeV-SCD was superior to that of MeV-VP22SCD. In Hep3B cells, the effect of MeV-SCD at multiplicity of infection (MOI) 0.01 was devastating, so that addition of 5-FC only slightly enhanced cytotoxicity. By contrast, at MOI 0.001, the effect of the virus was less pronounced, and the cytotoxic effect was clearly enhanced by 5-FC treatment ($P < 0.01$ on day 4 and $P < 0.001$ on days 5 and 6). HepG2 cells were found to be less sensitive towards the virus, so that combination with 5-FC significantly enhanced the cytotoxic effect at both MOIs ($P < 0.001$ on days 3–6). By comparison, MeV-VP22SCD mediated a toxicity dependent on MOI and prodrug concentration similar to MeV-SCD but to a weaker extent. Additional testing by lactate dehydrogenase release assays yielded analogous results with regard to kinetics and extent of cytotoxicity (Supplementary Figure S1). Treatment with prodrug alone did not cause cytotoxicity. Altogether, the vector MeV-SCD was found to be superior to MeV-VP22SCD.

5-FC is efficiently converted by MeV-expressed SCD and causes a strong bystander effect

We then sought to determine activation of the prodrug. First, we measured the toxicity elicited by the activated prodrug by transferring supernatants of infected and prodrug-treated HepG2

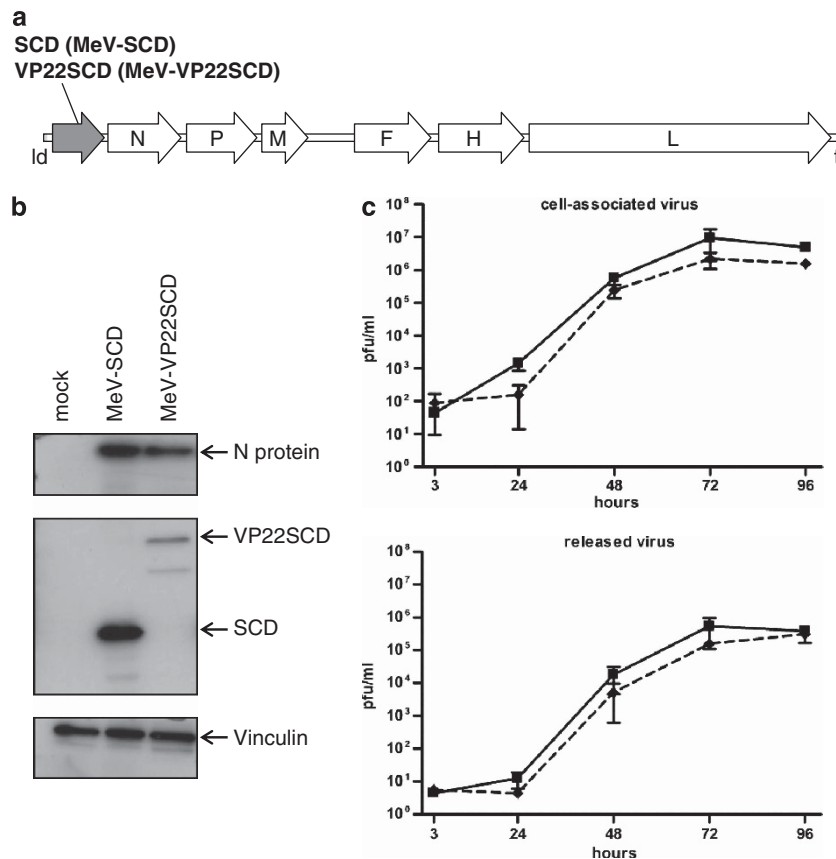


Figure 1. Generation and characterization of armed MeV. **(a)** Schematic drawing of a recombinant MeV cDNA containing an ATU encoding SCD or VP22SCD downstream of the leader (Id) position. **(b)** Hep3B cells were mock-infected or infected with MeV-SCD or MeV-VP22SCD at MOI 0.1 and harvested at 2 dpi. Cell lysates were subjected to immunoblot analysis for MeV N protein, SCD or vinculin. **(c)** Vero cells were infected at MOI 0.03 and cell-associated as well as released virus was quantified daily. Solid line: MeV-SCD; dotted line: MeV-VP22SCD. Values: mean of three independent experiments. Error bars: s.e.m.

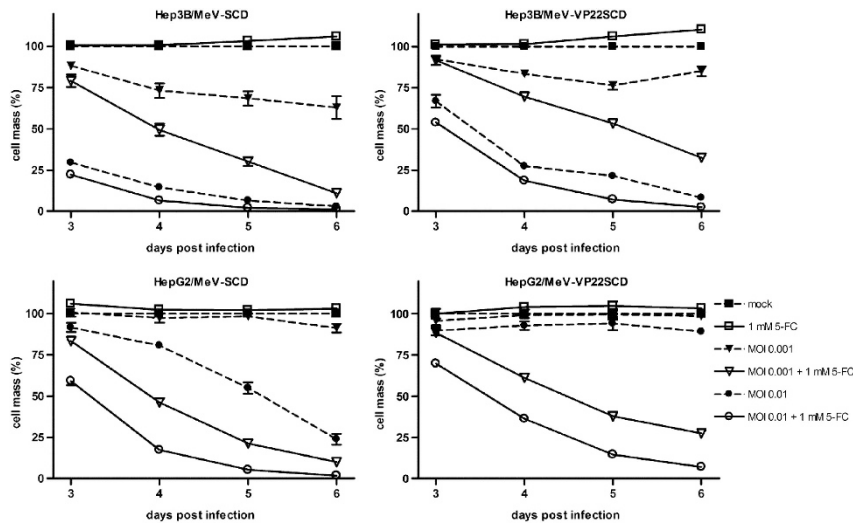


Figure 2. Enhanced cell killing by MeV-based suicide gene therapy. HepG2 and Hep3B cells were infected with MeV-SCD or MeV-VP22SCD and treated with 1 mM 5-FC at 2 dpi. The remaining cell mass was determined at the designated times post infection. Values: mean of three independent experiments performed in quadruplicates. Error bars: s.e.m.

cells to naive HepG2 cells. As controls, supernatants of mock-treated cells and of cells treated with virus or 5-FC only were used. Supernatants of the control-treated cells did not have any impact on survival of the recipient cells (Figure 3a). By contrast, supernatants of infected and prodrug-treated cells significantly reduced cell survival. Even at a 1:100 dilution, the remaining cell mass was reduced to <40% (Figure 3a). This effect was enhanced by higher concentrations of the conditioned media. Thus, even highly diluted supernatants of cells treated with the suicide gene therapy killed uninfected tumor cells, proving the strong bystander effect of the activated prodrug.

We then quantified the conversion rate of 5-FC into 5-FU by high-performance liquid chromatography (HPLC). In Hep3B cells, the prodrug conversion was highly efficient when vector MeV-SCD was used for infection: already at 24 h of incubation with 5-FC, 99–100% of 5-FC (at both 0.1 and/or 1 mM) were converted into its toxic metabolites (Figure 3b, left panel) so that no further testing was performed at any later time points. By contrast, when vector MeV-VP22SCD was used for infection of Hep3B cells, the 5-FC conversion rate at 24 h of incubation with 5-FC was found to be significantly lower ($P < 0.001$) than with MeV-SCD: 48% for 0.1 mM 5-FC and 28% for 1 mM 5-FC (Figure 3b, right panel). Only when the time of incubation with 5-FC was extended to 48 h, the conversion rate increased to >80% (Figure 3b, right panel). When HepG2 cells were infected with MeV-SCD, a much less efficient conversion was found to take place, both after 24 and 48 h incubation (Figure 3c). An even further diminished rate of 5-FC conversion was expected for vector MeV-VP22SCD in HepG2 cells (according to the weak oncolytic effectiveness of MeV-VP22SCD in HepG2 cells being demonstrated in Figure 2, panel to the lower right); therefore, testing of this setting was not undertaken.

Efficient therapy of Hep3B tumor xenografts

Next, the efficiency of the suicide gene therapy was investigated *in vivo*. Nude mice bearing subcutaneous Hep3B xenografts were treated with MeV-SCD or MeV-VP22SCD with or without application of 5-FC. All treatment regimens showed a strong oncolytic effect (Figure 4a) and significantly prolonged survival (Figure 4b); seven mice had a complete remission for ≥ 68 days; in three mice it lasted >200 days (Supplementary Table S1). However, the tumors of other mice reinitiated growth. To test whether this was due to an acquired resistance towards the therapy, tumors were

explanted from killed mice, cells were recultured, infected *in vitro* with MeV-SCD and treated with 5-FC. As a result, all tumor cells derived from explanted xenograft tumors were killed to a similar extent as the parental cell line Hep3B: (i) MeV-SCD-mediated oncolysis without addition of 5-FC (Figure 5; white bars) led to a strong reduction of the tumor cell mass with one exception (tumor specimen obtained from animal No.39); (ii) however, when 5-FC was added (Figure 5; bars in grey and black), parental Hep3B cells as well as all tumor specimens (including No.39) exhibited a cell mass decrease of about 50% when treated with the highest amount of 5-FC. Thus, it seems that these tumor cells had not developed resistances towards the SCD-enhanced MeV-based oncolytic therapy. Interestingly, reculturing of the explanted cells was not possible for some tumors, as typical cytopathic effects of MeV were observed after attachment of the cells to the culture dish, indicating presence of replication-competent infectious MeV in these samples. Such an ongoing virus replication in tumors has been observed before.²² To investigate whether this was the case in our investigations, we inoculated Vero cells with lysates of explanted tumors. In samples derived from control-treated mice, no viral effect was observed. However, in several samples from treatment groups, syncytia formation occurred in Vero cell cultures, proving that virus replication was still present in these tumors even >50 days after treatment (Figure 6a and Supplementary Figure S2). Interestingly, this virus infection was restricted to small areas within the tumors (Figure 6b and Supplementary Figure S3).

Inhibition of apoptosis does not abort the toxic effects of the suicide gene therapy

MeV-induced oncolysis has been ascribed to apoptosis. To investigate this for MeV-mediated suicide gene therapy, and to test if inhibition of apoptosis abrogates the cytotoxic effect, we analyzed activation of this cell death program. Hep3B and HepG2 cells were infected with MeV-SCD and treated with 5-FC 2 dpi. A subset of cells was treated with the pan-caspase inhibitor Q-VD-OPh *ab initio*. As a positive control for apoptosis induction, CD95 ligand and actinomycin D were applied. To show that no signal is induced in the assay if cells die by mechanisms other than apoptosis, verapamil was used, which induces necrotic cell death at the applied concentration. At 4 dpi, archetypical apoptotic low-molecular DNA degradation was measured (Figure 7a). Induction

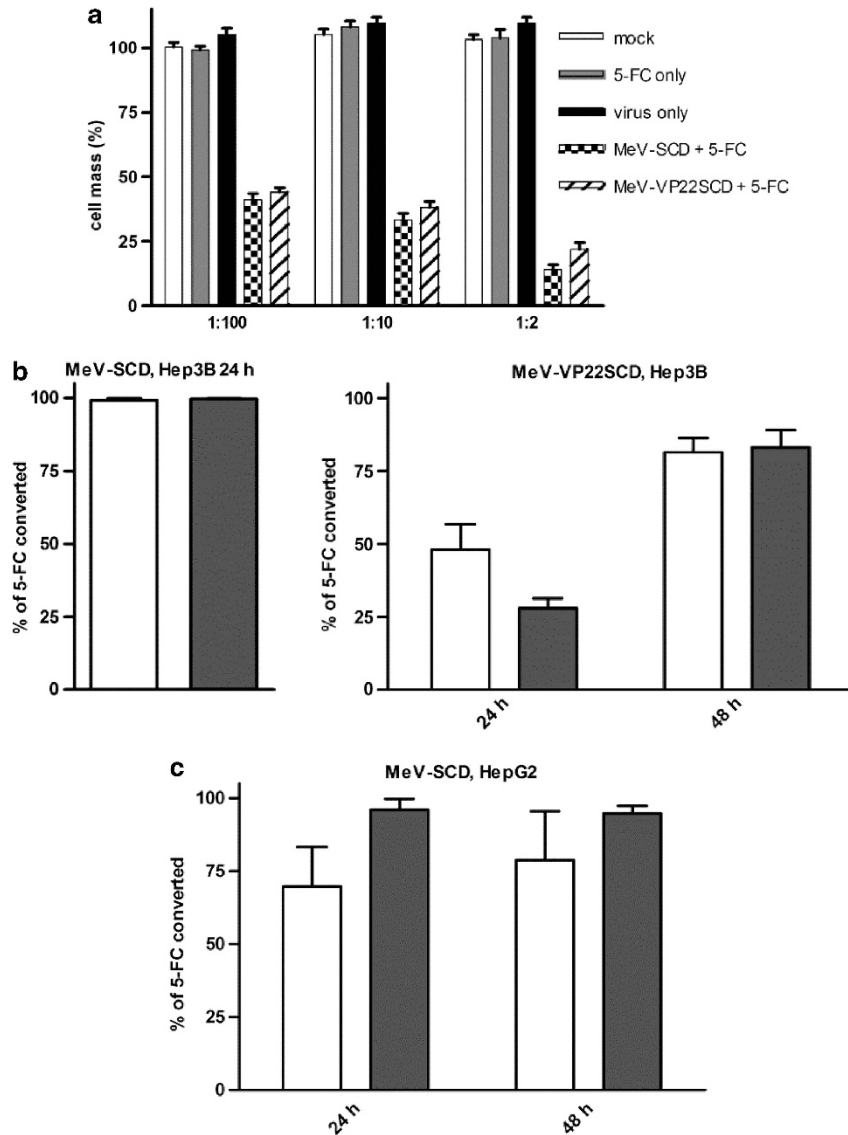


Figure 3. Bystander effect and prodrug conversion. (a) Measurement of bystander effect. HepG2 cells were control-treated or treated with the suicide gene therapy. Supernatants were harvested at 3 dpi, heat-inactivated and transferred in different dilutions (1:100, 1:10, 1:2) to naive HepG2 cells. The remaining cell mass of these recipient cells was determined after 3 days. For ‘virus only’ treatment, both viruses were tested, a mean of these results is shown. Values: mean of three independent experiments performed in quadruplicates. Error bars: s.e.m. (b, c) Prodrug activation. Hep3B (b) and HepG2 (c) cells were infected with MeV-SCD or MeV-VP22SCD at MOI 0.01 and treated with 5-FC (white bars: 0.1 mM, grey bars: 1 mM) at 48 h post infection. The amounts of 5-FC and 5-FU in cell culture supernatants were quantified by reverse-phase HPLC 24 and 48 h later. The percentage of 5-FC converted was determined as the concentration of 5-FU divided by the total concentration of 5-FU and 5-FC. Values: mean of three independent experiments. Error bars: s.e.m.

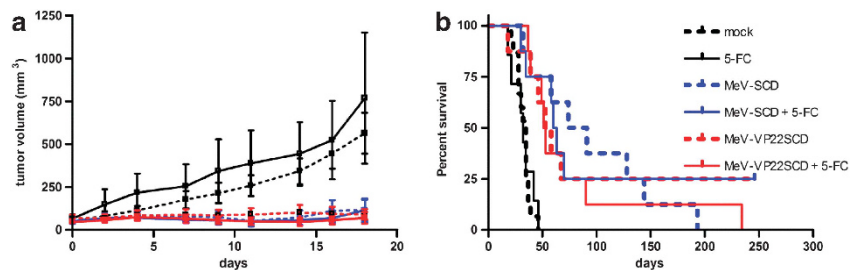


Figure 4. *In vivo* efficacy of the MeV-based suicide gene therapy. Nude mice bearing subcutaneous Hep3B tumors were treated with five intratumoral injections of MeV-SCD or MeV-VP22SCD (days 0–4; 2×10^6 plaque-forming units daily) followed by seven injections of 5-FC intraperitoneally (days 5–11; one daily dose of 500 mg 5-FC kg⁻¹ body weight). (a) Mean tumor volumes. Error bars: s.e.m. (b) Survival.

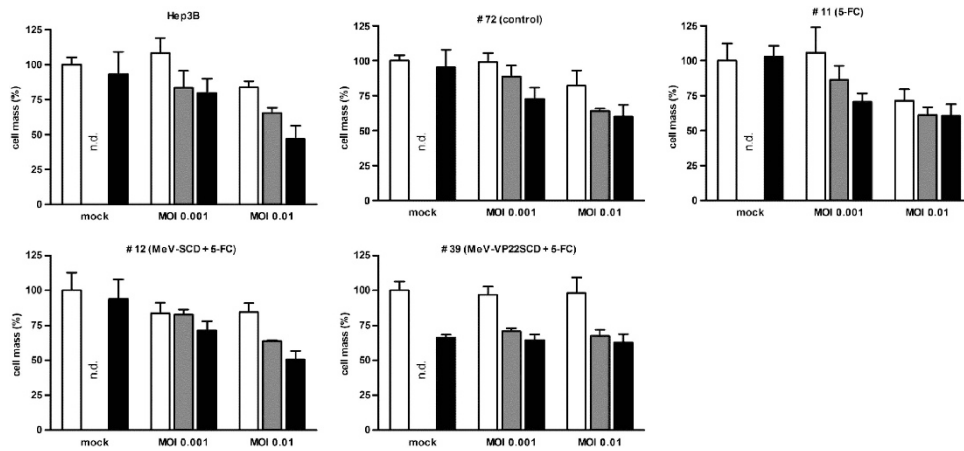


Figure 5. Treatment failure is not due to acquired resistances towards MeV. To test for acquired resistances towards the suicide gene therapy, recultured tumor cells were infected with MeV-SCD and treated with 5-FC at 2 dpi. The remaining cell mass was determined at 4 dpi. Graph titles designate ID-numbers and treatment groups of the respective mice. White bars: 0 mM 5-FC; grey bars, 0.1 mM 5-FC; black bars: 1 mM 5-FC. Values: mean from one representative experiment performed in triplicates (for each cell line subclone, a total of 1–3 independent experiments were performed). Error bars: s.d. ND, not determined.

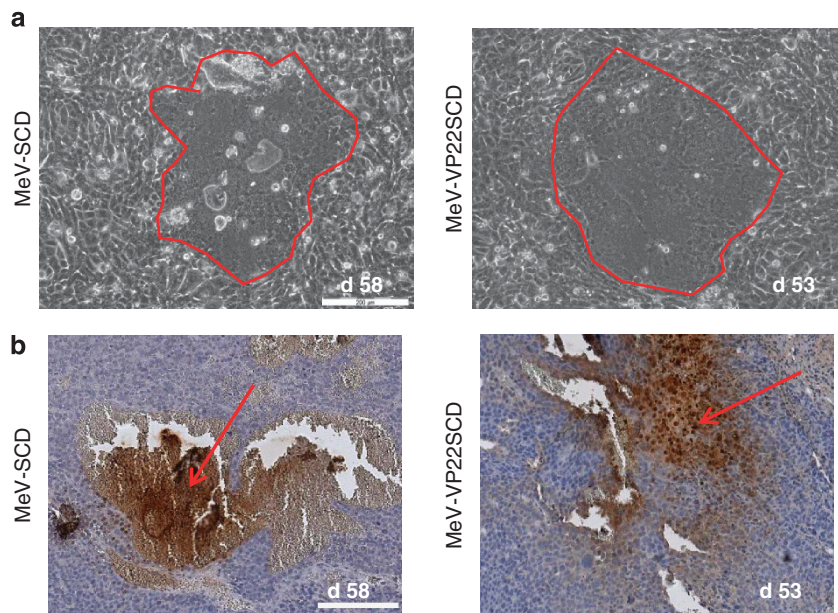


Figure 6. Long-term replication of virus in tumors. (a) To test for ongoing virus replication in tumor tissue, Vero cells were treated with tumor lysates. Three days later, pictures were taken. Syncytia as the typical MeV-mediated cytopathic effect are framed in red. Treatment groups and days after treatment initiation at which the respective mice were killed (for example, day 58) are specified. The scale bar in the left panel (200 μm) applies to both panels. (b) Histological analysis of tumor tissue. After mice were killed, tumors were explanted and a piece of tumor tissue was prepared for histological analysis and probed with an antibody binding to MeV N. Nuclei were stained with hematoxylin. Red arrows point to areas positively stained for MeV N. Treatment groups and days after treatment initiation at which the respective mice were killed (for example, day 58) are specified in the figure. The scale bar in the left panel (200 μm) applies to both panels.

of apoptosis was verified by measurement of caspases 3 and 7 activity and Nicoletti assay (data not shown). In parallel to the enzyme-linked immunosorbent assay, cells treated in exactly the same set up were evaluated by sulforhodamine B (SRB) assay. In both the cell lines, treatment with MeV-SCD with or without 5-FC caused a significant ($P < 0.001$) induction of DNA degradation (Figure 7a). Induction of apoptosis was inhibited by treatment with Q-VD-Oph. In spite of this effect of the caspase inhibitor on DNA degradation, the cytotoxic effect of MeV-SCD with or without 5-FC was not invalidated by treatment with Q-VD-Oph (Figure 7b). Thus, although apoptosis was successfully inhibited by treatment of hepatoma cells with the caspase inhibitor, this did not spare

these tumor cells from the cytotoxic effects of the SCD-enhanced oncolytic therapy.

DISCUSSION

Due to detection at late stages of disease and its pronounced chemoresistance, HCC remains a clinical challenge with poor prognosis. Oncolytic MeV has been shown to be a candidate for a novel HCC therapy,⁵ but based on the heterogeneity of tumors and the probability of therapy-resistant tumor cells, we aimed at developing armed MeV for the treatment of HCC. To achieve this, we generated armed oncolytic MeV encoding the SCD suicide

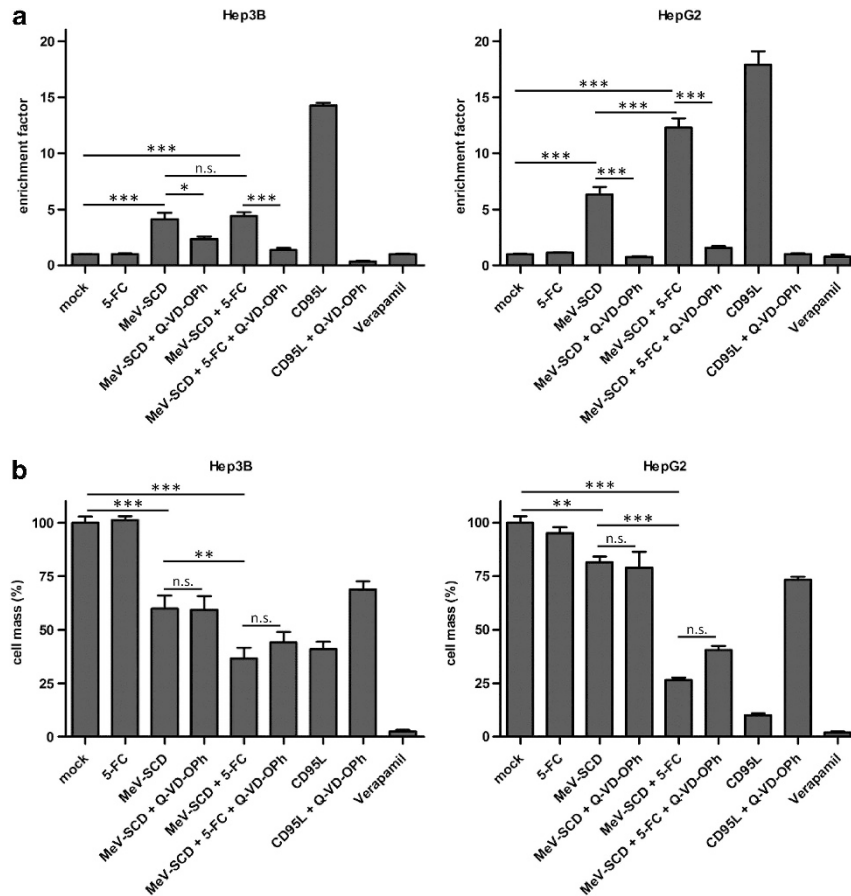


Figure 7. Apoptosis induction by MeV-mediated suicide gene therapy. **(a)** Hep3B and HepG2 cells were infected with MeV-SCD at MOI 0.01 and treated with 1 mM 5-FC at 2 dpi. A subset of the cells was treated with the pan-caspase inhibitor Q-VD-Oph (5 μ M) *ab initio*. At 4 dpi, apoptotic DNA fragmentation was quantified with a cell death enzyme-linked immunosorbent assay. **(b)** Cell mass of cells treated as described in **(a)** was quantified by SRB assay. CD95L: positive control (CD95 ligand + actinomycin D). Values: mean of three independent experiments performed in triplicates. Error bars: s.e.m. Positive control and verapamil treatment were performed only in one experiment. NS, not significant ($P > 0.05$). Statistically significant differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

gene or a SCD with an enhanced bystander effect (VP22SCD). Like other armed viruses, this vector kills tumor cells by two mechanisms: (i) the oncolytic effect of the virus, and (ii) the conversion of a non-toxic prodrug (5-FC) into a toxic drug (5-FU). Both viruses efficiently killed human hepatoma cells, which was significantly enhanced by 5-FC.

Like other activated prodrugs, 5-FU has a strong bystander effect.²³ To enhance this, we tested a prodrug convertase able to migrate into neighboring cells. In our group, this protein (VP22SCD) has been shown to be superior to SCD when encoded in adenoviral vectors.²¹ However, when expressed by oncolytic MeV, VP22SCD exhibited weaker *in vitro* effects than SCD (Figure 2). This might be due to the fact that MeV-infected cells express the fusogenic glycoproteins of MeV on their surface, leading to fusion with neighboring cells. MeV-encoded proteins (including the prodrug convertase) will thus be present in the cytoplasm of cells in proximity to the initially infected cell. In addition, the weaker effect of VP22SCD could be due to a partial masking of the active site of SCD by VP22 or to a slight change in the enzyme's structure caused by the fusion to VP22. As previous investigations on VP22SCD were performed with replication-deficient vectors,²¹ which have no inherent bystander effect, the enhancement by the intercellular transport of the prodrug convertase was obviously highly beneficial and outweighing the putative negative effects of the fusion to VP22, in contrast to a system with a strong bystander effect on its own (MeV).

In a mouse model of human HCC, we observed a significant reduction of tumor volume and prolongation of survival by treatment with the armed vectors, mirroring the results obtained with Hep3B cells *in vitro*. Several mice had a complete remission. Unfortunately, some of these mice died due to causes known before for this mouse strain.²⁴ However, we did not observe a difference between treatment with and without 5-FC. This could be partly due to the high efficiency of MeV in the Hep3B xenograft model, but as we observed some tumors that did not respond to the therapy, there are obviously additional factors influencing therapy outcome. It has been shown before that timing of prodrug treatment is a crucial factor in suicide gene therapy.^{11,25–27} In most of these investigations, it was observed that starting prodrug administration with delay after virus injection is more efficient. Thus, a tool enabling measurement of virus replication would be highly beneficial allowing the determination of the optimal time for prodrug administration. Such tracking tools have been tested with oncolytic MeV^{22,28} but have not been combined with suicide gene therapy so far. Furthermore, due to the heterogeneity of human tumors and potential adverse effects of the patient's immune system, a 'toolbox' will be required in future that will enable successful applications of oncolytic virotherapeutics in the clinic. At best, this toolbox should contain different features and possibilities to use and combine state-of-the-art oncolytic viruses with suicide gene therapies or other therapeutic features. As we have been able to

demonstrate a highly efficient conversion of the prodrug 5-FC and the independence of the MeV-based suicide gene virotherapy on intact apoptosis pathways, our suicide gene-armed measles virus MeV-SCD is indeed functional and should constitute a highly valuable addition to that toolbox.

In our investigations, we observed long-term replication of MeV in tumors (Figure 6a), a finding which possibly could be exploited for further treatment cycles, including additional rounds of prodrug application. However, it is not known if long-term replication of MeV observed in our current study is an artifact of immunodeficient models. In a clinical study of intraperitoneal application of oncolytic MeV in patients with ovarian cancer who all were immune to measles, no such long-term replication of MeV was found (being indicated by only transient expression of the carcinoembryonic antigen marker protein encoded in the MeV genome⁷); thus it might be that MeV regularly is cleared in immunocompetent cancer patients. However, when a different virotherapeutic, a recombinant vaccinia virus (GL-ONC1), recently was applied intraperitoneally to one gastric cancer patient exhibiting peritoneal carcinomatosis (NCT01443260; phase I/II study in patients with therapy-resistant peritoneal carcinomatosis currently performed at the University Hospital Tübingen), effective intraperitoneal replication of GL-ONC1 was demonstrated for >3 weeks (our unpublished results). Interestingly, this gastric cancer patient had not been prevaccinated with the vaccinia virus. Thus, when summarizing the data on both MeV- and VACV-based virotherapy, one cannot conclude at this early time of clinical virotherapeutic studies that long-term persistence of virotherapeutics would constitute an artifact and only can take place in highly immunodeficient animals and/or cancer patients. In addition, there are not enough data so far allowing a speculation on the efficacy of virotherapy (for example, MeV-based virotherapy) in patients having been pre-immunized to MeV versus patients exhibiting no immunization to MeV. By combining tracking with arming, it would not only be possible to determine the optimal time for the first administration of prodrug, but, considering repeated treatment cycles, also to decide whether a reapplication of virus is necessary before reapplication of prodrug.

As we observed a heterogeneous *in vivo* response, we tested explanted recultivated tumor cells for resistances towards the suicide gene therapy. All cell line subclones under investigation were as sensitive towards MeV and the suicide gene therapy as the parental cell line Hep3B. To our knowledge, such investigations have not been performed with any oncolytic virus and it is not known whether tumor cells develop resistances towards these therapies or whether therapy failure is only due to factors like suboptimal timing and delivery.

It has been shown that wild-type and vaccine strain MeV induce apoptosis.⁹ For MeV under investigation as oncolytic vectors, induction of apoptosis has been verified.⁸ Since then, MeV-induced cell killing has been attributed to this cell death program.^{29–31} However, as no cross-resistances of oncolytic viruses with chemotherapy or radiotherapy have been observed,¹⁰ as viruses affect a multitude of cellular functions and as many tumors have defects in their apoptosis pathways,³² we investigated the effect of apoptosis inhibition on MeV-mediated oncolysis and SCD-suicide gene therapy by treating infected or infected and 5-FC-treated cells with a pan-caspase inhibitor. Interestingly, these cells died to a similar extent as cells not incubated with the inhibitor. Thus, MeV and the suicide gene therapy induce apoptosis, but the therapy is not dependent on this cell death program and can thus be expected to be functional in tumors exhibiting defects in their apoptosis pathways. For oncolytic herpes simplex virus, it has been shown that treatment of infected cells with a pan-caspase inhibitor can enhance the oncolytic effect.³³ This was attributed to the fact that under normal circumstances herpes simplex virus quickly kills infected cells, thereby limiting its own replication. Inhibition of apoptosis allowed the virus to

replicate more efficiently, thus increasing the oncolytic effect. Whether treatment of MeV-infected cells with a caspase inhibitor also enhances viral progeny production remains to be investigated; however, we have shown that intact apoptosis pathways are not prerequisites for MeV-mediated oncolysis.

In this study, we present armed oncolytic MeV as a promising novel therapeutic for HCC. *In vitro*, we observed a strong enhancement of the oncolytic effect by treatment with the prodrug 5-FC. The virus-encoded SCD efficiently converted 5-FC into the toxic drug 5-FU. Investigation of the therapy regimen *in vivo* showed pronounced anti-tumor effects. We found that MeV-mediated oncolysis and the suicide gene therapy induce apoptosis but are not dependent on this cell death pathway. This raises hopes for the treatment of patients exhibiting such cell death defects in their tumor cells.

MATERIALS AND METHODS

Cell culture

Vero African green monkey kidney cells were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Human hepatoma cell lines Hep3B and HepG2 were obtained from the European collection of cell cultures (ECACC, Salisbury, UK). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Munich, Germany) supplemented with 10% fetal calf serum (FCS; PAA Laboratories, Pasching, Austria) for a maximum of 3 months after resuscitation. DSMZ and ECACC employ analysis of short tandem repeat polymorphisms for authentication of cell lines.

Infection of cells

Cells were infected in Opti-MEM (Life Technologies, Darmstadt, Germany) for 3 h at 37 °C. Afterwards, the inoculum was replaced with culture medium. 5-FC (Roche, Mannheim, Germany) was added in fresh medium at the specified time. Treatment with CD95 ligand/actinomycin D and verapamil was started 24 h before the respective measurement by addition of the substances into culture supernatants.

Viral growth curves

Vero cells (10^5 per well in a six-well plate) were infected with MOI 0.03. After infection, the cells were washed three times with phosphate-buffered saline (PBS, PAA Laboratories), and 1 ml DMEM with 5% FCS was added. At the designated times, supernatants and cells were harvested. Virus was released by one freeze/thaw cycle and viral titers were determined by 50% tissue culture infectious dose titration on Vero cells.³⁴

Immunoblotting

2×10^6 cells in a 10 cm dish were infected with MOI 0.1 and harvested at 2 dpi. Cells were washed $1 \times$ with cold PBS, scraped into 300 μ l lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40) and the proteins were released by three freeze/thaw cycles. The lysates were mixed with $6 \times$ Laemmli loading buffer and the proteins were separated on a 12% polyacrylamide gel and blotted on a polyvinylidene difluoridemembrane (Hybond-P, Amersham Biosciences/GE Healthcare, Munich, Germany). Membranes were probed with antibodies binding to MeV N protein (rabbit antibody, Abcam, Cambridge, UK; 1:6000 in 5% milk in TBS-Tween), SCD (rat antibody, kind gift from Transgene S.A., Illkirch Graffenstaden Cedex, France; 1:1000 in Roti-Block, Carl Roth, Karlsruhe, Germany) or human vinculin (mouse antibody, Sigma-Aldrich; 1:10 000 in Roti-Block). After removal of free primary antibody, membranes were incubated with horseradish peroxidase-coupled secondary antibodies: goat anti-rabbit (Bio-Rad, Munich, Germany; 1:8000 in 5% milk in TBS-Tween), goat anti-rat (Chemicon/Millipore, Schwalbach, Germany; 1:8000 in Roti-Block), and goat anti-mouse (Bio-Rad; 1:8000 in Roti-Block). Detection was performed with enhanced chemiluminescence (Immobilon Western Chemiluminescent HRP Substrate, Millipore) according to the manufacturer's instructions.

SRB assay

Cytotoxicity was determined by SRB assay.³⁵ 5×10^4 cells per well in 24-well plates were treated as above. Then, cells were washed $2 \times$ with PBS (4 °C) and fixed with trichloroacetic acid (10% w/w, 4 °C for 30 min).

After washing 4 × with water and drying, cells were stained with 0.04% SRB dye (Sigma-Aldrich, Taufkirchen, Germany) in 1% acetic acid for 10 min at room temperature. Unbound SRB was removed with 1% acetic acid. After drying, stained cells were solubilized in 10 mM Tris base (pH 10.5), and the optical density was measured at 550 nm.

Measurement of bystander killing *in vitro*

HepG2 cells (3×10^5 cells per well in six-well plates) were treated with the suicide gene therapy. Twenty-four hours after addition of 5-FC, supernatants were harvested and incubated at 60 °C for 30 min to heat-inactivate virus particles. Non-infected HepG2 (10^5 cells per well in 12-well plates) were incubated with different dilutions of the conditioned media for 72 h. The remaining cell mass was determined by SRB assay.

HPLC

3×10^5 cells per well in 6-well plates were mock infected or infected with MeV-SCD or MeV-VP22SCD at MOI 0.1. Forty-eight hours post infection, the supernatants were replaced with medium with or without 1 mM 5-FC. After an incubation of 24 h or 48 h, supernatants were harvested and incubated at 60 °C for 30 min to heat-inactivate the virus. To precipitate proteins, samples were mixed with an equal volume of acetonitrile (HPLC grade), vortexed for 2 min, incubated at room temperature for 2 min and centrifuged in a table-top centrifuge at 13 000 r.p.m. and 4 °C for 20 min. The supernatants were transferred into new reaction tubes and dried in a rotational vacuum concentrator (Alpha RVC, Christ, Osterode, Germany) at 30 mbar without heating for 16 h. The cold trap (Alpha 2–4, Christ) was set to –80 °C. To determine the concentration of 5-FC and 5-FU, the samples were resuspended in 100 µl mobile phase (5 mM tetrabutylammonium hydrogen sulfate, 1.5 mM potassium dihydrogen phosphate, pH 8). Reverse-phase HPLC was performed with a system from Merck Hitachi (Darmstadt, Germany) (auto sampler L-7200, L-6200 pump, L-4000 UV detector). 5-FC and 5-FU were separated by isocratic elution on a 5-µm C18 reverse-phase column (Hypersil ODS C18 250 × 4.6 mm, 5 µm, Thermo Scientific, Waltham, MA, USA) and the signals were monitored at $\lambda = 254$ nm. Data acquisition were performed with Easyline software (Chromsystems, Munich, Germany). To calculate the concentrations of 5-FC and 5-FU in samples, standards containing defined concentrations of 5-FC (Roche) and 5-FU (Fluka/Sigma-Aldrich, Munich, Germany) were measured. Based on these measurements, a calibration curve was created and used to calculate the concentrations in the experimental samples. All concentrations were calculated based on the height of the respective peaks and the percental conversion was calculated by dividing the concentration of 5-FC in a sample by the total concentration of 5-FC + 5-FU of that sample.

In vivo experiments

All animal experiments were approved by the local authorities and performed according to the German Animal Welfare Act. Tumors were established by injecting 10^6 Hep3B cells in 100 µl PBS into the right flank of 6-week-old Hsd:ATHymic Nude-Foxn1^{nu} mice (Harlan Laboratories, Boxmeer, Netherlands). Tumors were measured with a caliper three times per week and tumor volume was calculated by length × width × width × 0.5. After tumors had grown to a maximum diameter of 5 mm, animals were randomized to the treatment groups: (i) untreated control (15 animals), (ii) 5-FC (7 animals), (iii) MeV-SCD (8 animals), (iv) MeV-SCD + 5-FC (8 animals), (v) MeV-VP22SCD (8 animals) and (vi) MeV-VP22SCD + 5-FC (8 animals) and were treated by five intratumoral injections of virus (2×10^6 pfu per dose) or Opti-MEM (medium control) with one injection daily, followed by seven intraperitoneal injections of 5-FC (500 mg kg⁻¹ body weight per dose in PBS) on days 5–11. Animals were killed when tumor volumes reached 2000 mm³, weight loss >20% occurred or ulcerating tumors were observed.

Isolation of virus from tumor tissue

Pieces of tumors were frozen in liquid nitrogen, thawed for 3 min at 37 °C, worked through a 45-µm cell strainer with 1 ml Opti-MEM and centrifuged at 1890 g, 4 °C for 10 min. Vero cells in six-well plates (3×10^5 cells per well) were inoculated with the centrifugation supernatant.

Reculturing of explanted tumor cells

To reculture tumor cells from explanted tumor tissue, pieces of tumor were worked through a 45-µm cell strainer into a 6-cm culture dish. Cells were cultured in RPMI + 10% FCS + Penicillin/Streptomycin (Lonza, Basel,

Switzerland). On the next day, the culture was washed 3 × with PBS, and fresh medium was added. Presence of a significant amount of contaminating cells of non-tumor origin, for example, fibroblasts, was excluded by thorough microscopical examination.

Immunohistochemistry

Pieces of tumor were fixed in 4% paraformaldehyde overnight, and paraffin-embedded sections were subjected to immunohistochemistry using an antibody against MeV N (Novus, Cambridge, UK) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Nuclei were stained with hematoxylin.

Measurement of DNA laddering

To quantify apoptotic mono- and oligonucleosomes, cells in 24-well plates (5×10^4 cells per well) were infected and treated as above. At 4 dpi, DNA laddering was quantified with the Cell Death detection ELISA^{PLUS} (Roche) according to the manufacturer's instructions. The enrichment factor is the absorbance of a sample divided by that of the untreated control.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on Gene Therapy website (<http://www.nature.com/gt>)