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ORIGINAL ARTICLE

Lister strain of vaccinia virus armed with endostatin–angiostatin fusion gene as a novel therapeutic agent for human pancreatic cancer

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Survival after pancreatic cancer remains poor despite incremental advances in surgical and adjuvant therapy, and new strategies for treatment are needed. Oncolytic virotherapy is an attractive approach for cancer treatment. In this study, we have evaluated the effectiveness of the Lister vaccine strain of vaccinia virus armed with the endostatin–angiostatin fusion gene (VVhEA) as a novel therapeutic approach for pancreatic cancer. The Lister vaccine strain of vaccinia virus was effective against all human pancreatic carcinoma cells tested in vitro, *especially those insensitive to oncolytic adenovirus. The virus displayed inherently high selectivity for cancer cells, sparing normal cells both* in vitro *and* in vivo, *with effective infection of tumors after both intravenous (i.v.) and intratumoral (i.t.) administrations. The expression of the endostatin–angiostatin fusion protein was confirmed in a pancreatic cancer model both* in vitro *and* in vivo, *with evidence of inhibition of angiogenesis. This novel vaccinia virus showed significant antitumor potency* in vivo *against the Suit-2 model by i.t. administration. This study suggests that the novel Lister strain of vaccinia virus armed with the endostatin–angiostatin fusion gene is a potential therapeutic agent for pancreatic cancer.*
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Introduction

Pancreatic cancer is currently a leading cause of cancer-related deaths in the Western countries as most patients present with advanced or metastatic disease and few are suitable for curative surgical resection. Palliative chemotherapy has poor results and the overall 5-year survival rate is $<5\%$.[1] New treatment strategies are clearly imperative. With increasing knowledge of the molecular genetics of pancreatic cancer,[2] gene therapy has become an attractive option, although clinical trials to date have shown only moderate efficacy.[3]

Replication-selective oncolytic viruses are rapidly expanding as a class of therapeutic agents for cancer. Replication-selective oncolytic adenovirus is the most well-researched, and a first generation virus (*dl*1520, or H101 in China) has been approved as the world's first oncolytic virotherapy for head and neck cancer therapy.[4] This virus has been administered by intratumoral (i.t.) injection into patients with locally advanced pancreatic tumors in phase I–II trials. Although treatments were well tolerated, no objective responses were seen in patients after virus alone, and only two of 21 patients showed objective responses when gemcitabine was used in combination.[5] One major hurdle affecting oncolytic adenovirus potency is the genetic make-up in cancer cells, which affects virus infectivity. For example, the expression of adenovirus receptor, CAR (coxsackie-adenovirus receptor) is often low on many tumor types, including pancreatic cancer.[6] To make progress, new strategies are needed to overcome this obstacle. One of them could be to the use other oncolytic viruses such vaccinia virus.

Oncolytic vaccinia virus represents an attractive alternative, as it has several features that make it suitable for use as an oncolytic agent.[7–11] A defining feature of vaccinia virus as an oncolytic agent is that it relies mostly on its own encoded proteins to carry out replication and transcription in the cytoplasm, with few (if any) host proteins required. In addition, the vaccinia virus has a wide host range and tissue tropism, which provides an ability to infect almost all types of mammalian cells. These may overcome the difficulty encountered with adenovirus and allow vaccinia virus to replicate in many tumor types. The vaccinia virus has been safely injected into patients through subcutaneous,[12] intramuscular,[13] intratumoral[14] and intravesical[15] administration. The oncolytic capacity of vaccinia virus, as well as the safety record of this virus, make it a potentially ideal choice for cancer treatment.[11,16,17]

Oncolytic vaccinia virus is inherently tumor selective in part because of the low production of interferons by tumor cells in response to vaccinia infection, whereas these cytokines inhibit vaccinia replication in normal cells.[18]

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However, tumor selectivity is enhanced because of the overexpression of EGFR (epidermal growth factor receptor) by many tumor types. Vaccinia viruses express vaccinia growth receptor early during replication. Vaccinia growth receptor is homologous to cellular EGF and, therefore, binds to the ErbB family of receptors.[19] The signaling through these tyrosine kinase receptors results in DNA synthesis and cell proliferation, including thymidine kinase-induced nucleotide production, thereby increasing vaccinia replication.[20] As pancreatic cancers frequently overexpress EGFR, this tumor type represents a promising model for targeted oncolytic vaccinia therapy.[21]

Pancreatic tumors are often well vascularized and high microvessel density has been shown to correlate with poor prognosis after curative resection.[22] Therefore, inhibition of angiogenesis is a rational target for the treatment of pancreatic cancer and many angiogenesis inhibitors have been investigated in animal models of pancreatic cancer,[23] resulting in recent clinical trials.[24,25] Despite these efforts, efficacy has been limited. Endostatin and angiostatin are the two potent endogenous angiogenesis inhibitors that have displayed antitumor efficacy in various animal models,[26–29] including pancreatic cancer.[27,29] They act synergistically when used in combination, which led to the construction of an endostatin–angiostatin fusion gene.[30] This has been successfully delivered by both viral[30–33] and non-viral vectors.[34]

The combination of an oncolytic vaccinia virus that also expresses the endostatin–angiostatin fusion gene may provide potent, targeted angiogenesis inhibition on a continuous basis in the local tumor environment to slow tumor growth, allowing the virus to spread through and destroy the tumor. We therefore constructed an oncolytic vaccine strain of vaccinia virus expressing a human endostatin–angiostatin fusion gene (VVhEA). In this report, we tested our hypothesis and show that VVhEA is a potential therapeutic agent targeting human pancreatic cancer.

Results

Lister strain of vaccinia virus is more potent than adenovirus and replicates in human pancreatic cancer cell lines insensitive to adenovirus

To show the potential of the Lister vaccine strain of vaccinia virus as a therapeutic agent for human pancreatic cancer, we compared the cytotoxicity of VVLister and Ad5 in a panel of human pancreatic cancer cell lines *in vitro* (Figure 1a). VVLister displayed a greater potency than Ad5 in all cell lines tested, including cancer cells insensitive to adenovirus, such as PaTu8988s, Suit-2, HS766T and Capan1. To determine whether vaccinia virus-induced cell killing was indicative of replication induction in cancer cells, replication of VVLister was confirmed in PaTu8988s and Suit-2 (Figures 1b and c).

Lister vaccine strain of vaccinia virus displays selectivity between tumor cells and normal cells in vitro *and* in vivo

The Lister strain vaccinia virus has been used for visualization of tumors and metastases and cancer treatment,[35–37] however, the selective resistance of normal human cells was not definitively showed, especially between normal human cells and cancer cells. Therefore,

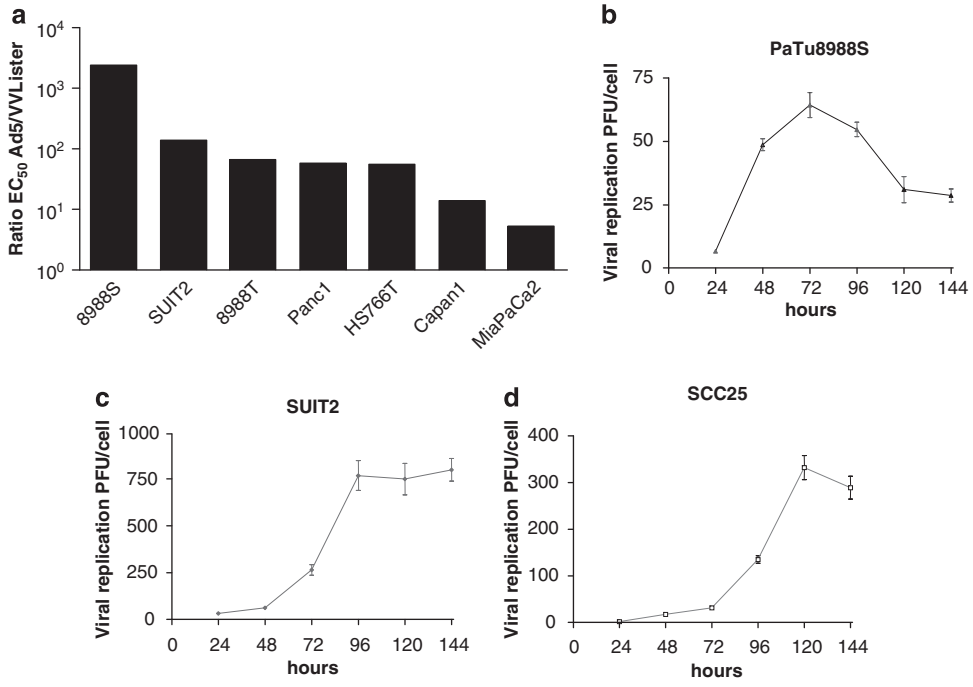

Figure 1 Cytotoxicity and replication of Lister strain of vaccinia virus in human cancer cells and normal epithelial cells. (**a**) Comparison of cytotoxicity of Lister strain vaccinia virus and adenovirus in a panel of human pancreatic cancer cell lines. Mean EC50 values were derived by MTS assay 6 days after infection of the Lister strain of vaccinia virus and adenovirus, displayed in order of Ad5/VVLister ratio (a value greater than 1.0 indicates that VV Lister was more potent); (**b** and **c**) the viral replication of vaccinia virus in human pancreatic cancer cells insensitive to adenovirus; (**d**) viral replication in human squamous epithelial cells. Cells were infected with 1 PFU per cell (5 PFU per cell for NHEK) of VVLister and cell lysates harvested over a time course. Mean viral replication values ± SEM were determined by the $TCID_{50}$ assay.

the tumor selectivity *in vitro* was evaluated by comparing the replication of VVLister in non-immortalized normal and malignant squamous epithelial cell lines. Despite infecting normal human epithelial keratinocytes (NHEKs) cells with five times the dose of VVLister as that used for SCC25 tumor cells, no significant viral replication was seen in NHEK (data not shown) in contrast to the marked viral replication in SCC25 (Figure 1d).

The VVlister vaccinia virus has shown tumor tropism *in vivo* in mouse models;[35–38] therefore, we investigated whether the virus still has selectivity in our human pancreatic cancer xenograft model by real-time fluorescence imaging and immunohistochemistry (IHC), which confirmed the tumor selectivity of the vaccinia virus *in vivo*. We first confirmed dose-dependent green fluorescent protein (GFP) expression in Suit-2 cells infected with VVRG *in vitro* (supplementary Figure 1) before administering BALB/c nude mice bearing Suit-2 xenografts with an i.t. or i.v. injections of VVRG (Figures 2a and b). GFP expression was seen in all tumors after delivery by either route from 24 through to 240 h. Expression after i.t. virus administration increased to a peak at 72 h, whereas after i.v. delivery, levels were still rising 10 days later, suggesting that i.v. delivery of the Lister strain of vaccinia virus may be superior to that of i.t. delivery. Only background activity was observed in tumors of control mice injected with phosphate buffered saline (PBS). Vaccinia virus displayed excellent tumor selectivity with extra-tumoral fluorescence only observed in the tails of three mice after i.v. and one after i.t. deliveries, and the paws of two mice after i.t. delivery, although this resolved by 240 h.

The tumor selectivity of the parental VVLister was also confirmed by IHC of tumors and organs harvested from nude mice bearing Suit-2 xenografts after single i.v. virus injection (Figure 2c). VVLister was seen in all tumors from 24 to 480 h after delivery. Selectivity over normal tissues was confirmed, as only monocytes in the spleen were positive for VV coat protein in one of three mice at 24 and 72 h and all three mice from 120 to 480 h. Ovaries, brains, liver, kidneys, lungs and adrenal glands were all negative, including PBS-treated controls (not shown).

Construction, potency and replication of a novel vaccinia virus expressing human endostatin–angiostatin fusion protein

To enhance the efficacy of oncolytic vaccinia virus, the endostatin–angiostatin fusion gene was inserted at the *Not*1 restriction site of the Lister vaccine strain of vaccinia virus (VVhEA, Figure 3a). Another recombinant virus (VVlacZ) was constructed with the marker gene at the same genomic site serving as a control (Figure 3a). The sequences of inserted genes were confirmed by PCR and sequencing (data not shown). The potency and replication of both recombinant viruses *in vitro* were consistently less than that of the parental VVLister (Figures 3b, c and d). The potency of VVhEA was also less than that of VVlacZ in all cell lines tested, but there was no significant difference in the levels of peak replication between VVhEA and VVlacZ in PaTu8988s or Suit-2 cells (Figures 3c and d).

The endostatin–angiostatin fusion protein is expressed in Suit-2 cells infected with VVhEA and inhibits HUVEC cell tube formation and proliferation in vitro

The expression of the human endostatin–angiostatin fusion gene in Suit-2 cells infected with VVhEA was confirmed by western blotting (Figure 4a). The anti-angiogenic function of the endostatin–angiostatin fusion protein was confirmed by the inhibition of human umbilical vein epithelial cell (HUVEC) tube formation and proliferation (Figures 4b, c and d). HUVEC tube formation was significantly inhibited by supernatant from Suit-2 cells infected with VVhEA when compared with VVlacZ ($P<0.001$) or mock-infected controls (Figure 4c). The inhibition of HUVEC proliferation was also significantly greater when treated with VVhEA supernatant than either VVlacZ ($P<0.0001$) or mock-infected controls (Figure 4d).

The endostatin–angiostatin fusion protein is expressed and inhibits angiogenesis in Suit-2 xenografts treated with VVhEA in vivo

The distribution of VVhEA and VVlacZ and human endostatin–angiostatin fusion protein expression were confirmed by IHC in Suit-2 tumors harvested from nude mice after i.v. or i.t. virus injections. The expression of human endostatin–angiostatin in animals after i.t. (Figure 5a) and i.v. (not shown, similar to i.t.) therapies was confirmed from 48 h onwards, with high-intensity immunoreactivity correlated with Lister coat protein staining in spatially similar areas in serial sections. PBS-treated tumors were negative for both proteins (not shown). Fusion protein expression was also confirmed in plasma after i.v. (Figure 5b) and i.t. deliveries (supplementary Figure 2). The level of endostatin expression in plasma after i.v. injection of VVhEA was significantly higher and lasted longer than those after i.t. injection of VVhEA, consistent with the result of reporter gene expression in tumor tissues (Figures 2a and b). The microvessel density in tumors from VVhEA-treated mice was significantly lower than those from VVlacZ-treated mice from 120 h onwards after i.t. treatment (Figure 5c) and 72 h onwards after i.v. delivery (Figure 5d), indicating the inhibition of angiogenesis by the fusion protein.

VVhEA prolongs survival of nude mice bearing Suit-2 tumor xenografts after i.t. delivery

The Suit-2 xenograft human pancreatic cancer model was established in BALB/c nude mice and treated with i.t. injections of vaccinia viruses to assess the antitumor potency of the novel vaccinia virus *in vivo* (Figure 6). Tumors regressed in three of five mice treated with low-dose (three doses at 1×10^7 plaque-forming unit (PFU), Figure 6a) VVhEA therapy, which resulted in significantly longer survival than mice in other groups (Figure 6b). Two of these mice remained alive at the end of the experiment, that is, 91 days after treatment.

High-dose (six doses of 5×10^7 PFU) therapy with all vaccinia viruses resulted in significantly slower rates of tumor growth than in PBS-treated controls (Figure 6c). However, VVLister-treated mice survived for a significantly shorter period as all mice had to be killed as a result of weight loss and sickness by 18 days after their first treatment. Post-mortem analysis found pock lesions

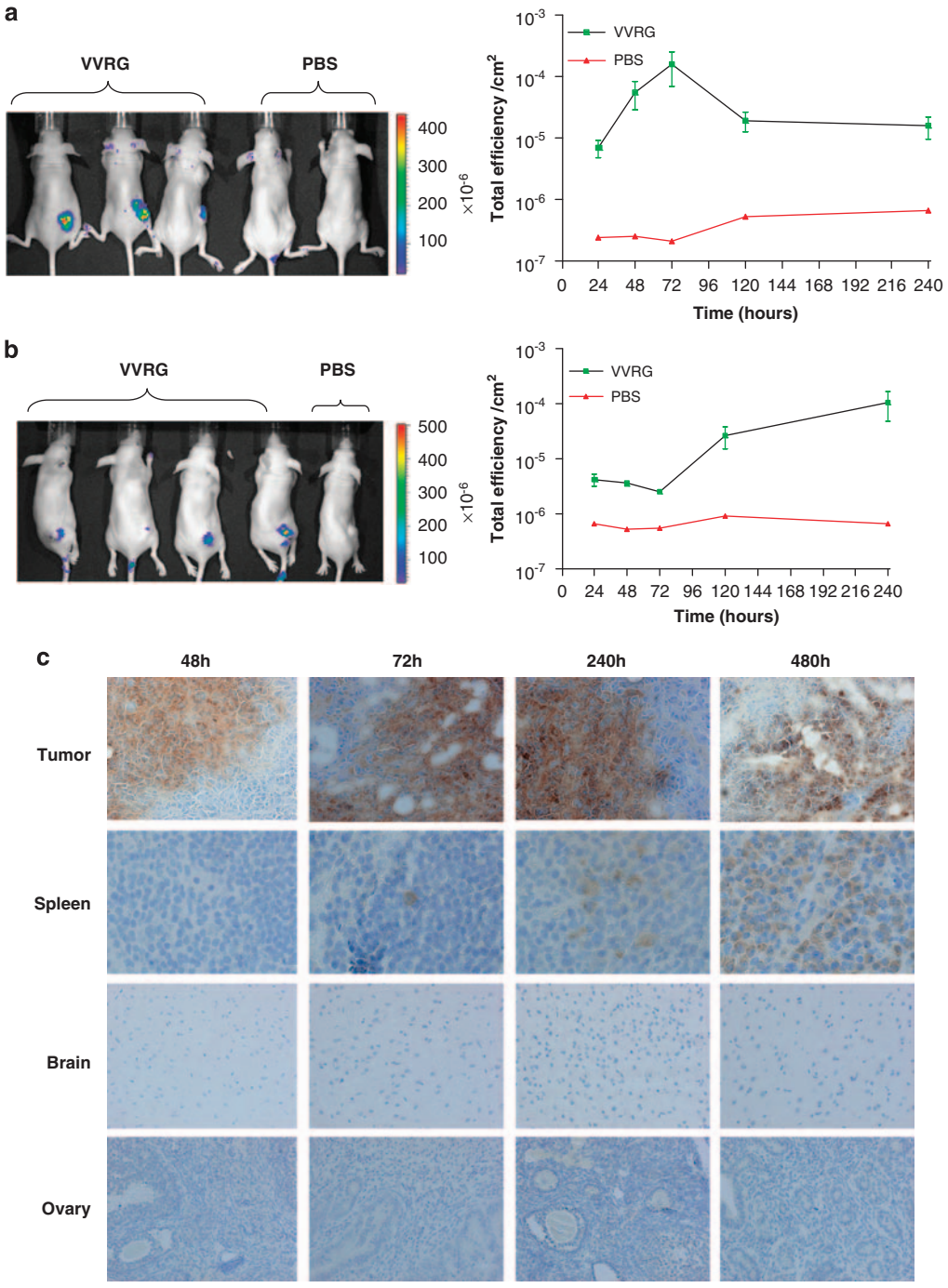

Figure 2 Biodistribution of vaccinia virus in BALB/c nude mice with Suit-2 subcutaneous tumors. (**a** and **b**) Biodistribution of VVRG *in vivo*. Biodistribution of VVRG was ascertained by IVIS imaging under inhalation anesthesia 24, 48, 72, 120 and 240 h after virus administration. Representative images are shown at 120 h (left panel) and combined mean efficiency ± SEM (right panel). A total of 1×10^6 cells Suit-2 were seeded by subcutaneous injection into the right flank of 10 BALB/c nude mice. When tumors reached 4–5 mm in diameter, in (**a**) three mice were injected once i.t. with 1×10^7 PFU VVRG and two mice with PBS as controls and in (**b**) four mice were injected once i.v. with 1×10^7 PFU VVRG and one mouse with PBS as a control; (**c**) biodistribution of the Lister strain of vaccinia virus after i.v. injection of 1×10^7 PFU VVRG to BALB/c nude mice with Suit-2 tumors by IHC for Lister coat protein in tumors, spleen, brain and ovaries (× 200).

on their feet, tails and oral cavities. Mice treated with VVhEA or VVlacZ did not suffer from the same severity of systemic side effects and survived longer than controls (Figure 6d). The VVhEA-treated mice survived significantly longer compared with the VVLacZ-treated mice, although the rate of tumor growth was no different between two groups.

In terms of clinical application, i.v. injection may be more feasible than i.t injection. Therefore, 1×10^7 PFU of VvhEA and VVLacZ were injected intravenously into the Suit-2 bearing nude mice. Unfortunately, all animals treated with recombinant vaccinia viruses developed tumor ulceration and pock lesions on their tails, and had to be sacrificed before showing efficacy.

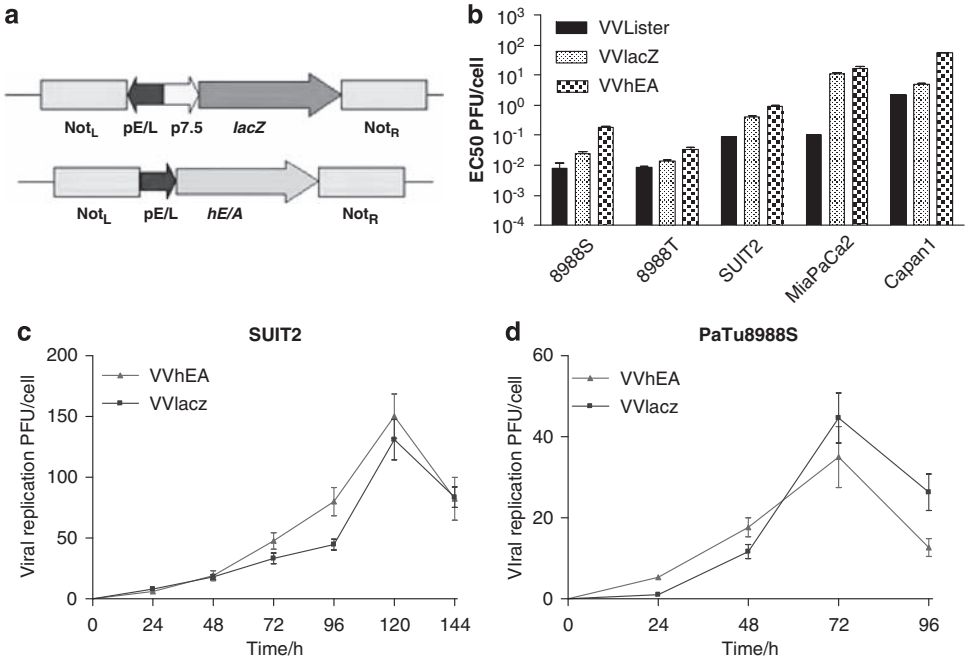

Figure 3 Potency and replication of recombinant vaccinia viruses armed with human endostatin–angiostatin fusion gene and LacZ gene. (**a**) VVlacZ and VVhEA constructions by insertion of the lacZ and human endostatin–angiostatin fusion genes, respectively, into the *Not*1 restriction site of VVLister; (**b**) potency of VVhEA and VVLacZ in human pancreatic cancer cell lines. EC50 values+SEM calculated by MTS assay 144 h after infection with different vaccinia viruses; (**c** and **d**) replication of recombinant viruses in pancreatic cell lines infected with 1 PFU per cell VVlacZ or VVhEA. Mean viral replication ± SEM was determined by $TCID_{50}$ assay.

Discussion

In this study, we first showed that the Lister vaccine strain of vaccinia virus was more potent at tumor cell killing than Ad5 in all cell lines tested, supporting our hypothesis that vaccinia virus represents a promising alternative to adenovirus for cancer gene therapy. The WR strain of vaccinia virus has been used most commonly for the construction of oncolytic vaccinia viruses, based on its superior lytic activity *in vitro*.[17] However, it is a laboratory-based strain, without the advantage of clinical experience as a vaccine. The Lister vaccine strain is an attractive oncolytic virus as its good safety profile has already been proved in humans during the vaccination program against smallpox in Europe and Asia. The recent complete sequencing of the Lister strain of vaccinia virus facilitated its further development and construction of more advanced vectors for gene therapy.[39]

In addition to its wide tissue tropism, oncolytic vaccinia virus must display tumor selectivity. The Lister vaccine strain does not cause any known human disease.[23] However, 80 per million vaccinia vaccinations produced lysis of skin epithelial cells, especially in immunosuppressed individuals, resulting in spreading tissue necrosis known as vaccinia necrosum.[40,41] Therefore, we selected normal squamous cells NHEK (more likely than other tissue origin to support vaccinia virus replication) and compared VVLister replication with SCC25, a human tumor cell line also of squamous cell origin. Only SCC25 supported the replication of vaccinia virus, confirming the selectivity of the Lister stain *in vitro*.

In a human pancreatic cancer model, we have also showed that the Lister vaccinia virus is inherently tumor-selective after delivery *in vivo*, evidenced by live fluorescence imaging of VVRG and the biodistribution of i.v. VVLister by IHC. This selectivity was also shown in murine tumor xenograft models (Tysome *et al*. unpublished data). The Lister vaccine strain vaccinia virus presents a superior safety profile *in vivo* as the Lister strain, in contrast to the WR strain,[9,18] has not been detected in the ovaries or brains of mice. Most surprisingly, we, for the first time, showed that i.v. delivery of the Lister strain vaccinia virus might be superior to i.t. delivery. This finding may have important implications for the future use of oncolytic vaccinia virus as i.v. therapy could target not only primary tumors, but also metastases. This observation may be strain-dependent, as tumor-specific transgene expression by a TK-deleted WR strain of vaccinia virus expressing cytosine deaminase[7] and a Wyeth strain virus expressing GM-CSF (granulocyte-macrophage colony-stimulating factor),[42] did not show this feature. Unfortunately, tumor specificity after i.v. delivery in our study did not translate into antitumor efficacy using the standard dose of 1×10^7 PFU, as the routine dose used for oncolytic vaccinia virus in this study was limited by tumor ulceration and pock lesions on the tail, paws and in oral cavities of the nude mice. These side effects may result from the huge viral replication in the 'factory' of tumor cells as we have observed that reporter gene and endostatin–angiostatin expressions were significantly higher after i.v. injection than i.t. injection (Figures 2b and 5b). Further, experiments are needed to achieve the optimal regime. In addition, although pock lesions have been reported after vaccinia virus administration to nude mice,[43–45] they have not been observed in immunocompetent mice.[44–47] Therefore, further studies are needed to clarify our findings using immunocompetent tumor models, where

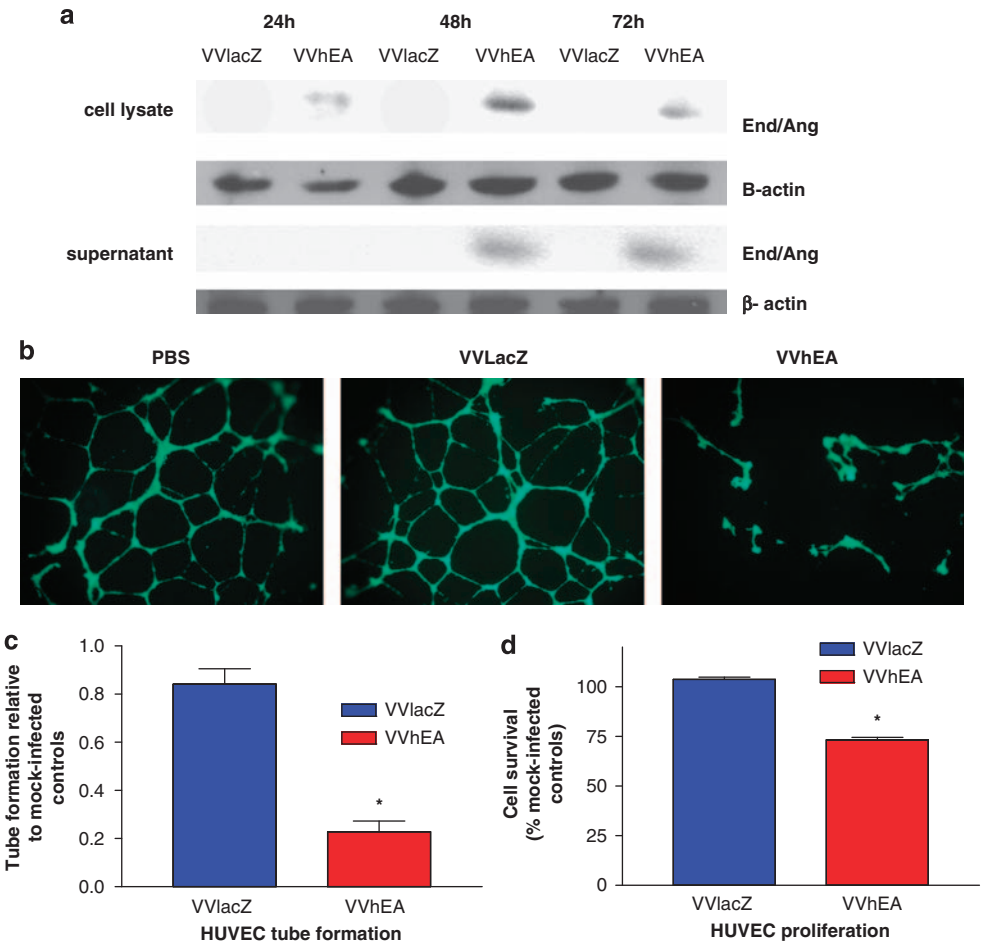

Figure 4 Expression and angiogenesis inhibition by the human endostatin–angiostatin fusion protein in Suit-2 cells infected with VVhEA *in vitro* (**a**) endostatin–angiostatin fusion protein expression in Suit-2 cells. Suit-2 cells were infected with 1 PFU per cell of VVhEA or VVlacZ. Cells and supernatant were harvested 24, 48 and 72 h later. Western blots were performed as described in Materials and methods. All fusion protein bands were between 70 and 80 kDa and β-actin around 45 kDa. Although intracellular transgene expression was found in the cells by 24 h after infection, endostatin–angiostatin fusion protein was detected in the supernatant only after 48 h; (**b** and **c**) inhibition of HUVEC tube formation. HUVEC were seeded in a 96-well BD Biocoat angiogenesis plate containing Matrigel (BD Bioscience, Bedford, MA, USA) with 10 μg of supernatant harvested and concentrated 72 h after Suit-2 cells were infected with 1 PFU per cell of VVhEA or VVlacZ. At 16 h later, 400 μg Calcein AM (Invitrogen, Eugene, OR, USA) was added and fluorescence microscopy used to assess the number of fully formed HUVEC tubes, which were compared with mock-infected controls and each other by one-way ANOVA; (**d**) inhibition of HUVEC proliferation, mean cell survival ± SEM as a percentage of uninfected HUVEC by MTS assay 96 h post-treatment with 10 μg ml^{-1} of supernatant harvested from Suit-2 cells infected with 1 PFU per cell of VVhEA or VVlacZ for 72 h. Mean results were compared by one-way ANOVA with Bonferonni's *post hoc* testing.

the potential for systemic treatment may not be hampered by systemic side effects.

Anti-angiogenic agents, which target activated endothelial cells, represent a promising strategy for the treatment of tumors. However, their development has been hampered by several factors including manufacturing difficulties, stability and solubility issues.[48] In addition, the majority of these inhibitors of angiogenesis are not directly cytotoxic to tumor cells so they need to be expressed on a continuous basis. Given the tumor-specific replication of oncolytic vaccinia virus, it is timely to explore their use to engineer inhibition of angiogenesis. We constructed a novel oncolytic Lister strain of vaccinia virus armed with a human endostatin–angiostatin fusion gene targeting the process of neovascularisation. The continuous expression and angiogenesis inhibition of the endostatin–angiostatin fusion protein was confirmed in the Suit-2 pancreatic cancer model both *in vitro* and up to 20 days after one treatment *in vivo*. We found that the potency and replication of both F14.5L-deleted viruses (VVhEA and VvlacZ) were attenuated when compared with the parental VVLister virus. We anticipate that this was because of insertional inactivation at the *Not*I site of the hypothetical protein F3 encoded by a small gene located between F14L (List049) and F15L (List050) of the genome.[39] The vaccinia F3 protein may be involved in immune evasion, accounting for viral attenuation after deletion. This concept was recently confirmed by others using VVRG (described by the authors as GLV-ld27).[38]

The VVhEA therapy resulted in significantly longer survival when compared with VVlacZ-treated mice *in vivo*, with regression in three of five tumors after low dose, and all tumors after high-dose therapy. However, tumor regression was also seen after high-dose treatment of all vaccinia viruses, although survival was only prolonged with VVhEA and VVlacZ, when compared with PBS. The attenuation of both recombinant viruses resulted in fewer side effects in immunodeficient mice *in vivo*, as VVLister-treated mice developed more pock

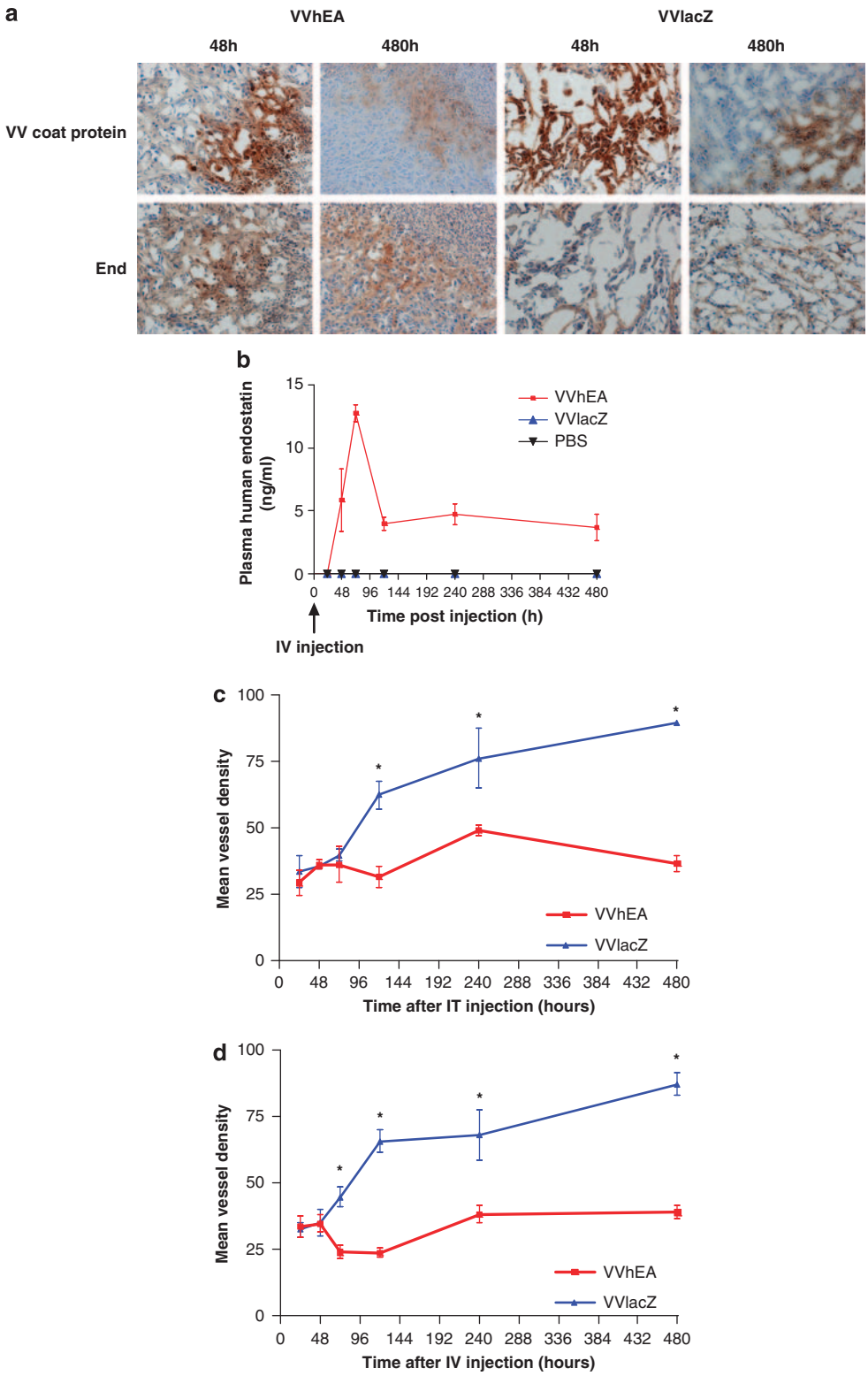

Figure 5 Expression and angiogenesis inhibition by the human endostatin–angiostatin fusion protein after treatment of Suit-2 tumor model *in vivo*. (**a**) IHC for Lister vaccinia virus coat protein and human endostatin was performed on Suit-2 tumors; (**b**) ELISA for human endostatin performed on plasma harvested from animals after 1×10^7 PFU i.v. VVhEA or VVlacZ with representative images at 48 and 480 h ($\times 200$); (**c** and **d**) IHC for murine CD31 to assess mean microvessel density ± SEM in five random $\times 200$ fields of i.t. (**c**) or i.v. (**d**) VVhEA-, VVlacZ- or PBS-treated tumors compared by *t*-tests.

lesions and rapidly lost weight, requiring their sacrifice even before PBS-treated controls. The survival advantage of VVhEA over VVlacZ after high-dose therapy may have been because of the VVhEA-mediated inhibition of angiogenesis affecting i.t. propagation of the virus and thus spread and distribution of the virus throughout the

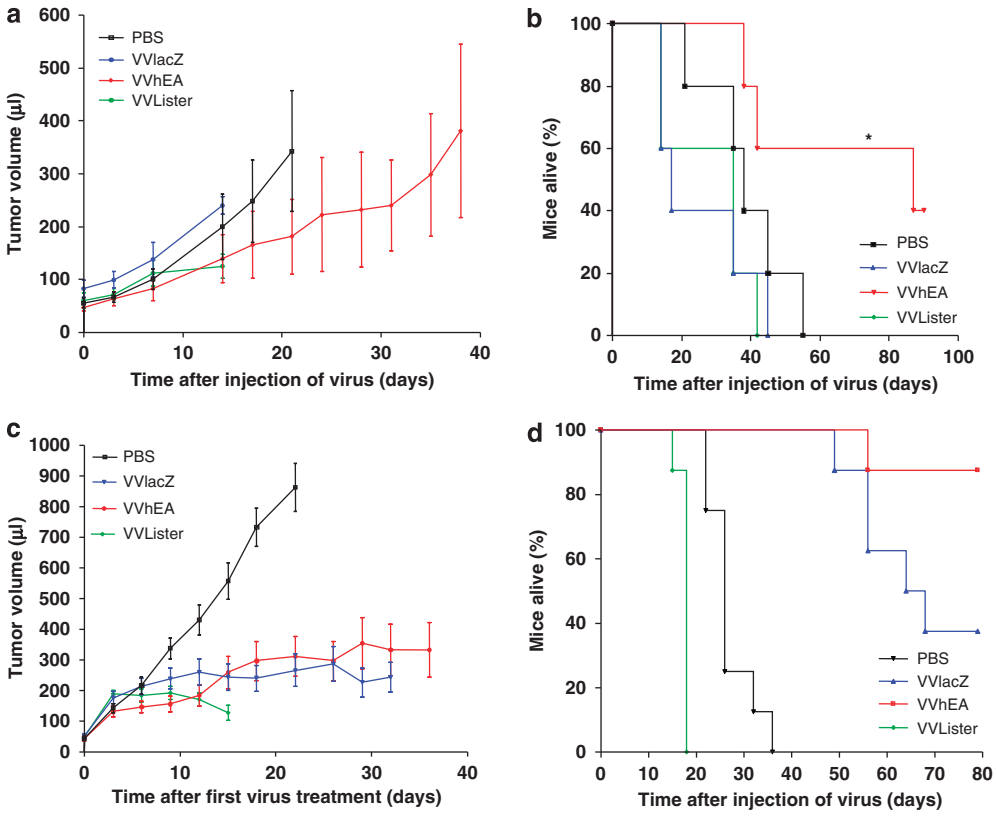

Figure 6 Antitumoral efficacy of different vaccinia viruses in established Suit-2 tumors *in vivo*. Suit-2 human pancreatic carcinoma xenografts implanted subcutaneously in BALB/c nu/nu mice as described in Materials and methods. (**a** and **b**) PBS or 1×10^7 PFU of different viruses were injected on days 0, 2 and 4 ($n = 5$ per group); (**c** and **d**) 5×10^7 PFU of different viruses and PBS were injected on days 0, 3, 6, 9, 12 and 15 ($n = 8$ per group); (**a** and **c**) show the tumor growth curves (until death or killed because of tumor ulceration or weight loss of first animal in each group); (**b** and **d**) show the survival rate of nude mice by Kaplan–Meier survival analysis (log-rank test for statistical significance).

mice, which may have reduced the side effect. In addition, VVhEA may reverse VEGF-induced cancer-associated systemic syndrome and prevent death in tumor-bearing mice without significantly compromising tumor growth.[49] Although the added benefit of the fusion gene in VVhEA over VVlacZ was not as great as might have been expected, these results obviously warrant further investigation in an immunocompetent model as anti-angiogenesis therapy can overcome endothelial cell anergy, promote leukocyte-endothelium interactions and inhibit tumor growth and microvessel density significantly, with remarked infiltration of leukocytes (CD45), as well as the number of CD8+ cytotoxic T lymphocytes in tumors.[50]

In conclusion, we show the superior antitumor potency of the Lister strain of vaccinia virus over wild-type adenovirus against pancreatic cancer, especially for tumor cell lines insensitive to adenovirus. The virus also displays inherently high selectivity for cancer cells, sparing normal cells both *in vitro* and *in vivo*. Through arming the Lister strain with the human endostatin–angiostatin fusion gene, we combined the anti-angiogenic properties of the fusion protein with the oncolytic properties of the Lister virus. This novel recombinant virus has shown efficacy in terms of tumor regression and prolonged survival in a human pancreatic cancer xenograft model and shows potential for translation to clinical therapy in the future.

Materials and methods

Cell lines

CV1, the African Green Monkey normal kidney cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 10% fetal calf serum (FCS). HUVECs and NHEKs were obtained from Cambrex (Cambridge, UK) and maintained in epithelial growth medium-2 (EGM-2) and keratinocyte growth medium (KGM), respectively.

Human pancreatic carcinoma cell lines Suit-2, PaTu8988S, PaTu8988T, MiaPaCa2, PANC1 and Capan1 were maintained in DMEM with 10% FCS. The human HNSCC cell line, SCC25, was obtained from Cancer Research UK Cell Culture Service (CRUKCCS) and maintained in DMEM:HAMSF12 in the ratio of 1:1 with 10% FCS and 400 ng ml^{-1} hydrocortisone (Sigma-Aldrich, St Louis, MO, USA).

Viruses

The Lister vaccine strain (LIVP) of vaccinia virus was used as a parental virus for the construction of recombinant viruses.[43] The fused human endostatin and angiostatin genes were inserted following the method described earlier[51] into the non-essential *Not*I restriction site, located between F14L and F15L of the LIVP and encoding a hypothetical protein, F3. VVlacZ

was constructed in the same way after insertion of the *E. coli lacZ* gene, to serve as a control for VVhEA. PCR and DNA sequencing were used to verify recombinant viruses. In an earlier study, the Renilla luciferase-GFP fusion gene was inserted into the same region to create VVRG.[43] Wild-type adenovirus Ad5 was described earlier.[52]

Evaluation of viral cytotoxicity in vitro

Cells were seeded at 1 and 5×10^3 cells per well, depending on growth rates, in 96-well plates, and infected with viruses 16–18 h later. Cell survival on day 6 after viral infection was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay and EC_{50} value (viral dose killing 50% of tumor cells) was calculated as described earlier.[52] All assays were performed at least three times.

Viral replication

Cells were seeded at $2–4 \times 10^5$ cells per well, depending on growth rates, in three wells of 6-well plates in media with 10% FCS, and infected with 1 PFU per cell of vaccinia viruses 16–18 h later. Samples were harvested in triplicate at 24-h intervals up to 144 h. Viral replication was detected by $TCID_{50}$ (50% tissue culture infective dose) as described earlier.[52]

Biodistribution of VVRG by fluorescence imaging in vivo

A total of 1×10^6 Suit-2 cells were implanted subcutaneously into the right flank of 10 female BALB/c nude mice (Harlan UK Ltd, Bicester, UK). When tumors reached 0.4–0.5 cm in diameter, mice received 100 μl i.t. or i.v. tail vein injections of 1.0×10^8 PFU VVRG or PBS. The biodistribution of VVRG was determined in mice anesthetized (2% halothane by inhalation in O_2 1 l min^{-1} and NO 1 l min^{-1}) 1, 2, 3, 5 and 10 days after treatment by measuring fluorescence with the IVIS camera (Xenogen Corp., Alameda, CA, USA) in tumors defined as region of interest (ROI) after imaging. All animal experiments were performed in accordance with the regulations of the UK Home Office and the local animal ethics committee.

Western blotting

A total of 2×10^5 Suit-2 cells were seeded in 60 mm dishes in conditional media and infected with 1 PFU per cell VVhEA or VVlacZ in DMEM with 2% FCS after 16–18 h. Cells and supernatant were harvested separately 24, 48 and 72 h after infection and supernatants concentrated using Centricon 10 kDa columns (Millipore, Bedford, MA, USA). A total of 30 μg aliquots of total protein were used to detect endostatin–angiostatin fusion protein expression by western blotting as described earlier.[53]

HUVEC proliferation and tube formation assay

A total of 1×10^4 HUVEC cells were seeded in 96-well plates in EGM-2 and treated 16–18 h later with 10 μg ml^{-1} of supernatant concentrated from Suit-2 cells infected with VVlacZ or VVhEA for 72 h. After another 96 h, cell viability was measured by MTS assay to determine HUVEC proliferation.

The inhibition of HUVEC tube formation was observed using the BD Biocoat angiogenesis system (BD Biosciences, Bedford, MA, USA) according to the manufacturer's instructions. Tube formation by HUVEC treated with supernatant was compared with mock-infected HUVEC alone by counting the number of completely formed tubes/well by fluorescence microscopy.

Histopathology and immunohistochemistry staining

When established Suit-2 tumors reached 0.4–0.5 cm in diameter, mice were stratified into groups of 18. Each received one 100-μl i.v. or i.t. injection of 1.0×10^7 PFU of VVLister, VVhEA, VVlacZ or PBS. Groups of three mice in each cohort were killed 24, 48, 72, 120, 240 and 480 h later, tumors removed, snap-frozen in isopentane and stored at −80 °C. Blood was collected into a heparinized tube and plasma stored at −80 °C. From animals that had received i.v. VVLister or PBS, the spleen, liver, kidneys, brain, ovaries, lungs and adrenal glands were also collected and stored. All tissues were processed for histopathology and IHC analyses for viral coat protein (1:2000 rabbit anti-vaccinia virus coat protein polyclonal antibody (MorphoSys UK Ltd, Bath, UK)), endostatin expression (1:250 rabbit anti-human endostatin polyclonal antibody (Abcam, Cambridge, UK)) and CD31 (1:200 rat anti-murine CD31 antibody (BD Biosciences, Oxford, UK)) to determine microvessel density as described earlier.[54]

ELISA

Human endostatin levels in the plasma of BALB/c nu/nu mice bearing Suit-2 xenografts treated with i.v. VVhEA, VVlacZ or PBS were obtained using the Duoset ELISA for human endostatin (R and D Systems, Abingdon, UK unless otherwise stated) according to the manufacturers' instructions.

In vivo *efficacy experiments*

As described above, when established Suit-2 xenografts of 20 mice reached 0.4–0.5 cm in diameter, mice were regrouped by tumor size and received 100 μl i.t. injections of 1.0×10^7 PFU VVLister, VVlacZ, VVhEA or PBS on days 0, 2 and 4. Tumor volumes were estimated (volume = (length × width^2 × π)/6) twice weekly until mice were killed when tumor volume reached 1.00 cm^3 or had been present for 3 months. This was repeated in experiments, in which 36 mice with Suit-2 xenografts received 5.0×10^7 PFU i.t. injections on days 0, 3, 6, 9, 12 and 15, and 20 mice with Suit-2 xenografts received 1.0×10^7 PFU i.v. injection on day 0, when tumor xenografts reached 0.8–0.5 cm.

Statistical analysis

All statistical analyses were performed as described in figure legends or results. Differences were considered significant where $P < 0.05$.

Conflict of interest

The authors declare no conflict of interest.

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