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ORIGINAL ARTICLE

A strategy for systemic delivery of the oncolytic herpes virus HSV1716: redirected tropism by antibody-binding sites incorporated on the virion surface as a glycoprotein D fusion protein

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We report on the ability of single-chain variable fragment (scFv) incorporated into the viral envelope to alter the tropism of herpes simplex virus (HSV) 1716. Using recombinant viruses expressing fusion proteins comprising cell-surface antigen-specific scFvs N terminus linked to amino acids 274–393 of gD, we demonstrated that the tropism of these HSV1716 variants was modified such that infection was mediated by the cognate antigen. Thus, an HSV1716 variant that expressed an anti-CD55 scFv targeting moiety linked to these gD residues was able to infect non-permissive Chinese hamster ovary cells expressing CD55 and this infection was specifically blocked by an anti-CD55 monoclonal antibody. Similarly, the infection efficiency of an HSV1716 variant for semi-permissive human leukaemic, CD38-positive cell lines was greatly improved by an anti-CD38 scFv targeting moiety linked to gD residues 274–393, and this enhanced infectivity was abrogated specifically by an anti-CD38 monoclonal antibody. Finally, intravenous/intraperitoneal injection of an HSV1716 variant displaying an anti-epidermal growth factor receptor (EGFR) scFv linked to residues 274–393 of gD enhanced destruction of subcutaneous EGFR-positive tumours in nude mice compared to unmodified HSV1716. Therefore, targeting of HSV1716 oncolysis to specific cell types through the display of entry mediating scFv/gD fusion proteins represents an efficient route for systemic delivery.
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Introduction

Harnessing the cytopathic effects of virus infection is rapidly becoming established as an anti-cancer treatment and replication-competent mutants of the α-herpesvirus, herpes simplex virus type 1 (HSV-1) are showing great potential as oncolytic therapeutic agents.[1,2] The HSV-1 mutant 1716, lacking the ICP34.5 gene, has greatly reduced lethality in mice but replicates as efficiently as wild-type virus in actively dividing tissue culture cells.[3,4] The ICP34.5 open reading frame (ORF) is a neurovirulence gene and its protein product has been proposed to condition post-mitotic cells for viral replication, probably by an interaction with proliferating cell nuclear antigen.[5,6] ICP34.5 deletion mutants cannot replicate in terminally differentiated cells but will lytically infect dividing cells and this has proved to be an effective tumour destruction strategy. In recent clinical trials, injection of HSV1716 has been shown to be safe in treating patients with recurrent glioma, metastatic melanoma and squamous cell carcinoma of the head and neck[7] and proof of principle of selective replication within tumours has been obtained.[8–11]

An essential strategy for the improved effectiveness of oncolytic viral therapies depends upon systemic delivery of targeted viruses that seek out and destroy all cancerous cells.[2,12,13] Reprogramming viral tropisms has thus received much attention, an approach requiring redirection of the natural tropism from native receptors to a receptor of choice. Targeted infection by vaccinia virus, retrovirus and measles virus displaying single-chain antibody-binding sites (scFv) incorporated into their structure by fusion with viral envelope proteins has been described.[14–22] Bridging, bispecific targeting molecules comprising either an anti-adenovirus scFv or the Coxsackievirus-adenovirus receptor linked to a targeting scFv, have been used for targeting oncolytic adenoviruses.[23–29] Results from a number of studies with HSV-1 have shown that it is possible to alter the tropism by incorporating ligands such as erythropoietin, interleukin (IL) 13, human hepatitis B virus preS1 peptide, the N-terminal fragment of urokinase-type plasminogen activator or 6-His into the viral envelope as glycoprotein fusion proteins.[30–35] Targeting has also been achieved using a soluble adapter molecule comprising an anti-epidermal growth factor receptor (EGFR) scFv linked to the HSV-1 gD-binding domain of nectin-1 and more

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recently, a scFv against HER2/neu incorporated into the N terminus of gD has been shown to redirect the tropism of HSV-1 to this mammary tumour receptor.[36,37]

Initiation of infection by HSV-1 requires cells to display the appropriate receptors to permit viral access, a process requiring the complex interplay of a number of cellular and viral membrane components.[38,39] Four virus membrane glycoproteins, gB, gD and the heterodimer comprising gH and gL (gH/gL) have been shown to be necessary and sufficient for HSV-1 entry into cells. Initial contact is between gB and cellular heparan sulphate, gD then interacts specifically with the cellular receptors for HSV-1 entry that include herpesvirus entry mediator (HVEM), nectins-1 and -2 and 3-*O*-sulphated heparan sulphate. Membrane fusion requires the concerted activities of gB and gH/gL so that the nucleocapsid gains access to the cell and infection is initiated. Nectin-1 is probably the principal entry receptor for infection of central and peripheral nerve cells whereas HVEM expression is more restricted and limited to cells of lymphoid origin.[40–44] Wide bioavailability of HSV-1 entry mediators will create problems for the efficient systemic administration of oncolytic HSV1716 and we have investigated therefore the possibility of redesigning the viral tropism by scFv embedded in the viral envelope by fusion with the C-terminal tail of gD. Our results demonstrated that membrane-displayed scFv linked to gD specifically influenced the tropism of HSV1716 thus allowing development of effective tumour-targeted HSV1716 for systemic delivery.

Results

Preliminary data obtained using viruses that displayed scFvs linked to 15 sequentially deleted N-terminal truncations of HSV-1 gD (scFv/gD) identified a minimal fragment of the protein, comprising residues 274–393, which functioned optimally to redirect the tropism of the virus. Analysis of the effects of scFv/gD glycoprotein fusion proteins on tropism used infection of non-permissive Chinese hamster ovary (CHO) cells by viruses propagated on stable Vero cell lines expressing the scFv/gD glycoprotein fusion proteins. As gangliosides are ubiquitous components of cell membranes and GD3 was present on CHO cells as determined using the anti-GD3 monoclonal antibody (mAb) R24 (data not shown), an scFv derived from the R24 mAb was used in this initial study. The DNA encoding each fusion protein construct was assembled in the pEL4-mammalian-expression vector, containing the R24scFv such that the expressed protein consisted of a secretory leader sequence, an N-terminal scFv domain for binding to a targeting ligand linked to viral glycoprotein D domains for virus incorporation and a myc-tag appended to the C terminus of the glycoprotein D domains for detection by immunofluorescence and western blotting. Linear representations of HSV-1 gD and a typical scFv/gD fusion protein are shown in Figure 1a(i) and (ii), respectively. All 15 gD deletions were linked to the C terminus of the R24 scFv and stable Vero cell lines expressing the scFv/gD fusion proteins were created by antibiotic selection. Vero cell localization and expression of the myc-tagged fusion proteins was demonstrated by immunofluorescence and western blotting and incorporation into virus was assessed by western blotting of purified virion preparations obtained after HSV1716gfp infection of the cell lines. The results are summarized in Table 1. In total, 2 of the 15 R24scFv/gD fusion protein constructs, with the R24scFv linked to residue 58 of gD (R24/58gD) and the R24scFv linked to residue 105 of gD (R24/105gD), were not expressed. Of the expressed fusion proteins, only the R24scFv linked to residue 164 of gD (R24/164gD) and the R24scFv linked to residue 231of gD (R24/231gD) were not incorporated into virus. All expressed R24scFv/gD fusion proteins, including R24/164gD and R24/231gD, which were not incorporated into virus, demonstrated a distinctive perinuclear accumulation with some immunofluorescent patches present at the plasma membrane (data not shown). No fluorescence was observed in cells that failed to express R24/58gD and R24/105gD. The cellular localization of the expressed fusion proteins is consistent with an accumulation in the nuclear membrane/endoplasmic reticulum and further trafficking of the fusion proteins probably through the Golgi apparatus to the outer membrane of the cell.

CHO cells, normally resistant to HSV1716 infection when the virus is propagated on Vero cells, were infected with 1 PFU per cell HSV1716gfp derived from infection of R24/gD fusion protein-expressing cell lines and the percentages of infected cell determined by fluorescence microscopy (Table 1). HSV1716gfp propagated on Vero cells (HSV1716V) failed to infect CHO cells and this contrasts with HSV1716gfp propagated on Baby-hamster kidney (BHK) cells (HSV1716B) that infects approximately 30% of CHO cells. A number of HSV1716gfp viruses derived from propagated on Vero cell lines expressing R24/gD fusion proteins were able to infect CHO cells at levels similar to or greater than those obtained with HSV1716B. Thus, the R24scFv linked to gD residues 37 (R24/37gD), 78 (R24/78gD), 92 (R24/92gD), 128 (R24/128gD), 260 (R24/260gD) and 274 (R24/274gD) infected 27, 11, 20, 18, 60 and 55%, respectively, of CHO cells compared to only occasionally infected CHO cells for the other R24/gD fusion proteins.

On the basis of these observations, we produced a series of targeted recombinant viruses with expression cassettes for anti-CD20 (HSV1716CD20), anti-CD38 (HSV1716CD38), anti-CD55 (HSV1716DAF) or anti-EGFR (HSV1716EGFR) scFvs fused to residues 274–393 of gD inserted in the RL1 loci. Expression of the scFv/gD fusion proteins was controlled by the constitutively active CMV-IE promoter and the fusion proteins comprised a secretory leader sequence, N-terminal scFv for binding to a targeting ligand linked to C-terminal viral glycoprotein D residues 274–393 for virus incorporation with a myc-tag appended at the C terminus for detection by immunofluorescence/western blotting (see Figure 1a(ii)). These targeted HSV1716 variants were generated using a novel HSV1716GateRed site-specific recombination system that also inserted a PGK-gfp-expression cassette in tandem with and in the same orientation as the scFv/gD-expression cassette. Linear representations of the scFv/274gD fusion protein-expression cassette, the scFv/274gD fusion protein-expression cassette inserted in the Gateway entry vector pENTR1Agfp, the destination vector HSV1716GateRed at the RL1 loci and the viral product of recombination between the entry and destination vectors are shown in

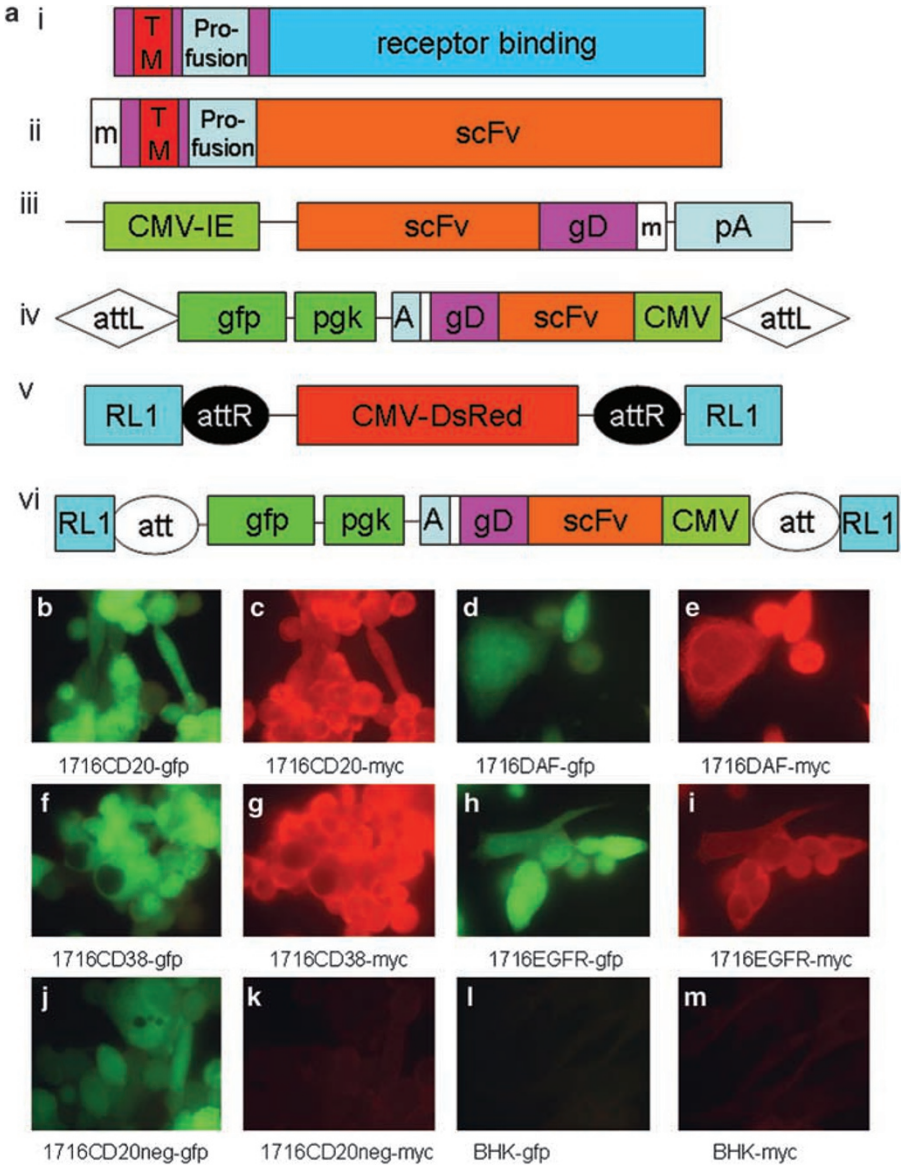

Figure 1 (**a**) Linear representations (not to scale) of (i) HSV-1 gD structure showing the regions of the protein involved in receptor binding, profusion activity and insertion in the viral membrane (TM), (ii) the single-chain variable fragment (scFv)/274gD fusion protein structure relative to gD showing the location of the scFv-binding domain and regions involved in profusion activity, membrane insertion and the myc tag (**m**), (iii) the scFv/274gD fusion protein-expression cassette used for recombinant virus production, CMV-IE: CMV immediate early promoter and pA: bovine growth hormone polyadenylation signal, (iv) the multiple cloning site located between the attL site-specific recombination sequences of the vector pENTR1Agfp showing pgk-gfp and CMV-scFv/274gD-BGHpA-expression cassettes, (v) the HSV1716GateRed genome within the RL1 loci showing the attR site specific recombination sequences flanking the CMV-DsRed-expression cassette within the deleted region of the ICP34.5 gene and (vi) the viral product from site-specific recombination between (iv) and (v) with pgk-gfp and CMV-scFv/274gD-BGHpA-expression cassettes now inserted in the RL1 loci. (**b**–**m**) Immunofluorescence/fluorescence microscopy of Baby-hamster kidney (BHK) cells infected with HSV1716CD20 (**b**, **c**), HSV1716DAF (**d**, **e**), HSV1716CD38 (**f**, **g**), HSV1716EGFR (**h**, **i**) or HSV1716CD20neg (**j**, **k**). Mock-infected BHK cells are shown in **l** and **m**. Staining with the anti-myc monoclonal antibody is shown in **c**, **e**, **g**, **i**, **k**, **m** and gfp is shown in **b**, **d**, **f**, **h**, **j**, **l**.

Figure 1a(iii)–(vi), respectively. Expression of the scFv/274gD proteins in infected cells was demonstrated by immunofluorescence using the anti-myc tag mAb (Figures 1b–m) with cytoplasmic/perinuclear distributions observed in BHK cells infected with HSV1716CD20 (Figure 1c), HSV1716DAF (Figure 1e), HSV1716CD38 (Figure 1g) and HSV1716EGFR (Figure 1i). Expression of gfp was also observed in cells infected with these viruses (Figures 1b, d, f and h). Additionally, an HSV1716 variant in which the expression cassette for the anti-CD20 scFv linked to residue 274 of gD was in the opposite orientation relative to PGK-gfp was created (HSV1716CD20neg) and this variant failed to express a protein product as only gfp expression was observed (Figures 1j and k). Mock-infected BHK cells are shown in Figures 1l and m. Expression of the scFv/274gD fusion proteins following infection of BHK cells was also confirmed by western blotting of whole-cell extracts using an anti-myc tag mAb (data not shown). A 45 kDa protein was detected with HSV1716CD20, HSV1716DAF,

Table 1 Expression and CHO cell infection data from R24 scFv linked to 15 N-terminal deletions of HSV-1 gD

Construct	*Approx MWt (kDa)*[a]	*Vero cell expression*[b]	*Virus incorp*[b]	*Localization in Vero cells*	*CHO cell infectivity*[c]
R24/37gD	70	++	++	Perinuclear	27%
R24/58gD	67	ND	ND	ND	<1
R24/79gD	65	++	++	Perinuclear	11%
R24/92gD	64	++	++	Perinuclear	20%
R24/105gD	61	ND	ND	ND	<1
R24/128gD	60	++	++	Perinuclear	18%
R24/139gD	59	++	+	Perinuclear	<1
R24/164gD	56	++	ND	Perinuclear	<1
R24/179gD	55	++	+	Perinuclear	3
R24/191gD	54	++	+	Perinuclear	<1
R24/207gD	52	++	+	Perinuclear	<1
R24/231gD	49	++	ND	Perinuclear	<1
R24/239gD	48	++	+	Perinuclear	<1
R24/260gD	46	++	++	Perinuclear	60%
R24/274gD	44	++	++	Perinuclear	55%

Abbreviations: Approx, approximately; CHO, Chinese hamster ovary; incorp, incorporation; ND, not detected.
[a]Fusion protein MWt = scFv (30 kDa)+gD amino acids.
[b]Band intensity from western blots; high, +++; intermediate, ++; low, +.
[c]Percentage of CHO cells infected per field of view, two fields of view counted per virus.

HSV1716CD38 and HSV1716EGFR that was absent from mock infected or from BHK cells infected with HSV1716 or HSV1716CD20neg. SDS–polyacrylamide gel electrophoresis (PAGE)/western blotting using the anti-myc mAb on 1×10^6 PFU HSV1716CD20, HSV1716DAF, HSV1716CD38 and HSV1716EGFR confirmed that the scFv/274gD fusion protein was incorporated into virus (data not shown). Virus stocks of HSV1716CD20, HSV1716DAF, HSV1716CD38, HSV1716EGFR and HSV1716CD20neg were prepared by propagation in Vero cells in T175 flasks and the yields of each virus ($c4 \times 10^9$ PFU per flask) were within the range of yields for HSV1716 propagation on Vero cells suggesting that the expression and incorporation of the targeting moiety did not influence replication efficiency.

Using fluorescence microscopy, the abilities of these viruses to infect CHO, CHO/decay-accelerating factor (DAF), SupT, THP-1 and TolB cells were assessed either in the presence or absence of mAbCD20, mAbCD38, mAbDAF or mAbEGFR, the mAbs from which the anti-CD20, anti-CD38, anti-DAF or anti-EGFR scFvs were cloned. THP-1 and SupT are monocyte and T-cell lines, respectively, that express CD38 but not CD20 and TolB is a B-cell line that expresses both CD38 and CD20, although levels of CD20 expression in TolB cells were apparently low as only weak fluorescence was observed with TolB/mAbCD20. We also created a CHO cell line (CHO/DAF) that constitutively expressed human DAF as demonstrated by immunofluorescence with mAbDAF (Figure 2a) and a recombinant minibody version of mAbDAF (rmAbDAF; Figure 2b) with intense membrane staining of DAF in the CHO/DAF cells but not with normal CHO cells (Figure 2c).

Only occasional CHO cells were infected by HSV1716CD20, HSV1716CD38 or HSV1716DAF (Figures 2d–f , respectively) compared to approximately 20% of CHO cells infected with HSV1716gfp propagated in BHK cells (HSV1716B; Figure 2g). HSV1716CD20 (Figure 2h) and HSV1716CD38 (Figure 2i) were also unable to infect CHO/DAF cells although, as with CHO cells, HSV1716B infected approximately 30% of CHO/DAF cells (Figure 2k). Significantly, HSV1716DAF was able to infect approximately 60% of CHO/DAF cells (Figure 2j) and, by pre-incubating the CHO/DAF cells in mAbDAF (Figure 2m) or the rmAbDAF (Figure 2n), this level of infectivity was reduced to approximately 5%. Pre-incubation of CHO/DAF cells in mAbCD20 (Figure 2l) had no effect on the infectivity of the HSV1716DAF with approximately 70% of cells infected and pre-incubation of CHO/DAF in mAbDAF did not inhibit HSV1716B infectivity (Figure 2o).

The fluorescence microscopy data was confirmed by western blotting using an anti-HSV-1 R1 antiserum to probe whole-cell extracts from CHO/DAF cells. R1 expression was only detected in CHO/DAF cells infected with HSV1716DAF with no expression detected in CHO/DAF cells infected with HSV1716CD20 or HSV1716CD38 (data not shown). Thus, viruses that displayed a targeting moiety against DAF were better able to infect CHO cells with membrane-exposed DAF than viruses that displayed targeting moieties directed against CD20 or CD38, not present on CHO cells.

SupT, THP-1 and TolB cells were semi-permissive for HSV1716 infection with between 10 and 30% of SupT, THP-1 or TolB cells infected by HSV1716gfp in three separate experiments and this level of infection was unaffected by pre-incubation of the cells in mAbCD20, mAbCD38 or control medium (Figures 3a–c, respectively). HSV1716CD20 or HSV1716CD20neg were also only able to infect similar numbers of SupT (Figure 3a), THP-1 (Figure 3b) or TolB (Figure 3c) cells and, again, these levels of infection were unaffected by pre-incubation of the cells in mAbCD20, mAbCD38 or control medium. In contrast, HSV1716CD38 was able to infect 60–90% of the CD38-positive SupT (Figure 3a), THP-1 (Figure 3b) or TolB (Figure 3c) cells and, significantly, these higher levels of infection were reduced to 5–30% by pre-incubating the cells in mAbCD38 but were unaffected by pre-incubating the cells in mAbCD20 or control medium. Analysis of variance (ANOVA) generated highly significant P-values ($0.004 < P < 0.012$) only for HSV1716CD38 infection of SupT, THP-1 and TolB cells in the presence of mAbCD20 or control medium but not in the presence of mAbCD38 indicating that the CD38-

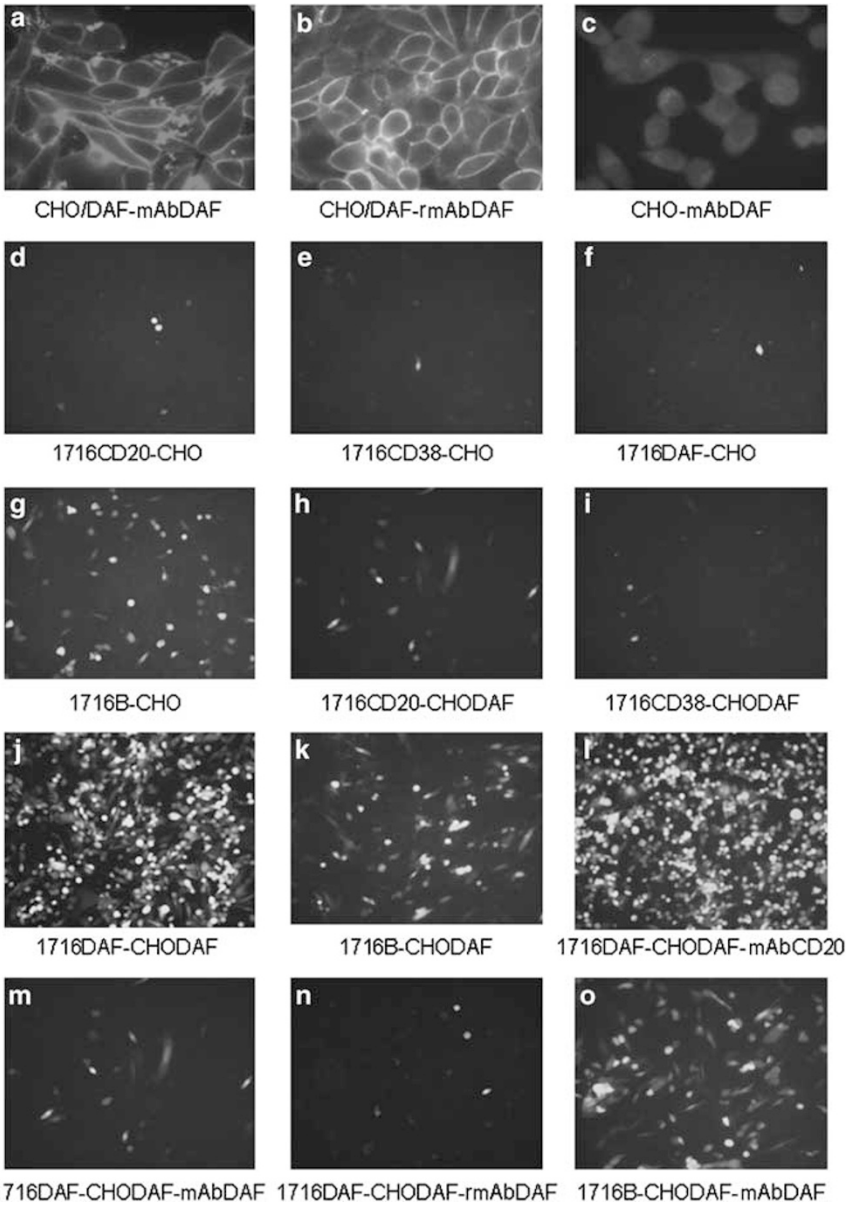

Figure 2 Immunofluorescence with monoclonal antibody decay-accelerating factor (mAbDAF; **a**, **c**) and a recombinant minibody version of mAbDAF (**b**) demonstrating expression of DAF by Chinese hamster ovary (CHO)/DAF (**a**, **b**) but not by normal CHO (**c**) cells. Fluorescence microscopy demonstrating levels of CHO (**d–g**) or CHO/DAF (**h–o**) cell infection by HSV1716B (**g**, **k**, **o**) or by Vero cell propagated HSV1716CD20 (**d**, **h**), HSV1716CD38 (**e**, **i**) or HSV1716DAF (**f**, **j**, **l–n**). CHO/DAF cells were pre-incubated in mAbDAF (**m**, **o**), recombinant mAbDAF (**n**) or mAbCD20 (**l**).

binding sites displayed on the HSV1716CD38 envelope significantly influenced tropism. Additionally, the similarities in the levels of HSV1716 or HSV1716CD20 infection of SupT, THP-1 or TolB cells indicates that the presence of the targeting moiety in the viral envelope does not influence the normal route of infection.

Unfortunately, we were unable to identify a human tumour cell line that was EGFR-positive and resistant to HSV1716 infection for *in vitro* analysis of HSV1716EGFR. For example, the human epidermoid carcinoma cell line A431 expresses EGFR as demonstrated by mAbEGFR binding to the cell surface (Figures 4b and d) and showed similar levels of susceptibility to HSV1716gfp or HSV1716EGFR infection, as assessed by gfp expression (Figures 4a and c). Further, duplicate infections of A431 cells in 60 mm dishes with 20 PFU HSV1716 or HSV1716EGFR produced similar yields of 1.1×10^6 and 1.25×10^6 PFU or 1.0×10^6 and 1.45×10^6 PFU, respectively. Thus, *in vitro*, there was no apparent difference in replication of either HSV1716 or HSV1716EGFR on A431 cells again indicating that the expression and incorporation of the targeting moiety did not impair virus replication.

Nude mice bearing subcutaneous A431 tumours were systemically injected in the tail vein with phosphate-buffered saline (PBS) or 1×10^6 PFU HSV1716 or HSV1716EGFR on days 1 and 3, tumours were measured daily and mice were killed once the tumours had reached 15 mm diameter. Mice that received the control PBS injection had more rapidly growing tumours than mice

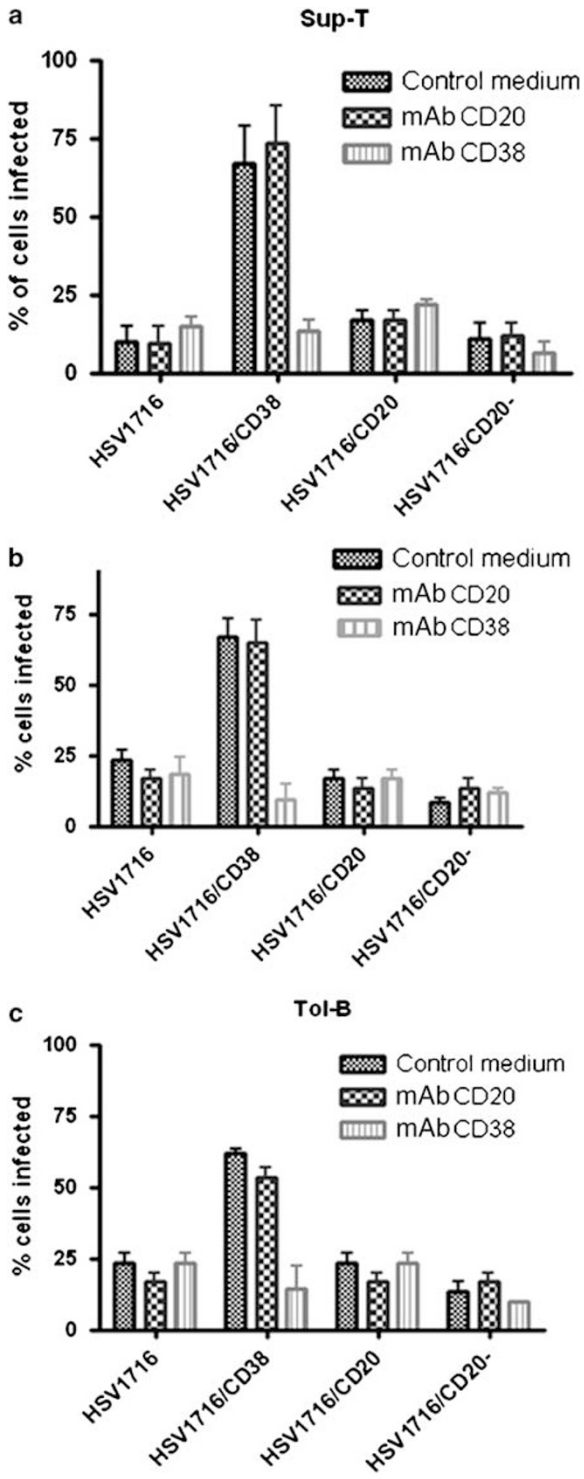

Figure 3 Graphs showing percentages of SupT (**a**), THP-1 (**b**) or TolB (**c**) cells infected by HSV1716gfp, HSV1716CD20, HSV1716CD38 or HSV1716CD20neg. Cells were pre-incubated in control medium, mAbCD20 or mAbCD38. Data are from a single representative field of view from three different experiments.

that received HSV1716 and were killed sooner. Mice that received HSV1716EGFR had more slowly growing tumours and survived longer than those that were injected with HSV1716 (Figure 5a). However, in this initial experiment, the tumours from several mice receiving intravenously injected HSV1716EGFR were actually much smaller than measurements suggested as they formed a capsule-like structure, the majority of which was cellular debris, presumably a consequence of extensive viral replication and, consistent with this, large amounts of virus were extracted from tumours of mice injected with HSV1716EGFR compared to HSV1716-injected mice. Thus, 3.1×10^5, 5.2×10^6 or 8.7×10^6 PFU were extracted from tumours of mice killed on days 16, 23 or 24, respectively, after HSV1716EGFR injection compared to 0, 0 or 4.1×10^3 PFU extracted from HSV1716-injected mouse tumours on days 16, 17 or 17, respectively. Immunohistochemical analysis of excised tumours clearly demonstrated more extensive virus antigen expression following injection of HSV1716EGFR (Figures 4d and e) compared to the smaller, more localized focus of unmodified HSV1716 antigen expression (Figure 4f) with no viral antigen detected in the PBS-injected control tumours (Figure 4g). The failure to detect any virus in two out of three tumour extracts from HSV1716-injected mice is consistent with the small localized foci of virus replication observed immunohistochemically in these tumours or, alternatively, after intravenous injection, little or no unmodified HSV1716 lodged in these tumours. In a subsequent experiment using intravenous injection, HSV1716EGFR was again more effective at reducing tumour burdens and prolonging survival compared to HSV1716 (Figure 5b) with mean survival times for no virus, HSV1716 or HSV1716EGFR of 10, 19 or 25.5 days, respectively. No virus was extracted from the skin, heart, liver, gut, lung, brain, bone, spleen or kidney of several mice injected with HSV1716EGFR and this lack of virus in these organs was confirmed using an HSV-1-specific PCR with DNA extracted from these tissues as template.

Further evidence of HSV1716EGFR tumour targeting was provided by intraperitoneal injection of nude mice bearing subcutaneous A431 tumours. On days 1, 3, 5, 6 and 9 the mice were injected with PBS or 2×10^6 PFU HSV1716 or HSV1716EGFR. When injected by this route, HSV1716 was ineffective as the median survival time for both PBS- and HSV1716-injected mice was 18.5 days. Tumour growth was significantly reduced in mice receiving HSV1716EGFR (Figure 5c, Table 2), resulting in a prolonged median survival of 28 days for the HSV1716EGFR-injected mice. Thus, by intraperitoneal injection, presumably, insufficient HSV1716 must reach the subcutaneous tumours to have any effect on tumour growth whereas targeting the virus to EGFR on the tumour cell surface promoted improved tumour uptake and prolonged survival.

Discussion

Recent phase 1 clinical trials have demonstrated an excellent safety profile following direct intratumoural injection of oncolytic HSV1716 and the efficacy of this virus is being investigated currently in a phases II/III trial for the treatment of recurrent glioma. It is clear however that the therapeutic usefulness of HSV1716 will be greatly facilitated if the virus can be administered by the circulation. In addition, the platform of tumour indications *viz* haematological tumours and metastatic disease, susceptible to oncolytic viral therapy could be

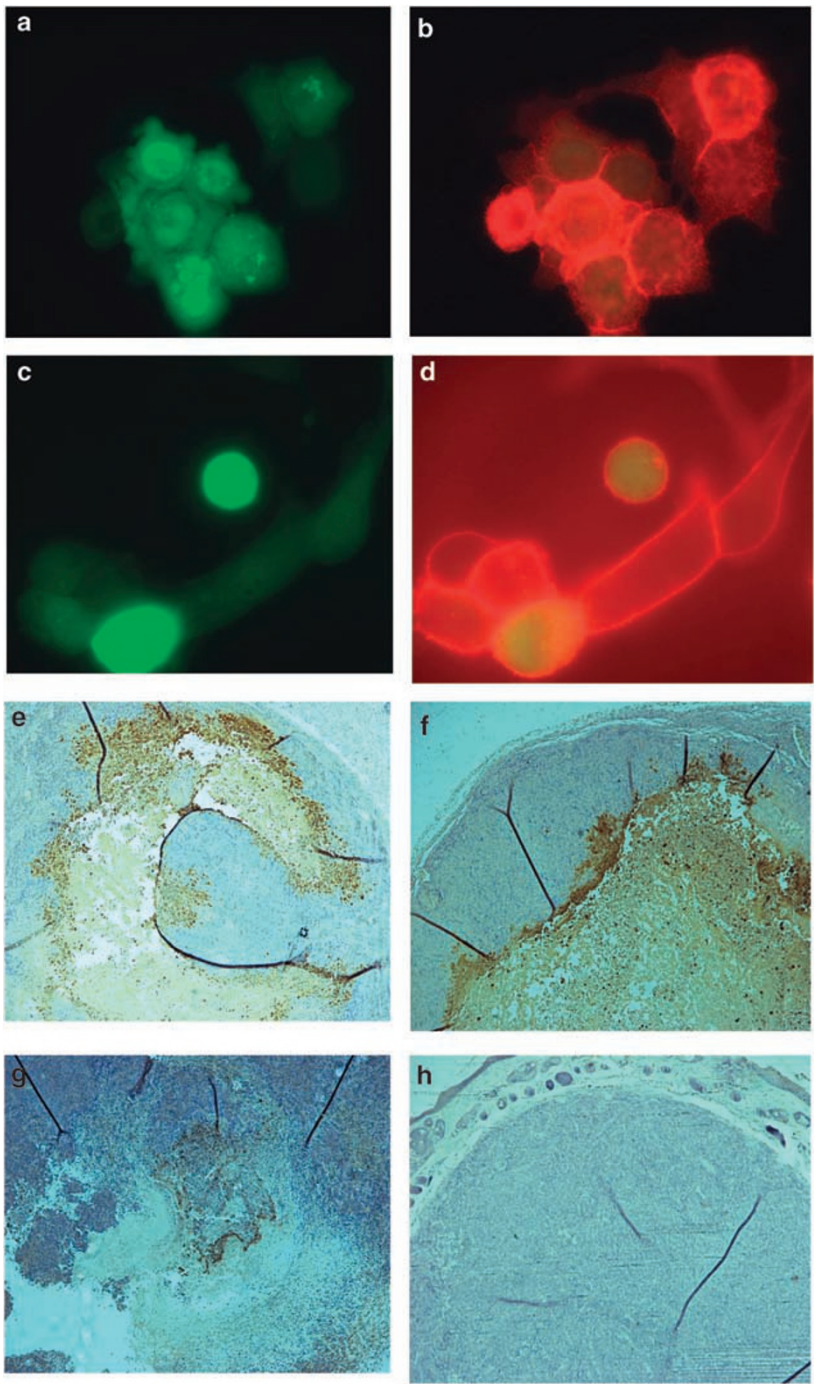

Figure 4 (**a**–**d**) Immunofluorescence/fluorescence microscopy of A431 cells infected with HSV1716EGFR (**a**, **b**) or HSV1716 (**c**, **d**). Gfp fluorescence is shown alone in (**a**, **c**), and is shown combined with mAbEGFR immunofluorescence in (**b**, **d**). (**e**–**h**) Immunohistochemistry using an anti-HSV-1 antiserum on tumours from nude mice bearing subcutaneous A431 tumours after intravenous injection of HSV1716EGFR (**e**, **f**), HSV1716 (**g**) or no virus (**h**). Tumours were excised on day 23 for HSV1716EGFR, day 18 for HSV1716 and day 9 for no virus.

greatly expanded. Incorporation of tumour-targeting moieties into the virus structure is a prerequisite for such strategies.

A number of reports have shown that various ligands linked to gC have been able to influence the tropism of HSV-1. Laquerre *et al.*,[30] using a virus deleted for the heparan sulphate-binding region of gB, substituted gC with modified versions in which erythropoietin replaced residues 1–161, 83–161 or 1–375 of the protein. The fusion protein with erythropoietin inserted between residues 83 and 161 was able to mediate uptake but not infection of a non-permissive erythropoietin receptor-bearing cell line. Viruses that lacked the heparan sulphate-binding region of gB and with either the cytokine IL13 both replacing residues 1–148 of gC and inserted between residues 24 and 25 of gD or with residues 20 to 155

of the urokinase plasminogen activator inserted between gD residues 24 and 25 were able to infect specifically cells expressing the cognate receptor.[31,32,34] Infection of a hepatoblastoma cell line by viruses in which the human hepatitis B virus hepatocyte-binding preS1 protein or its minimally active peptide respectively replaced gC amino acids (aa) 149–213 or 149–442 was blocked by anti-preS1 antibodies and replacing the heparan sulphate-binding region of gC with a 6-His tag improved virus binding to permissive 293 cells expressing a pseudo-His-tag receptor.[33,35]

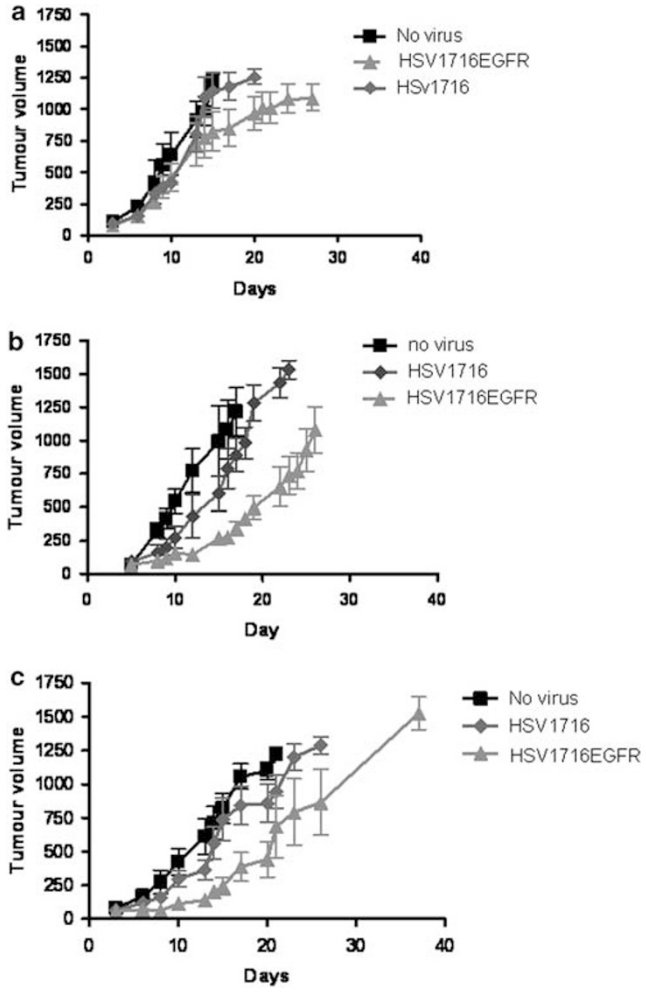

Figure 5 Graphs showing average tumour volumes in an A431 mouse tumour model after intravenous (**a**, **b**) or intraperitoneal (**c**) injections of PBS (no virus control), HSV1716 or HSV1716EGFR.

Initially, we analysed the suitability of various glycoprotein D fragments to direct the incorporation of N terminally fused scFv into the HSV-1 envelope and, assessed the abilities of incorporated fusion proteins to alter the tropism of the virus. Although scFv/glycoprotein fusion proteins have been used to redirect the tropism of various different viruses including HSV-1, these studies have used the scFv linked to almost full-length glycoproteins through the N terminus and optimization of incorporation and targeting ability have not been analysed.[37] We linked a scFv from the well-characterized anti-GD3 ganglioside mAb, R24 to 15 sequentially deleted gD fragments and all of the constructs were created in mammalian-cell-expression vectors for stable cell line production. The resultant R24/gD fusion proteins were incorporated into virus by infecting the cell lines with HSV1716-expressing green fluorescent protein, and epitope tagging of the fusion proteins allowed estimations of their levels of expression and incorporation into virus. The infectivity of the viruses derived from stable cell lines was assessed using fluorescence microscopy to monitor gfp expression in CHO cells that stained positively with R24, and results identified that the R24scFv linked to residues 274–393 of gD was very efficient at altering HSV1716 tropism. As CHO cells are non-permissive for Vero cell propagated HSV1716 infection, they provided an excellent system to assess alterations to viral tropism.[45]

Our initial analysis using infection of Vero cell lines to achieve viral assimilation of targeting moieties identified an efficient scFv mediated route to influence HSV1716 tropism. However, although this cell-based system was a convenient method to analyse a large number of different fusion protein constructs, production of targeted viruses is restricted to propagation on the appropriate cell line. Recombinant viruses expressing the targeting moieties during infection will be required and we produced a series of recombinant HSV1716 viruses expressing anti-CD20, anti-CD38, anti-DAF or anti-EGFR scFv linked to gD residue 274. These viruses were created using an efficient and rapid site-specific recombination protocol based on the Gateway cloning system. PCR confirmed the genomic structure at the RL1 loci of the HSV1716CD20, HSV1716CD38, HSV1716DAF and

Table 2 Comparisons of average tumour volumes from nude mice with subcutaneous A431 tumours injected intraperitoneally with either HSV1716EGFR or HSV1716

Day	*HSV1716EGFR average tumour volume ± s.d.*	*HSV1716 average tumour volume ± s.d.*	P-*value*	*Significant?*
8	71.4 ± 35.4	120.1 ± 24.3	0.259	No
10	62.9 ± 22.8	160.0 ± 32.4	0.051	No
13	113.2 ± 18.8	296.9 ± 57.6	0.030	Yes
14	140.6 ± 27.6	363.2 ± 70.1	0.032	Yes
15	203.6 ± 46.8	563.0 ± 117.2	0.038	Yes
17	244.4 ± 60.7	736.8 ± 156.2	0.033	Yes
20	387.7 ± 107.3	843.0 ± 142.1	0.040	Yes

Statistical analysis (ANOVA) was not possible after day 20 as most of the HSV1716-injected mice had died.

HSV1716EGFR, and immunofluorescence/western blotting confirmed both expression of the 45 kDa fusion proteins and their incorporation into virus. The orientation of the scFv/274gD-expression cassette relative to the PGK-gfp-expression cassette was important as one virus, in which the expression cassette for the anti-CD20scFv linked to residue 274 of gD was in reverse orientation, resulting in the close juxtaposition of the CMV-IE and PGK promoters, failed to express the scFv/gD fusion protein. Elements of the PGK promoter immediately adjacent to the CMV-IE promoter must inhibit its transcriptional ability.

HSV1716DAF demonstrated redirected tropism of the virus with infection of the non-permissive, DAF-positive CHO cells mediated specifically through an interaction with cell-surface DAF. The infection of CHO/DAF cells was mediated specifically by the anti-DAFscFv linked to residue 274 of gD as mAbDAF or a recombinant mAbDAF minibody blocked infection by HSV1716DAF whereas the unrelated mAbCD20 or mAbCD38 had no effect. HSV1716CD38 showed enhanced infectivity for the semi-permissive CD38-positive SupT, THP-1 and TolB cells as, although HSV1716 can infect up to 30% of THP-1, SupT or TolB cells, suggesting the limiting presence of an HSV-1 receptor, most likely HVEM, the presence of a CD38-binding moiety displayed on the virus coupled with the cognate antigen on the cellular membrane, must have increased the proportion of specific virus/cellular interactions resulting in at least a doubling of infection rates. Significantly, these increased infection levels of SupT, THP-1 and TolB cells by HSV1716CD38 were specifically inhibited by mAbCD38 but not by mAbCD20.

Although an HSV1716 variant expressing an anti-CD20scFv linked to 274gD was produced, no data demonstrating its abilities to alter tropism was generated. Unfortunately, the HSV1716CD20 did not enhance infection of the CD20-positive TolB cells although, as mAbCD20 only reacted weakly with TolB cells, possibly there was insufficient CD20 available on the cells to mediate viral penetration or, alternatively, as the minibody version of mAbCD20 failed to bind TolB cells (data not shown), perhaps mAbCD20 was unsuitable for conversion to the scFv format.

Using the EGFR-positive human squamous cell carcinoma cell line A431 to form subcutaneous tumours in nude mice, we were able to demonstrate that intravenously delivered HSV1716 targeted to EGFR by an anti-EGFR scFv linked to 274gD was better able to destroy tumours and promote survival than unmodified HSV1716. Indeed, the targeted viruses performed better than our initial data suggested as a number of mice that received HSV1716EGFR were killed prematurely because, although their apparent tumour diameters were 15 mm, when the tumours were excised after killing, the bulk of the mass consisted of cellular detritus surrounding a much reduced tumour. Presumably, the EGFR-binding capability of the targeted HSV1716 variants augmented greatly the number of viruses locating to the tumour compared to the untargeted HSV1716 and this initial higher dose of virus adsorbed from the circulation was responsible for enhanced tumour destruction. Immunohistochemical and virus titration analysis of excised tumours confirmed that levels of virus replication in mice injected intravenously with HSV1716EGFR were much higher than in mice receiving unmodified HSV1716. Further evidence of the efficacy of tumour targeting was provided using intraperitoneal injection. By this route of administration, HSV1716 had no discernable effect on tumour growth whereas HSV1716EGFR significantly reduced tumour growth and prolonged survival.

These studies were performed to identify a reliable method for redesigning the tropism of oncolytic HSV such that systemically delivery will effectively target tumour cells. We have developed a plasmid-based system to link a mAb-derived scFv to gD residue 274 and recombinant viruses that display the targeting scFvs are produced rapidly using a novel *in vitro* site-specific recombination system. ScFvs have been used previously to target HSV-1 with an anti-EGFR scFv linked to the gD-binding domain of nectin-1 creating a soluble bridging molecule capable of attaching the virus to CHO cells displaying EGFR on their surface and insertion of an anti-HER2/neu scFv at gD residue 24 targeted the virus to CHO cells expressing the HER2/neu receptor.[36,37] The soluble bridging molecule required gD to link virus and cell but the virus expressing the anti-HER2/neu scFv/gD fusion protein was gD negative. However, insertion of the scFv at residue 24 will only ablate HVEM binding and the chimaeric protein was still able to use nectin-1 as a receptor. Here, HSV1716 variants that express an anti-DAF scFv or an anti-CD38 scFv linked to gD residue 274 mediated infection by cognate antigen binding, although the recombinant viruses retained gD, and apparently, their abilities to infect cells using either HVEM or nectin-1 was unaffected. Possibly, the fusion proteins can function as the sole receptor-binding protein to mediate infection of targeted cells as the scFv will mimic the specific interaction required to tether the virus closely to the cellular membrane and membrane fusion will be elicited by the remaining membrane-proximal residues of gD that form a profusion domain required for recruitment of gB and gH/gL.[46] Currently, we are attempting to create a gD-negative HSV1716 variant but, as gD is an essential protein, such a virus will be severely attenuated or even replication incompetent and it is interesting to speculate whether a gD-negative virus is mandatory to achieve optimal tumour targeting. Certainly, HSV1716 variants displaying both the targeting moiety and native gD will still be able to infect off-target cells expressing HSV-1 receptors but, as demonstrated by HSV1716EGFR, the targeting moiety enhanced tumour uptake and destruction compared to unmodified HSV1716 suggesting that adsorption by non-tumour cells of systemically injected HSV1716 variants is not a major issue. Neutralizing antibodies that target gD may also be a problem for such systemically administered HSV1716 variants and ablation of the gD ORF will prevent this. However, once a tumour has been infected with a targeted, gD-negative HSV1716 variant, spread to other tumour cells within the mass will be totally dependent on the specific scFv/antigen interaction. As heterogeneity is a feature of many tumour types, not all cells will display the specific membrane protein and will therefore avoid infection. Thus, the presence of both the scFv/gD fusion protein and gD in the virus envelope is possibly a more effective configuration allowing an initial high level of tumour uptake followed by optimum infection rates.

Materials and methods

Cells and viruses

BHK, CHO-K1 and Vero cells were grown as previously described.[45] Hybridomas mAbR24, mAbIF5 (mAbCD20), mAbTHB-7 (mAbCD38) and mAb108 (mAbEGFR), expressing mAbs against the ganglioside GD3, CD20, CD38 or EGFR, respectively, and the human leukaemic SupT, THP-1 and TolB cell lines, were obtained from ATCC/LGCpromochem (London, UK) and were cultured in RPMI1640 medium supplemented with 10% NCS. Hybridoma HD1B (mAbDAF) that expresses a mAb that recognizes human DAF/CD55, a kind gift from Dr Claire Harris, Department of Medical Biochemistry, University of Wales College of Medicine, Cardiff, UK, was also maintained in RPMI1640 medium supplemented with 10% NCS. HSV1716gfp is described in Harland *et al*.[6] and production of virus stocks is described in Conner *et al*.[45]

Preparation of scFvs from hybridoma cells

Hybridoma cells were grown to high density and cell pellets obtained by centrifugation at 1000 *g* for 5 min. Total RNA was extracted using the SV Total RNA kit (Promega, Southampton, UK) and cDNA prepared using the ImProm II Reverse Transcription System (Promega). Recombinant scFv variants of the mAbs were derived from these cDNAs using the protocols and PCR primers described by Pope *et al*.[47] Briefly, scFv-encoding DNAs for each mAb were prepared by RT–PCR amplification and linkage of antibody VH and VL sequences with the scFv-encoding DNAs then amplified using PCR primers that incorporate *Sfi*I and *Not*I restriction enzymes sites at the 5′ and 3′ ends, respectively, for cloning in the phagemid vector pCANTAB6. Competent *Escherichia coli* cells (strain HB2151) were transformed with the phagemid vectors and scFv expression by IPTG induction was confirmed by western blotting (data not shown); scFvs expressed from pCANTAB6 have *myc* tags for detection.

Construction of pEL4, a vector for scFv/glycoprotein fusion protein production

Plasmid pEL4 for expression of scFv/glycoprotein fusion proteins was created to allow PCR-amplified scFv DNAs to be cloned in-frame downstream of an immunoglobulin G (IgG) heavy chain leader sequence as *Sfi*I/*Not*I fragments and in-frame upstream of a PCR-amplified HSV-1 glycoprotein DNA fragment. Initially, using pCANTAB6 with an R24 scFv insert as template, the scFv DNA was amplified using primers ELsfifor and R24notrev.

Primer ELsfifor is a 99-base oligonucleotide (**GAATTC**ATGGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCACTCCGC**GGCCCAGCCGGCC**GATGTGCAACTGGTGGAGTCT) that has a 5′ *Eco*R1 site (in bold) then 63 bases (underlined) encode the R24 IgG heavy chain leader sequence followed by the R24 scFv 5′ sequence with an intervening *Sfi*I site (in bold and underlined). PCR with ELsfifor and R24notrev (GAGTCATTCT**GCGGCCGC**CCGTTTTATCTCCAGCTTGGT), the anti-sense primer exactly matching the 3′ end of the R24 scFv with a *Not*I site (in bold), generated the R24 scFv DNA for cloning into the *Eco*R1/*Not*1 sites in the mammalian-expression plasmid pCDNA4myc/HisA (Invitrogen, Paisley, UK) to generate pEL4.

The presence of the IgG leader in this plasmid was confirmed by sequencing. The R24 scFv component in pEL4 can be readily exchanged with an alternative scFv, amplified by PCR using primers that provide flanking *Sfi*I/*Not*I sites.[47] As each of the scFvs created in this study has a unique *Rsa*I fingerprint, any scFv exchange was confirmed by PCR amplification of the scFv DNA and *Rsa*I digestion (data not shown).

Construction of vectors for the expression of scFv/glycoprotein fusion proteins

For HSV-1 gD, which comprises 393 aa with the TM domain located between aa 342 and 364, 15 random deletions sequentially removing aa 1–273 were PCR amplified using the 15 forward and common reverse primers shown in Table 3. The gD forward primers are identified by the extent of the N-terminal deletion (for example, primer 108gD amplified a gD DNA fragment with 36 codons deleted from the N terminus and starts at nucleotide 109/codon 37). An additional A nucleotide between the *Not*1 site (in bold) and the gD sequences (underlined) ensured that the gD and scFv DNAs were cloned in frame; the *Not*1 site plus the additional A inserts an Ala-Ala-Ala linker between the scFv and glycoprotein domains of the fusion protein. The gD reverse primer lacks a stop codon and the *Xho*I site used for cloning ensured that the inserted fragment was in frame with the Myc/His tags provided by the pCDNA4 parent vector. After PCR amplification from viral DNA, the gD fragment was sequentially digested with *Not*I, followed by *Xho*I and ligated into the likewise digested pEL4 containing the scFv DNAs. The gD PCR fragments were also cloned into pGEMT-easy and sequenced to confirm their identity (data not shown).

Production of stable cell lines expressing scFv/glycoprotein fusion proteins

Vero cells were transfected with 50 μg scFv/gD fusion protein-encoding plasmids mixed with 10 μl lipofectamine 2000 (Invitrogen) in 250 μl of serum-free Dulbecco's modified Eagle's medium (DMEM)/F12 medium. After

Table 3 Sequences of primers used for the PCR amplification of sequentially deleted gD fragments. Restriction sites are in bold and HSV-1 glycoprotein sequences are underlined

gD forward primers

108gD GGATCC**GCGGCCGC**AGCCGACCCCAATCGCTTTCGCGGC
171gD GGATCC**GCGGCCGC**AGGGGTCCGGCGCGTGTACCACATC
231gD GGATCC**GCGGCCGC**ACTCCCGATCACGGTTTACTACGCC
273gD GGATCC**GCGGCCGC**ACGCAGCGTGCTCCTAAACGCACCG
312gD GGATCC**GCGGCCGC**AATTGT CCGCGGGGCC TCCGAAGAC
381gD GGATCC**GCGGCCGC**AGGAGGCAACTGTGCTATCCCCATC
414gD GGATCC**GCGGCCGC**AGAGTA CACCGAATGC TCCTACAAC
489gD GGATCC**GCGGCCGC**AGACAGCTTCAGCGCCGTCAGCGAG
534gD GGATCC**GCGGCCGC**ACACGCCCCCGCGTTTGAGACCGCC
570gD GGATCC**GCGGCCGC**ACGGCTCGTGAAGATAAACGACTGG
618gD GGATCC**GCGGCCGC**AGAGCACCGAGCCAAGGGCTCCTGT
690gD GGATCC**GCGGCCGC**ACAGGCCTACCAGCAGGGGGTGACG
714gD GGATCC**GCGGCCGC**AGTGGACAGCATCGGGATGCTGCCC
777gD GGATCC**GCGGCCGC**AAGCTTGAAGATCGCCGGGTGGCAC
819gD GGATCC**GCGGCCGC**AACGAG CACCCTGCTG CCCCCGGAG

gD reverse primer

GGCCAAGCTT**CTCGAG**TCTAGAGTAAAACAAGGGCTGGTGCGAGGA

48–72 h of transfection, cells were trypsinized and plated out with growth medium containing 1 mg ml^{-1} zeocin (Invitrogen). Cells were selected with the zeocin antibiotic for 2–3 weeks after which time individual clones were clearly visible. Cells were trypsinized and cloned by limiting dilutions in 24-well plates. Clones were expanded and maintained in appropriate medium containing 1 mg ml^{-1} zeocin. Expression of scFv/glycoprotein fusion proteins was confirmed either by western blotting or by immunofluorescence using an anti-myc mAb (New England Biolabs, Hitchin, UK).

For production of virus stocks, confluent monolayers of stable cell lines were infected with 1×10^5 PFU HSV1716gfp and, after 3–4 days of infection, total virus was harvested, purified by high-speed centrifugation and titrated on BHK cells. All yields were within the range obtained for HSV1716gfp infection of normal Vero cells (data not shown). The presence of scFv/glycoprotein fusion proteins in the virion was confirmed by western blotting using the anti-myc mAb.

Production of viruses expressing scFv/gD fusion proteins by site-specific recombination

A series of targeted viruses was constructed with scFvs linked to residue 274 of gD using a novel *in vitro* site-specific recombination system. Central to this method was production of HSV1716GateRed, an HSV1716 variant, which contained Gateway destination sites located within each of the ICP34.5 deleted regions. The Gateway Vector Conversion system (Invitrogen) provided DNA with attR site-specific recombination sequences for insertion into a vector of choice and was blunt-end ligated into plasmid sp73 to create sp73gate. Once inserted in the plasmid, the DNA between the attR sites, encoding chloramphenicol resistance and the *ccdB* gene, was removed by *Not*1/*Bst*XI digestion. The blunt-ended vector backbone was then ligated with a blunt-ended 1.3 kbp CMV-DSred-expression cassette, excised from the plasmid pCMV-DsRed-Express (BD Biosciences, Cowley, UK) by *Afl*II/*Nsi*I digestion, to create the plasmid sp73gatered. The DNA consisting of the attR sites flanking the CMV-DSred-expression cassette was excised from sp73gatered by *Bgl*II/*Xho*I digestion, blunt ended with Klenow and ligated into the blunt-ended, alkaline phosphatase-treated *Bgl*II site in the plasmid RL1-del used for the production of HSV1716 variants by homologous recombination. RL1-del consists of the HSV-1 *Bam*HI k-DNA fragment (123459–129403) that includes the RL1 gene and its flanking sequences cloned into the *Bam*HI site of plasmid pGem-3Zf (Promega). The 477 bp *Pfl*MI/*Bst*EII fragment from the RL1 ORF (125292–125769) has been removed to inactivate the ICP34.5 gene and replaced with a multi-cloning site (MCS) providing unique *Bgl*II, *Nru*I and *Xho*I restriction enzymes sites. The transgene to be inserted into the RL1 loci is ligated into the MCS of RL1-del and homologous recombination with HSV-1 DNA, driven by the RL1 flanking sequences, results in concomitant deletion of the ICP34.5 gene and incorporation of the desired transgene. Southern blotting confirmed that the Gateway destination sites flanking the DsRed-expression cassette was cloned into both RL1 loci (data not shown).

The Gateway entry vector, pENTR1A was modified for use in our HSV1716 site-specific recombination system as follows. The DNA between the attL sites in pENTR1A was removed by *Eco*R1 digestion and the resulting blunt-ended vector backbone was ligated with a green fluorescent protein-expression cassette comprising the 1.3 kbp blunt-ended *Eco*RI/*Afl*II fragment that contains the PGK promoter/gfp gene excised from the vector pSNRG (a kind gift from Dr B Singh (MSKCC, New York, USA)). Although *Eco*R1 digestion removed the *ccdB* gene from pENTR1A, a number of other restriction sites between the attL flanking sequences were retained for insertion of additional gene/DNA sequences of interest to be cloned into HSV1716 alongside the PGK-gfp-expression cassette.

Novel HSV1716 variants were created *in vitro* by incubating HSV1716GateRed DNA with the appropriate gene/DNA sequence of interest inserted in pENTR1A-gfp. HSV1716GateRed DNA was obtained by phenol/chloroform extraction of BHK cells 24 h after infection with the HSV1716GateRed virus and, after 70% isopropanol precipitation, the DNA was resuspended in 1 ml nuclease-free water. The expression cassettes for the scFv/274gD fusion proteins were excised directly from the appropriate pEL4 constructs as 2.3 kbp *Ssp*I/*Pvu*II fragments that contained the CMV-IE promoter, the scFv/gD encoding DNA and the BGH polyadenylation signal. These fragments were ligated directly into the plasmid pENT1A-gfp, which had been *Bam*HI digested, blunt ended with Klenow and alkaline phosphatase treated. For site-specific recombination reactions using these pENTR1A-gfp constructs, 5 μg plasmids and 3 μg HSV1716GateRed DNA were incubated overnight with LR clonase. After enzyme inactivation by Proteinase K digestion, the entire reaction mix (11 μl) was added to 250 μl of serum-free DMEM/F12 (Invitrogen) medium containing 10 μl lipofectamine 2000 and used to transfect 50% confluent BHK cells in a 60 mm dish. After 4–6 h, the cells were shocked in 25% dimethyl sulfoxide/PBS, washed and cultured in 5 ml of Glasgow minimum essential medium at 37 °C for 48–72 h. Cells were harvested by scraping into the medium and, after 2 min sonication, 5 sequential 10-fold dilutions were plated out on Vero cells. The *in vitro* site-specific recombination reaction was very efficient and converted up to 90% of viral DNA from DsRed to gfp expression such that, a single plaque picked from the serially diluted plates was 100% pure with no DsRed virus contamination and was used for HSV1716 recombinant stock production. PCR, with primers that amplify across the ICP34.5 deleted region was used to confirm the appropriate genomic structure at the RL1 loci of these viruses with viral DNA prepared using a Wizard SV genomic DNA kit from BHK cells 24 h after infection with the relevant viruses at 5 PFU per cell (data not shown).

Production of CHO cell lines expressing human CD55 and a recombinant mAbB minibody

Plasmid pDAF, which contains a mammalian-expression cassette for human CD55 with a hygromycin selectable marker, was a kind gift from Dr Claire Harris and was used to transfect CHO cells. Plasmid (50 μg), mixed with 0.25 ml serum-free DMEM/F12 medium containing 10 μl lipofectamine 2000, was added to CHO cells and, after 48 h in culture, cells were trypsinized and plated out in a

T75 flask in growth medium supplemented with 0.5 mg ml^{-1} hygromycin. After 14 days in culture individual colonies of cells were trypsinized and, after cloning by limiting dilution, a stable hygromycin-resistant CHO cell line, termed CHO/DAF, which expressed CD55, as assessed by western blotting using mAbDAF, was produced. The CHO/DAF cell line was maintained in DMEM/F12 supplemented with 10% NCS and 0.5 mg ml^{-1} hygromycin.

Using the anti-DAF scFv DNA cloned into pEL4, an expression cassette for a minibody construct comprising the scFv DNA linked to the human IgG1 CH2/CH3 (Fc) region DNA was prepared. The resultant expressed protein was able to dimerize by the IgG1 Fc region and was secreted into the medium from a stable CHO cell line isolated using the zeocin resistance gene of pEL4. The 5′ and 3′ primers for the amplification of the human IgG1 Fc DNA, which incorporate *Not*1 and *Xho*1 restriction sites (underlined), were respectively ACC TTGCAGCGGCCGCAAGACCCAAATCTTGTGACAAA ACTC and GATCACGTCTCGAGTTATCATTTACC CGGAGACAGGGAGAGGCTCTTTCTG and these were used to amplify the 700 bp IgG1 Fc region from peripheral blood mononuclear cell total RNA by RT–PCR. The amplified fragment was cloned directly after RT–PCR into the PCR cloning vector pGEM-Teasy (Promega) and was sequenced to confirm its identity. The PCR fragment was digested sequentially using *Not*1 followed by *Xho*1 and ligated into the pEL4 plasmid containing the anti-DAFscFv to generate a plasmid that expresses the DAFscFv linked to the human IgG1 Fc region. CHO cells were transfected and selected as described above except that the growth medium contained 1 mg ml^{-1} zeocin for selection rather than hygromycin. Medium from zeocin-resistant stable cell lines was harvested after 5 days in culture and expression of the mAbDAF minibody was confirmed by denaturing and non-denaturing PAGE/western blotting using an anti-human IgG Fc antibody which identified respective 55 and 110 kDa proteins (data not shown).

Fluorescence microscopy/western blotting/ immunofluorescence

As HSV1716 variants used in this study expressed gfp, the infectivity of progeny viruses was assessed by fluorescence microscopy as described in Conner *et al*.[45] For western blotting, cell lines to be infected with the various HSV1716 variants expressing scFv/274gD fusion proteins were plated out in 35 mm plates and incubated for 24 h at 37 °C in 5% CO_2 before infection. For harvesting, cells were washed once with 1 ml PBS and whole-cell extracts obtained by the direct addition of 0.2 ml SDS–PAGE sample buffer. After SDS–PAGE and transfer to nitrocellulose membranes, blots were probed with the anti-myc mAb diluted 1:1000. To determine the presence of scFv/glycoprotein fusion proteins in virions, 1×10^6 PFU purified virus were mixed with 0.1 ml SDS–PAGE sample buffer and analysed as described above. To assess infectivity of targeted viruses, confluent monolayers of cells were infected with virus and incubated for 8–24 h before cells were harvested as described above. Blots were probed with a polyclonal antibody to the R1 subunit of the viral ribonucleotide reductase.[48] For immunofluorescence to detect scFv/274gD fusion protein expression by HSV1716 variants, BHK cells were plated out on glass coverslips, cells were infected with the appropriate virus at 1 PFU per cell for 24 h and cells were fixed in 4% paraformaldehyde. After blocking for 1 h in 2% normal horse serum, cells were incubated overnight in the anti-myc mAb diluted 1:1000, washed four times in PBS with 0.05% Tween (PBST) and incubated for 2–4 h either in anti-mouse IgG/fluorescein isothiocyanate (FITC) or IgG/Texas Red conjugate (Vector Laboratories Ltd, Peterborough, UK) diluted 1:200. For immunofluorescence with the recombinant mAbDAF minibody, an anti-human Fc/FITC conjugate at 1:1000 was used. After four washes in PBST, coverslips were mounted and analysed by fluorescence microscopy.

In vivo *tumour reduction studies*

Female 6- to 8-week-old athymic nude mice (Charles River Laboratories, UK) were maintained under specific pathogen-free conditions. Actively growing A431 cells were harvested and, after resuspension in PBS, 1×10^6 cells per mouse were injected subcutaneously. When tumours reached 5 mm in diameter, the mice were injected either intravenously via the tail vein or intraperitoneally with PBS, HSV1716 or HSV1716EGFR. Mice were inspected daily, when tumour diameters reached 15 mm they were killed and the tumour xenograft removed for immunohistochemistry/viral extraction. The viral load in tumours and various organs was assessed by plaque-forming assay on BHK cells. Extracted intact tumours/organs were frozen immediately at −70 °C and, after thawing, the tissues were mechanically homogenized in a Retsch homogenizer in 1 ml PBS prior to titration.

For immunohistochemistry, tumour samples were fixed for at least 24 h in 4% paraformaldehyde before embedding and sectioning using standard protocols. Briefly, paraffin-embedded sections were dewaxed, dehydrated and endogenous peroxidase activity quenched. Sections were blocked with 10% normal goat serum before incubation in an anti-HSV-1 polyclonal primary antibody (DakoCytomation, Ely, UK) diluted 1:1000. A biotinylated secondary antibody at 1:500 dilution, an avidin/biotin complex solution and the chromagen DAB (all from Vector Laboratories Ltd) were then used to detect the primary antibody binding to tissue. The slides were counterstained, dehydrated mounted and analysed using light microscopy.

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