TITLE: A Novel RNA Virus System for Selective Killing of Breast Cancer Cells

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The goals of this project were to develop a novel method for targeting an SV5 infection to breast cancer cells and to control the virus mediated killing by: 1) engineering SV5 to express an anti-HER-2 single chain antibody in place of the normal HN attachment protein, and 2) to engineer SV5 to express the herpes simplex thymidine kinase. The gene encoding an anti-HER-2 single chain antibody (sFv) linked to HN was inserted into the SV5 infectious clone in place of the gene for HN and virus was recovered. Initial studies demonstrated expression of the sFv-HN chimeric protein in cells infected with the rSV5. To determine if SV5 could be used for controlled cell killing, a second rSV5 was generated to encode the thymidine kinase gene. Cells infected with rSV5-TK showed a time-dependent loss of viability when infected cells were cultured in the presence of the prodrug ganciclovir (GCV) while no significant toxicity was observed in the absence of prodrug. Titration experiments showed that rSV5-TK plus GCV resulted in cell death for all mouse and human cell lines tested. The recovery of an rSV5 with a novel attachment protein and controlled cell killing by a rSV5-TK support the further development of SV5 as a therapeutic vector for targeted killing of cancer cells.
Introduction

Viruses and virus-based vectors represent a powerful tool for the delivery of recombinant molecules to cells. However, a major drawback to current systems has been the inability to target and limit an infection by a recombinant virus to predetermined cell types or tissue. The goal of the proposed work is to develop a novel method based on recombinant SV5 (rSV5) for targeting and killing predetermined population of tumor cells. The hypothesis to be tested is that the cell-type specificity of an SV5 infection can be pre-determined by incorporating the appropriate foreign membrane protein into the viral envelope. In this work, recombinant SV5 (rSV5) will be engineered to incorporate a membrane-bound form of a single chain antibody (sFv) into its envelope. It is anticipated that the specificity of infection by this rSV5 will be limited to cells expressing the appropriate surface antigen that is recognized by this antibody. To test this hypothesis, the HN gene will be modified to be fused with a single chain antibody specific for HER2, a cell surface antigen that is over expressed in a large number of carcinomas. Cell lines will be created which express HER2. Recombinant virus expressing the Hnfull-sFv has been isolated. The cell-type specificity of infection by this recombinant virus will be tested to determine if the infection is limited to cancer cells expressing the cell surface HER2 antigen. This annual report describes the problems we have encountered in identifying a candidate attachment protein to insert into the rSV5 genome. I present data that I have overcome this problem and have recovered a novel rSV5 with a new attachment protein. The results support further work on targeting rSV5 to preferentially infect HER-2 cells.

Body

List of Approved Tasks

Task 1. To determine the requirements for incorporation of the membrane-bound HER2-specific sFv into the SV5 envelope.

a. Chimeric proteins containing portions of either the SV5 HN or the F protein link the sFv will be analyzed by flow cytometry and by indirect immunofluorescence to identify the construct which provides the most efficient cell surface expression.

b. Incorporation of the chimeric sFv proteins into SV5 virions will be confirmed by expression in SV5-infected cells and western blotting of virions.

Task 2. To determine if the tropism of rSV5-sFv-F is restricted to cells which express cell-surface HER2.

a. Cell lines which contain the HER2 gene under control of an inducible (tetracycline) promoter will be isolated by transfection of plasmids containing a drug resistant gene. Expression in the cell lines will be monitored by immunoblotting with anti-HER2 monoclonal antibodies

b. Recombinant SV5 viruses (rSV5-sFv and rSV5-sFv-stop) that contain the above chimeric genes will be generated. Viruses will be characterized by biochemical and virological approaches.

c. The specificity of infection by rSV5-sFv will be determined by flow cytometric analysis, western blotting of cell lysates and by virus neutralization assays.
Research Accomplished on Task 1.

1. Inefficient cell surface transport of proteins containing the anti-HER2 sFv linked to fragments of the HN protein. The original approach to changing the attachment protein of SV5 involved constructing a series of chimeric proteins which contained a portion of the HN protein linked to the anti-HER2 sFv as diagramed schematically in Fig. 1. The chimeric proteins were efficiently expressed in transfected cells as assayed by western blotting of cell extracts (not shown).

![Figure 1. A) Schematic diagram of chimeric proteins containing the anti-HER2 sFv linked to portions of the HN membrane protein. B) Western blot of cells expressing the unmodified sFv or one of the chimeric proteins.](image)

However, as stated in our previous annual reports, the level of cell surface expression of the chimeric proteins was very low. This was evident when we attempted to stain transfected cells with the antibody to the HA epitope tag at the c-terminal end of the molecules. This low level of cell surface expression is a problem for the recovery of infectious virus with a new attachment protein, since virus budding occurs at the cell surface and this is where the attachment protein is incorporated into the virion. We have determined that this low cell surface expression is not due to inefficient transfection efficiency, since transfected cells that are permeabilized with detergent show very clear staining patterns (not shown). As reported in our previous annual report, our attempts to overcome this problem by removing the naturally occurring HN internalization signal contained at glutamate 37 were not successful.

2. Efficient cell surface expression of a chimera linking the anti-HER2 sFv to the full length HN protein. Recently, a recombinant measles virus has been reported that had a new cell tropism by incorporation of a sFv onto the C-terminal end of the H attachment protein (Hammond et al., 2001). Thus, the rationale was that the full length HN protein would be able to fold properly and independently of the sFv, while the truncated HN fragments we had used were malfolded and were not competent for transport to the cell surface.

To determine if a similar approach could be used to overcome the problem with our truncated HN-sFv chimeras, we constructed a cDNA to encode a hybrid protein in which the anti-HER2 sFv was linked to the full length HN. As shown schematically in figure 2, the open-reading-frames for the HN and sFv were separated by a (Gly-Gly-Ser)₃ spacer region.
To monitor expression of the new chimeric protein, cells were transfected with plasmids encoding unmodified sFv, the truncated sFv-HN1 (see Fig. 1 schematic), or two clones encoding the full length HN linked to sFv. Cell extracts were prepared and analyzed by western blotting with the antibody to the C-terminal HA tag on the sFv. As shown in Figure 3, cells transfected with the HNfull-sFv plasmids expressed a high molecular weight protein that was not seen in mock transfected cells (lane M). The size of the HNfull-sFv protein was slightly higher in apparent MW relative to WT HN (not shown).

Immunofluorescence was used to determine if the HNfull-sFv was transported to the cell surface where it could be incorporated into budding virions. CV-1 cells were mock transfected or transfected with plasmids encoding WT HN or the HNfull-sFv chimera. At 20 h post transfection, cells were fixed and analyzed for cell surface staining using a monoclonal antibody specific for the native conformation of HN. As shown in Fig. 4, cells transfected with WT HN plasmid showed bright punctate cell surface staining that was not seen in mock transfected cells. Likewise, cells transfected with the HNfull-sFv plasmids showed bright cell surface staining, however the pattern of staining was not as clear and punctate as that seen in the case of WT HN. The number of cells expressing HNfull-sFv closely matched the WT HN sample. Together, these data are very important for our project, since they demonstrate that the chimeric HNfull-sFv is efficiently expressed at the cell surface where budding can occur.

Efficient cell surface expression of a chimera linking the anti-HER2 sFv to the SH protein. As an alternative approach to incorporating the sFv into an SV5 virion, we have constructed a cDNA encoding the sFv linked to the SV5 small hydrophobic (SH) protein. SH is a 44 amino acid protein expressed at the surface of infected cells (Hiebert et al., 1988). The orientation of SH is such that the N-terminal end is in the cytoplasm and only two residues extend into the extracellular space. We reasoned that a membrane protein with such a short ectodomain would have less stringent requirements for transport than that see for the 550 residue HN protein, and thus would be more likely to transport the sFv to the surface. SH is incorporated into budding virions, which would fulfill an additional requirement.
A cDNA clone was constructed to link the sFv open-reading-frame to SH. Western blotting of transfected cells showed efficient expression (not shown). Cell surface staining experiments similar to that shown in Fig. 4 have not yet been confirmed, but preliminary data indicate that transport is very efficient. We have not pursued this avenue, since we have recovered virus with the sFv linked to full length HN (see below).

**Figure 4.** CV-1 cells were mock transfected or transfected with plasmids expressing WT HN or the HNfull-sFv chimeric protein. Cells were fixed in paraformaldehyde and stained with anti-HN antibody followed by FITC-conjugated anti-mouse.
Problems encountered in accomplishing Task 1 and solutions that were within the approved SOW.

- Linking the sFv to truncated versions of HN did not result in efficient transport to the cell surface as originally hypothesized.

  **Solution:** Construct sFv-HN chimeric molecules that link the anti-HER2 sFv to full length HN. This HNfull-sFv molecule was very efficiently transported to the cell surface.

  **Solution:** We have also constructed chimeric molecules whereby the anti-HER2 sFv is linked to the SV5 SH protein, and these molecules are efficiently transported to the surface.

- The problem of internalization of HN from the cell surface could reduce incorporation into virions.

  **Solution:** Construct the E37K mutant shown previously to reduce internalization of full length HN into the truncated sFv. This mutation did not increase steady-state levels of the truncated HN at the surface.

- The problem of determining if the chimeric HN truncated-sFv proteins could be incorporated into virions was not possible to address, since without efficient cell surface expression the molecules are not in the position to be incorporated during budding from the plasma membrane.

  **Solution:** We have bypassed this question by constructing an rSV5 expressing the HNfull-sFv protein from the genome.

Our attempts to get cell surface transport of truncated HN-sFv chimeras was not successful. We feel that this is due to the fact that surface transport of sFv molecules is difficult and inefficient to begin with, and when this is coupled to a truncated and improperly folded HN, the result is that the molecule is not transported efficiently at all. Thus, our original plan of using truncated HN molecules was based on published data showing that the same truncated HN we have tested (HN1, Fig. 1) was able to transport the cytoplasmic protein pyruvate kinase to the cell surface (Hiebert and Lamb, 1988). We have shown that this is not true when the foreign molecule is an sFv.

The exciting result that we have obtained is that a linking the anti-HER2 sFv to the full length HN allows cell surface transport. As described below, we have incorporated this gene into a rSV5. It is important to note that the task in our original SOW was to identify the construct which provides the most efficient cell surface expression.
Research Accomplished on Task 2.

Isolation of cell lines that constitutively express human HER-2. As described in task 2a, we have set out to isolate cell lines that have tet-inducible expression of HER-2. The previous report described our success in isolating cells that have high expression of the tet repressor needed to block expression of a target gene under control of the tet repressor element. However, despite numerous attempts, we have been unable to isolate double-selected cell lines that express HER-2 under the tet operator. As an alternative approach to create a cell line to propagate and titer the rSV5 containing the HNfull-sFv attachment protein, we have isolated cell lines permissive for SV5 growth and plaque assay that express human HER-2. The HER-2 gene was inserted into pBabe under control of the CMV promoter. DNA was used to transfect CV-1 cells, a cell line that is used for plaquing and growing SV5 stocks. Cell colonies that were resistant to G418 (encoded on the plasmid) were isolated and grow up. As shown in Fig. 5, 4 cell lines were isolate that expressed high levels of HER-2 by western blotting. These are valuable cell lines for our experiments, since they can be used for growth and tittering rSV5. Importantly, along with the parental CV-1 cells, they can also be used to test the specificity of targeted infection by the rSV5-sFv vectors. Thus, Task 2a has been completed.

Figure 5. Cell lines constitutively expressing human HER-2. Stable cell lines (lanes 1-4) were isolated by tranfection with a cDNA expressing HER-2 and analyzed by western blotting with a polyclonal anti-HER-2 antibody. M, mock transfected cells; +, HER-2 overexpressed by vaccinia virus infection.

Construction of cDNA encoding HNfull-sFv in place of the WT HN gene. As a first step in recovering virus with an altered attachment protein, we have inserted the gene for HNfull-sFv in place of the WT HN gene. Clones were screened for the presence of a second XbaI site which was introduced as part of the HA tag at the end of the sFv open-reading-frame. Virus was recovered from A549 cells transfected with the resulting plasmid as described previously (He et al., 1997; Parks et al. 2002) and was designated rSV5-HNfull-sFv.

A. Anti-SV5 Anti-P
   M  WT  A  B  M  WT  A  B
   NP  -  -  -  -  -  -  -
   P  -  -  -  -  -  -  -

B. Anti-HA
   M  A  B  +  WT
   HN-sFv  -  -  -  -
   TK-HA  -  -  -  -

To determine if cells infected with the rSV5-HNfull-sFv virus could express the HA-tagged HNfull-sFv, A549 cells were mock infected (M) or infected with WT rSV5 (WT) or two cloned isolates (A and B) of rSV5-HN-sFv. Cells were radiolabeled with 35S-amino acids for 15 min at 15 h post infection and lysates immunoprecipitated with antibodies to SV5 or to the P protein before analysis by SDS-PAGE. Note that the titer of rSV5-HN-sFv is lower than WT rSV5, but the NP and P proteins can still be detected as evidenced in the autoradiogram in Figure 6. Cell lysates were also analyzed by western
blotting with an antibody to the HA epitope tag added to the C-terminus of the HN-sFv protein. As shown in panel B of Fig. 5, cells infected with rSV5-HNfull-sFv expressed an HA-tagged protein not seen in mock infected or WT rSV5-infected cells. These data support the contention that viable rSV5 can be recovered that expresses a novel HN attachment protein, and that C-terminal extensions to the SV5 HN protein do not prevent recovery of infectious virus.

It is important to note that the rSV5-HNfull-sFv protein has an HN with the normal sialic acid binding capacity seen with WT rSV5. Thus, the tropism of this virus is not restricted to HER-2 expressing cells. In the future we will generate rSV5-HNfull-sFv to have lost the natural receptor binding capacity of the WT HN protein.

Additional Studies Supported by DAMD170010488

Controlled cell killing by a recombinant negative strand RNA virus. In most tissue culture cell lines tested, infection with the paramyxovirus simian virus 5 (SV5) results in very little cell death. To determine if SV5 could be used as a vector for controlled killing of tumor cells and to improve the safety of any targeted vector, a recombinant SV5 (rSV5-TK) was constructed to encode the herpes simplex virus thymidine kinase (TK) gene inserted between the HN and L genes (see Fig. 7A). MDBK cells infected with rSV5-TK showed a time-dependent loss of viability when infected cells were cultured in the presence of the prodrug acyclovir or ganciclovir (Fig. 7B) while no significant toxicity was observed in the absence of prodrug. Cells infected with a control rSV5 expressing GFP and cultured with prodrug showed only a slight reduction in growth rate and little cell death (Fig. 7B). Time-lapse video microscopy of rSV5-TK infected MDBK cells that were cultured in the presence of ACV showed an accumulation of cells with morphological effects characteristic of apoptotic cell death (Fig. 7C). An MDBK cell line persistently infected with rSV5-TK retained sensitivity to prodrug-mediated cell killing, despite being passed for two months and having a lower overall level of TK expression than that found in an acute infection. Titration experiments showed that the rSV5-TK plus GCV combination resulted in cell death for all mouse and human cell lines tested, although the kinetics and efficiency of cell death varied between cell types. Our results demonstrating controlled cell killing by a recombinant paramyxovirus have implications for the use of negative strand RNA viruses as therapeutic vectors for targeted killing of cancer cells.
Figure 7. A. Structure of an rSV5 encoding the TK protein between the HN and L genes. B. Micrographs of controlled killing of MDBK cells. C. MDBK cells were infected and incubated in ACV. The number of apoptotic cells was determined by time lapse video microscopy at the indicated times p.i. The accumulation of rounded cells is expressed as a percentage of the total number of cells in the field. Data are representative of two independent experiments.
Key Research Accomplishments.

- Determined that sFv linked to truncated forms of HN will not be transported to the cell surface at a high enough efficiency to allow incorporation into budding virions.

- Constructed a chimeric protein consisting of the anti-HER2 sFv linked to the full length HN separated by a (Gly-Gly-Ser)3 spacer.

- Determined that the HNfull-sFv protein is very efficiently transported to the cell surface of transfected cells. This will form the basis for the rSV5 with altered attachment protein. (Task 1a finished)

- Constructed a chimeric protein consisting of the anti-HER2 sFv linked to the SV5 small hydrophobic protein SH. The SH-sFv protein is expressed at the cell surface as assayed by immunofluorescence. (Task 1a alternative)

- Isolated monkey kidney cell lines over-expressing human HER-2 for growing and titering rSV5, and for testing the specificity of infection (Task 2a finished).

- Constructed full length infectious cDNA for SV5 that has the HNfull-sFv inserted in place of the normal attachment protein. Recovery of infectious virus expressing the new HN attachment protein (Task 2b finished)

Reportable Outcomes

1) Construction of genes encoding membrane-bound, cell surface chimeric protein composed of the anti-HER2 sFv linked to paramyxovirus HN protein separated by Gly-Ser spacer region.
2) Isolation of monkey kidney cell lines constitutively expressing human HER-2.
3) Isolation of rSV5 virus harboring an HN-sFv attachment protein
4) Isolation of rSV5 expressing TK for controlled cell killing and for improved safety.

Conclusions

The overall goal of our work is to develop the paramyxovirus SV5 as a novel vector for controlled and targeted killing of tumor cells. Our model system is based on construction of a rSV5 with a novel attachment protein containing the anti-HER2 sFv linked to HN. The specificity of infection would be tested in cell lines with inducible expression of HER2. We have encountered two problems in our original plan: the proposed sFv-HN fusion proteins with truncated HN were inefficiently transported to the cell surface where budding occurs and we were unable to isolate double-drug resistant stable cell lines with inducible expression of HER2.

In order to move our work forward, we have overcome both of these problems by taking alternative approaches that are still well within the approved Statement of Work. We have constructed a new attachment protein whereby the sFv is linked to the full length HN, not to a fragment of HN. This molecule is efficiently expressed at the surface (see fig. 4 above). In addition, we have linked the sFv to the SH protein, a second SV5 protein that is incorporated into virions and we will use this as yet another
alternative approach. Again, this is still within the approved statement of work which was to determine the requirements for incorporating membrane bound anti-HER2 sFv into a virion.

Likewise, we have taken an alternative approach to isolate cell lines expressing HER2. The CV-1 cell lines described in this report are still within the Statement of Work, since they will allow us to test the specificity of infection with the rSV5-HNfull-sFv by using normal CV-1 cells and the HER2 expressing cells as negative and positive target cells.

We have requested a no-cost extension for this project, which was approved for one year. During that year we made substantial progress in this project, having recovered a novel rSV5 containing a new HN protein linked to sFv. Our work will be an important addition to the development of vectors that are capable of infecting predetermined populations of tumor cells and, together with a suicide gene (e.g., Parks et al, 2002), the rSV5 vectors will be used for controlled killing of tumor cells.

References


Final Report Bibliography of publications and meeting abstracts


List of personnel receiving pay for Research Effort.

Griffith D. Parks, Ph.D. Principal Investigator
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Controlled Cell Killing by a Recombinant Nonsegmented Negative-Strand RNA Virus

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INTRODUCTION

There has been remarkable progress in recent years on the development of reverse genetics systems for manipulating negative-strand RNA viruses. As such, a number of nonsegmented negative-strand RNA viruses have been engineered to express a variety of foreign proteins (reviewed in Conzelmann, 1998; Pekosz et al., 1999; Palese, 1995). The ability to recover paramyxovirus- and rhabdovirus-like cDNA has raised the possibility of using these viruses as therapeutic vectors for the delivery of genes or toxins for cancer therapy. In this report, we have engineered a noncytopathic negative-strand RNA virus to express an enzyme that activates a prodrug and demonstrated that this vector can be used for controlled cell killing.

The paramyxovirus simian virus 5 (SV5) is a member of the rubulaviruses, a group that includes mumps virus, human parainfluenza virus type 2, and SV41 (Lamb and Kolakofsky, 1996). The ~15-kb negative-sense RNA genome encodes seven tandemly linked genes (Fig. 1A) that code for the nucleocapsid protein (NP), which binds the viral RNA genome; the phospho- (P) and large (L) proteins, which together constitute the viral polymerase; the matrix protein involved in virus assembly; and the membrane proteins HN and F, which are responsible for cell attachment and fusion, respectively. The V and small hydrophobic (SH) proteins are thought to be involved in counteracting cellular innate immune responses to SV5 infection. The V protein is important for blocking interferon signaling in SV5-infected cells by targeting the degradation of cellular STAT1 protein (Didcock et al., 1999). The SH protein serves an unknown function in the viral life cycle, but infection of cells with a recombinant SV5 (rSV5) lacking the SH gene results in the induction of apoptosis (He et al., 2001).

SV5 has inherent properties that could be exploited to generate novel therapeutic vectors. One such property is that infection with SV5 results in very little cell death in most tissue cell lines tested (e.g., Choppin, 1964; He et al., 2001). We reasoned that a paramyxovirus that does not induce a dramatic cytopathic effect could serve as a vector for controlled killing of infected cells. We hypothesize that an rSV5 vector could be engineered to express a well-characterized enzyme that would induce cell death in a controlled manner and by a mechanism which is understood.

The herpes simplex virus (HSV) thymidine kinase (TK) protein in conjunction with acyclovir (ACV) or ganciclovir (GCV) is a prototype system for controlled cell killing and has been used extensively as a "suicide gene" in a variety of cancer therapies (reviewed in Mesnil and Yamashita, 2000; Moolten, 1994). Within a cell, TK selectively mediates the conversion of the prodrug ACV or GCV to the toxic triphosphate form, which results in cell death.
phosphorylates the nontoxic prodrugs ACV and GCV, converting them into potent intracellular compounds that can be incorporated into host DNA and ultimately induce cell death (Elion et al., 1977). Thus, cell killing is controlled with the TK/GCV combination, since it occurs in cells that express TK and are exposed to the prodrug. In addition, the “bystander effect” that results from the TK/GCV combination allows neighboring cells within a tumor microenvironment to also become susceptible to GCV-mediated killing (Mesnil and Yamasaki, 2000). To test our hypothesis on the use of rSV5 as a vector for controlled killing of cancer cells.

RESULTS

Recovery of rSV5 expressing the HSV TK protein

Previous work has indicated that extensions to the carboxy-terminus of TK do not affect enzyme activity (Loinas et al., 1998). Thus, to facilitate detection of the TK gene product, the 3’ end of the TK open reading frame was extended to include an 11-amino-acid epitope recognized by an anti-HA monoclonal antibody. The resulting TK-HA gene was modified by a PCR to be flanked by gene start and gene end signals from the SV5 NP–PA and HN–L junctions, respectively, and then inserted at the HN–L junction encoded in the SV5 infectious cDNA clone (Fig. 1A). Virus was recovered from cells transfected with the resulting plasmid as described previously (He et al., 1997) and was designated rSV5-TK.

Western blotting with anti-HA antibody was carried out to determine a time course of TK expression in cells infected with rSV5-TK. As shown in Fig. 1B, MDBK cells infected with rSV5-TK synthesized an ~45-kDa polypeptide (TK-HA) by 8 h postinfection (p.i.) that was not detected in mock infected cells (lane M). A faster migrating form of TK was also detected at lower levels that may represent a degradation product. The kinetics of TK detection in cells infected with rSV5-TK was slightly delayed relative to NP and P, consistent with the gradient of viral transcription and the position of the TK gene in a 3’ promoter-distal site in the SV5 genome.

For some negative-strand RNA viruses, the insertion of a foreign gene can influence viral growth characteristics (e.g., Sakai et al., 1999; Skiadopoulos et al., 2000). To determine if inserting the TK gene into the SV5 genome affected the rate of virus growth, a single-step growth analysis was carried out on cells infected at a high m.o.i. with rSV5 WT, rSV5-TK, or a control virus containing the GFP gene at the HN–L junction (rSV5-GFP; He et al., 1997). As shown in Fig. 1C, the insertion of the TK gene into rSV5 did not affect virus growth, since each of the rSV5 viruses showed very similar growth rates in MDBK cells and reached ~10^6 PFU/ml as a final titer.

ACV-mediated killing of MDBK cells infected with rSV5-TK

Cells infected with rSV5-TK were treated with varying concentrations of ACV to determine if they were sensitized to killing by the prodrug. Monolayers of MDBK cells were mock infected or infected with rSV5-TK or the control virus rSV5-GFP at an m.o.i. of ~50 and then cultured in the presence of ACV (0–20 μg/ml). After 48 h, cells were removed from the dishes and the number of viable cells was determined by counting cells that excluded trypan blue staining. As shown in Fig. 2A, the number of viable cells remaining after 48 h is expressed as a fold change relative to the number of cells in the culture before infection. In the absence of ACV, the number of viable MDBK cells increased six- to eightfold after infection with either rSV5-GFP or rSV5-TK (0 bars, Fig. 2A). This result is consistent with previous reports that SV5 infection is largely noncytopathic in MDBK cells (e.g., He et al., 2001). With the addition of ACV to the culture media, the number of mock- or rSV5-GFP-infected cells increased by approximately three- to fivefold. By contrast, adding as little as 1 μg/ml ACV to the growth media of cells infected with rSV5-TK prevented an increase in the number of viable cells over the 2-day assay period (black bars, Fig. 2A). At ACV concentrations higher than 1 μg/ml, the number of viable cells decreased from the original number of cells at the time of infection by two- to fourfold.

Figure 2B shows a time course of the change in the number of viable MDBK cells after mock infection or infection with rSV5-GFP or rSV5-TK. In the absence of ACV, the number of viable cells by 48 h p.i. increased approximately fourfold regardless of whether they were mock infected or infected with rSV5-GFP or rSV5-TK (dashed lines, Fig. 2B). For mock-infected or rSV5-GFP-infected cells, these increases in numbers of viable cells were not significantly affected by including ACV in the media (filled triangles and squares, Fig. 2B). However, in the presence of 20 μg/ml ACV, rSV5-TK-infected cell numbers were essentially unchanged after the first day p.i., and viable cell numbers decreased two- to threefold by 48 h p.i. (filled circles).

The changes in cell number shown in Fig. 2 reflect the proliferation, survival, and death of cells in the population. Time-lapse video microscopy was carried out to more closely monitor cytopathic changes to individual rSV5-TK-infected MDBK cells after exposure to ACV. Figure 3 shows pictures from a representative experiment in which MDBK cells were infected with rSV5-TK (Figs. 3A–3C) or rSV5-GFP (Figs. 3D–3F) and cultured in the
Fig. 1. Genome structure, protein expression, and growth of rSV5-TK. (A) Structure of rSV5-TK. The SV5 genome is depicted as a rectangle with vertical bars denoting the intergenic regions. The 3' leader (le) and 5' trailer (tr) are shown as dark boxes. The HSV TK open reading frame was modified to encode a 3'-end HA epitope tag and was flanked on the 3' and 5' sides by the gene end-intergenic-gene start signals from the NP-PV and the HN-L gene junctions, respectively. (B) TK expression from rSV5-TK. MDCK cells were mock infected (M lane) or infected with rSV5-TK. At the indicated hours p.i., cells were lysed and equal aliquots analyzed by Western blotting with polyclonal antisera specific for the SV5 NP and P proteins (top) or an HA-specific monoclonal antibody (bottom). (C) Growth of rSV5-TK. MDCK cells were infected at an m.o.i. of ~20 with rSV5 WT, rSV5-GFP, or rSV5-TK and medium was harvested at the indicated times p.i. Virus titers were determined by plaque assay on CV-1 monolayers. Data represent the averages of two experiments.
FIG. 2. Controlled killing of MDBK cells infected with rSV5-TK in the presence of ACV. (A) Titration of ACV effect. Monolayers of MDBK cells were mock infected or infected with rSV5-TK or rSV5-GFP. Growth media were supplemented with ACV to the indicated concentrations. Two days later, viable cell numbers were determined by trypan exclusion and are expressed as the fold change from the initial number of infected cells. Data are the means (± standard deviation, SD) of three independent experiments. (B) Time course of ACV effect. MDBK cell monolayers were mock infected or infected with rSV5-TK or rSV5-GFP. The growth media were supplemented with either 0 (dashed lines) or 20 μg/ml ACV (solid lines). At 1 and 2 days p.i., viable cell numbers were determined as described for A. Data are the means of three independent experiments. For clarity, the SDs are shown only for rSV5-TK plus ACV, but all other SD were within 20%.

did not undergo cell division was calculated as a measure of a defined cytopathic effect. The graph at the bottom of Fig. 3 shows the results from a representative experiment in which the accumulation of rounded cells is expressed as a percentage of the total number of cells in the field. When cultured with ACV, the rSV5-TK-infected cell population showed an initial lag in accumulation of rounded cells up to ~18–24 h p.i. (filled squares), consistent with the time course of cell viability shown in Fig. 1B above. However, by 48 h p.i., nearly 70% of the cells in the field had rounded up and many had disintegrated into cellular debris. By contrast, for rSV5-TK-infected cells without ACV (filled triangles) or for rSV5-GFP-infected cells exposed to ACV (open squares) only ~2 and ~5%, respectively, of the infected cells in these control samples had rounded up without undergoing cell division by 48 h p.i. Taken together, these data demonstrate controlled cell killing of MDBK cells by a negative-strand RNA virus vector, since only the combination of rSV5-TK infection and ACV treatment induces a significant loss of MDBK cell viability.

Susceptibility of various cell lines to GCV-mediated killing by rSV5-TK

A variety of cell lines were tested for their sensitivity to cell killing by rSV5-TK along with GCV, a prodrug that we have found to have a lower nonspecific effect on mock-
FIG. 3. Time-lapse video microscopy of MDBK cells infected with rSV5-TK or rSV5-GFP in the presence of ACV. Flasks of MDBK cells were infected with rSV5-TK (top row, A–C) or rSV5-GFP (bottom row, D–F) and incubated in medium containing 10 μg/ml ACV. Time-lapse video microscopy of a field of infected cells was carried out as described under Materials and Methods. Selected video frames are shown representing time 0 (A and D), 24 h p.i. (B and E), and 48 h p.i. (C and F). In the bottom graph, the number of rounded cells that did not undergo cell division was determined as an assay of cytopathic effects at the indicated times p.i. The accumulation of rounded cells is expressed as a percentage of the total number of cells in the field. Data are representative of two independent experiments.

Several features of rSV5-TK cell killing are evident in the results shown in Fig. 4. First, infection of some cell lines with either rSV5-GFP or rSV5-TK in the absence of added GCV resulted in a lower fold increase in cell number compared to mock infected cells (Fig. 4, 0 GCV bars). This reduction in growth of infected cells is most evident in the case of HeLa and A549 cells and is consistent with the report that SV5 infection slows progression through the HeLa cell cycle (Lin and Lamb, 2000). Second, culturing uninfected cells (Fig. 4, white bars) or rSV5-GFP-infected control cells (hatched bars) with the highest GCV concentration (200 μM) resulted in a slight decrease in cell growth compared to no added GCV, with the magnitude of this effect being dependent on the particular cell type assayed. Most importantly, the combination of infection with rSV5-TK and GCV treatment showed a significant reduction of viable cell numbers in each cell line tested. The efficiency of cell killing with
rSV5-TK and GCV varied between cell lines as evident by comparing the results with MDBK and 4T1 cells. MDBK cells were very sensitive to GCV-induced cell killing by rSV5-TK, and a significant loss of viability was seen with as little as 2 μM GCV. For the rSV5-TK-infected 4T1 murine breast cancer cell line, there was a lower overall loss of viability with increasing doses of GCV compared to MDBK cells and significant decreases in cell numbers from the starting culture were seen only at the higher GCV concentrations. The individual cell lines also showed a difference in the kinetics of cell killing. For example, the profile of rSV5-TK cell killing for human lung A549 cells was similar to that of U937 cells; however, the significant loss of viability shown in Fig. 4 for A549 cells was delayed until 4 days p.i. as opposed to the 2 days needed for killing of the other cell lines.

The sensitivity of various cell lines to rSV5-TK-mediated cell killing was not strictly related to cell growth
rate. As shown in Fig. 4, MDBK cells had the shortest doubling time (~11.5 h) and were the most sensitive to GCV-mediated killing by rSV5-TK. However, 4T1 cells had a similar doubling time (DT ~12 h) and were significantly less sensitive to rSV5-TK/GCV-mediated killing. U937 and A549 cells had similar doubling times (~20 and ~17.5 h, respectively), but A549 cells took 4 days to show a significant loss of viability. Likewise, Western blot analysis showed that the efficiency of rSV5-TK cell killing was not strictly linked to the level of SV5 or TK proteins expressed (not shown). Taken together, the most important conclusion from these data is that every cell line tested shows controlled cell killing by the rSV5-TK and GCV combination.

Effect of p53 levels on the efficiency of rSV5-TK cell killing

More than 50% of human cancers have been found to contain mutations in the tumor suppressor protein p53 (Greenblatt et al., 1994). Thus, preferential replication or killing of cells that are defective in p53 would be a desirable characteristic of a therapeutic viral vector. The H1299-derived human lung cancer cell line HT7, which contains a doxycycline-inducible p53 gene (Koumenis et al., 2001), was used to determine if rSV5-TK gene expression or growth was altered by different levels of p53. HT7 cells were mock treated or treated for 20 h with 0.5 μg/ml doxycycline to induce synthesis of p53 before infection with rSV5-TK. As shown in Fig. 5A, p53 levels were increased by doxycycline treatment (+ lanes) relative to control untreated samples (− lanes) and these levels were maintained in rSV5-infected cells at 24 h p.i. Western blotting of cell extracts prepared 24 h p.i. showed no significant difference in the accumulation of viral P and TK proteins (Fig. 5A, 24 h lanes). Likewise, the final yields of rSV5-TK from HT7 cells did not differ significantly between cultures that were induced to synthesize p53 and control uninduced cells (data not shown).

There are conflicting reports on whether p53 plays an important role in cell killing by the TK plus GCV combination (Krohne et al., 2001; Matsumura et al., 1999; Wallace et al., 1996). To determine the influence of p53 on the efficiency of rSV5-TK cell killing, doxycycline-treated or mock-treated HT7 cells were infected with rSV5-TK and cultured with various concentrations of GCV (0–200 μM). After 3 days, viable cell numbers were determined. In the absence of rSV5-TK infection, doxycycline treatment resulted in a slight decrease in cell numbers relative to control untreated cells (Fig. 5B, no virus bars), consistent with the induced p53 having a negative effect on cell growth. HT7 cells infected with rSV5-TK showed a dose-dependent loss of viability with increasing concentrations of GCV from 2 to 200 μM. At each GCV concentration, there were fewer cells remaining in cultures that were treated with doxycycline versus untreated control

![Graph](https://via.placeholder.com/150)

**FIG. 5.** Effect of increased levels of p53 protein on the growth and efficiency of cell killing by rSV5-TK. (A) Increased levels of p53 do not alter the level of protein expression from rSV5-TK. HT7 cells were mock treated (− lanes) or treated with 0.5 μg/ml doxycycline (+ lanes) for 20 h prior to mock infection or infection with rSV5-TK. Cell lysates were prepared immediately after infection (0 lanes) or 24 h p.i. in the presence or absence of 0.5 μg/ml doxycycline. Equivalent amounts of protein from each sample were assayed by Western blotting with antibodies specific for p53, P, or TK-HA. (B) Effect of increased levels of p53 on cell killing by rSV5-TK. HT7 cells were mock treated or treated with 0.5 μg/ml doxycycline (Dox) for 20 h prior to infection with rSV5-TK. Cells were then cultured with or without doxycycline and the indicated concentrations of GCV. After 3 days, viable cell numbers were determined as described under Materials and Methods. (C) Induction of p53 sensitizes HT7 cells to GCV. HT7 cells were treated for 18 h with 0.5 μg/ml doxycycline or 200 μM GCV for 2 days and numbers of viable cells were determined. Data represent the means of three independent experiments (±standard deviation) and are expressed as the fold change in the number of viable cells relative to the starting culture.
PARAMYXOVIRUS EXPRESSING THYMIDINE KINASE

A    NP
M    P
S

1:4
1:8
1:16
1:32

>1:32

TK-HA

M    P
S

©
©

Mock Infected
Persistent Cell Line
rSV5-TK

B

GCV Concentration (µM)

FIG. 6. Cells persistently infected with rSV5-TK retain sensitivity to GCV-mediated killing. (A) Expression of viral and TK proteins in acute and persistently infected MDBK cells. Lysates were prepared from rSV5-TK persistently infected MDBK cells (P lanes), from MDBK cells infected for 48 h with rSV5-TK (S lanes) or from mock-infected control cells (M lanes). After protein concentrations were normalized (starting with 0.2 and 2 µg of protein for NP and TK samples, respectively), serial dilutions of lysate were analyzed by dot-blot hybridization using antiserum specific for the SV5 NP protein or the HA-tagged TK protein. (B) Effect of GCV on the viability of MDBK cells persistently infected with rSV5-TK. The growth media on monolayers of mock-infected, rSV5-TK-infected, and the pass 15 MDBK PI cell line were supplemented with GCV to the indicated concentrations. After 2 days, viable cell numbers were determined as described in the legend to Fig. 2.

cultures. However, this loss of viability was not solely due to killing by rSV5-TK infection. This is evident in Fig. 5C, where doxycycline-treated cells were also more sensitive to GCV, even in the absence of virus infection. This can be attributed to a combined effect of p53-dependent inhibition of cell growth and an increased basal toxicity of GCV in the presence of p53. Together, these data suggest that p53 is not required for rSV5-TK/GCV cell killing but may increase the efficiency of prodrug action in human cells.

GCV-mediated killing of MDBK cells persistently infected with rSV5-TK

The above results indicate that cells acutely infected with rSV5-TK are susceptible to GCV-mediated killing. We established an MDBK cell line persistently infected (PI) with rSV5-TK to determine if these cells retained their sensitivity to GCV-mediated killing. Immunofluorescence microscopy with anti-P antibodies demonstrated the presence of SV5 antigen in >95% of the PI cells that had been maintained for over 15 cell passages (not shown). The PI cells showed no obvious difference in growth characteristics or morphology relative to uninfected cells or cells acutely infected with rSV5-TK. To determine the relative expression levels of viral and TK protein, cell lysates were prepared from mock-infected MDBK cells, the PI cell line, or MDBK cells at 48 h p.i. with rSV5-TK. Serial dilutions of lysates were analyzed by immunoblotting using a dot-blot format and antibodies for the SV5 NP and TK proteins. As shown in Fig. 6A, cells acutely infected with rSV5-TK (S lanes) accumulated slightly more NP and TK protein at 48 h p.i. than was found for the PI cell line (P). As shown in Fig. 6B, exposure of the PI cell line to 2 µM GCV resulted in a significant decrease in cell numbers relative to mock-infected cells, with larger decreases seen at higher GCV concentrations. The efficiency of GCV-mediated cell killing was slightly higher than that seen for cells infected with rSV5-TK for 2 days. Thus, the PI cell line appears to be more sensitive to GCV-mediated cell killing than the cells acutely infected with rSV5-TK.

DISCUSSION

In this report we have tested the hypothesis that a nonsegmented negative-strand RNA virus could be engineered as a vector for controlled killing of infected cells. Our rationale was that the inherently noncytopathic virus SV5 could express a foreign protein to kill cells in a controlled manner and by mechanisms that are relatively well understood. In most tissue culture cell lines tested, rSV6 infection results in very little cell death (e.g., Choppin, 1964; He et al., 2001). Using time-lapse video microscopy, we have found that by 48 h p.i. less than 5% of a population of MDBK cells infected with rSV5-TK have rounded up without undergoing cell division, a common cytopathic effect of viral infections. This result is consistent with the results of He et al. (2001), who showed by TUNEL assay that there was no significant difference between mock-infected and rSV5-infected MDBK cells in the fraction of cells displaying markers of apoptosis. While SV5 infection of some cell types, such as CV-1 and BHK, leads to extensive cell–cell fusion (e.g., Horvath
than the 2 days required for all other cell types. A number of viruses have been shown to encode components that act to block apoptotic pathways in infected cells (Griffin and Hardwick, 1997; Roulston et al., 1999, and references therein), we initially anticipated that SV5 might have a mechanism(s) that could inhibit apoptosis induced by the TK plus ACV/GCV combination. Recent data suggest that the SV5 SH protein may be involved in counteracting apoptosis during an SV5 infection (He et al., 2001). Our results indicate that any SV5-mediated block in an apoptotic pathway can be overcome by the combination of TK expression plus prodrug treatment. However, an effective therapeutic vector based on rSV5-TK will need to be more efficient and potent at cell killing. It remains to be determined if expression of the TK gene from the rSV5-ASH virus previously described by He et al. (2001) will enhance the rate or the overall extent of cell killing by rSV5-TK. Alternatively, it may be possible to enhance cell killing further by engineering rSV5 to express well-defined inducers of apoptosis (e.g., Bax).

The finding that p53 suppressor protein is nonfunctional in greater than 50% of human tumors (Greenblatt et al., 1994) suggests that it may be possible to design viral vectors that could selectively replicate in and kill p53-deficient cells but not p53+wt cells. Despite initial indications that this held true for a therapeutic E1B 55-kDa-deleted mutant adenovirus, recent data have shown that the growth properties of this vector do not always correlate with the status of p53 (Dix et al., 2001; Goodrum and Ornelles, 1998). Our results with a human lung cell line containing an inducible p53 gene indicate that protein expression and growth of rSV5-TK are not affected by large differences in expression of p53. While cells induced for overexpression of p53 in our experimental system were sensitized to GCV-mediated death in the absence of rSV5-TK infection, we conclude that p53 status is unlikely to be a major factor influencing the ability of our current rSV5-TK vector to kill tumor cells.

There are a number of advantages to using paramyxoviruses as therapeutic vectors, including the apparent lack of strict packaging constraints, cytoplasmic replication without DNA integration, and the ability to incorporate foreign glycoproteins into the virion envelope. A potential disadvantage of their use as safe therapeutic vectors is their capacity to establish persistent infections both in cell culture and in vivo (reviewed by Randall and Russell, 1991). We have shown that an MDBK cell line persistently infected with rSV5-TK retained long-term expression of TK and sensitivity to GCV-mediated cell killing similar to those found in an acute infection. This result supports the proposal that including the TK gene in recombinant negative-strand viruses could improve the safety of these vectors by providing a means to control or eliminate a persistent viral infection. In addition, these data indicate that the noncytopathic nature of rSV5 infection provides a means to obtain long-term...
expression of foreign genes without overt changes in the infected cells.

Controlled cell killing and selective cell targeting are two critical features for a virus to be useful in targeted therapy of cancer cells (reviewed in Verma and Somia, 1997; Anderson, 1998). The results reported here with rSV5-TK provide proof-of-concept addressing the first requirement, in which an inherently noncytopathic virus has been engineered to kill cells by a mechanism that can be controlled. SV5 has inherent properties that can be also exploited to address the second requirement of selective cell targeting. The SV5 F protein is capable of promoting membrane fusion without the SV5 HN attachment protein (e.g., Horvath and Lamb, 1992; Lamb, 1993). This raises the possibility that the SV5 HN protein with a binding specificity for sialic acid could be replaced with an attachment protein with specificity for a targeted cellular receptor. The approach of redirecting a viral infection has recently been presented for measles virus, another negative-strand RNA virus. Selective cell targeting has been achieved by using recombinant measles viruses that contain chimeric attachment proteins consisting of the viral H protein linked to epidermal growth factor or to a single chain antibody (Hammond et al., 2001; Schneider et al., 2000). Work is in progress to engineer rSV5 to encode both the TK gene for controlled cell killing and a chimeric attachment protein for targeting of the viral infection to selected cell types.

MATERIALS AND METHODS

Cells, viruses, and plaque assays

Cultures of cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) or RPMI 1640 containing 10% fetal calf serum (FCS). The 4T1 murine breast cancer cell line was kindly provided by Fred Miller (University of Michigan). The p53-inducible H7 human lung cells have been described (Koumenis et al., 2001). All other cell lines were obtained from the Comprehensive Cancer Center of Wake Forest University. To determine doubling times, 10^5 cells were plated in 3.5-cm dishes and at various times, cells were removed from the dish and counted using a Coulter counter. Vaccinia virus MVA expressing T7 RNA polymerase was a kind gift from B. Moss and was grown and titered on BHK cells (Wyatt et al., 1995). Single-step growth kinetics and plaque assays were carried out as described previously (Parks et al., 2001). An m.o.i. of 50 was used for all infections with rSV5 vectors. An MDBK cell line persistently infected with rSV6-TK was established by infecting 10^6 cells at an m.o.i. of 50 and passing the resulting cells every 4–5 days.

Recovery of rSV5 expressing TK

The full-length SV5 infectious cDNA clone pBH276 (He et al., 1997) was kindly provided by B. He and R. A. Lamb (Northwestern University). A SphI–SalI DNA fragment encoding the HN–L junction was modified by a PCR using Pwo polymerase (Boehringer Mannheim) and subcloned into pGem3 to generate pG3-HNL-MCS, such that the HN–L junction contained a multiple cloning site with EcoRV and SalI restriction enzyme sequences. A plasmid (pPEP78) encoding the herpes simplex TK gene was kindly provided by Dr. Peter Pertel (Northwestern University). The TK gene was modified by a PCR to remove the internal SphI site and add a 3'-terminal Asp718 site without changing the encoded amino acids. The resulting PCR product was inserted into pG3-HA such that the translation open reading frame was fused at the 3' end to a 10-amino-acid segment recognized by an anti-HA antibody (YPYDVPDYA). The TK-HA gene was inserted into pG3-HNL-MCS such that the gene was flanked on the 3' and 5' sides by transcription signals from the NP–P and HN–L junctions, respectively (Fig. 1A; Rassa and Parks, 1999), and the overall length was a multiple of 6 (Murphy and Parks, 1997). A SphI–SalI DNA fragment encoding the TK-HA gene was excised and then inserted into the corresponding sites in the SV5 full-length cDNA clone (He et al., 1997) to yield pRSV5-TK. SV5 virus expressing the HA-tagged TK protein was recovered from pRSV5-TK as described previously (He et al., 1997) with minor modifications (Parks et al., 2001). Virus stocks were generated from a single plaque by growth in MDBK cells. Wild-type (WT) rSV5 and rSV5-GFP were similarly isolated from pBH276 and pBH311, respectively (He et al., 1997).

Western blotting

MDBK cells plated in 24-well dishes were infected with rSV5 WT, rSV5-GFP, or rSV5-TK at an m.o.i. of ~20 for 1 h. Cells were washed and covered with DMEM/2% FCS. At each time point, cells were washed with PBS and lysed in gel loading buffer and equal aliquots of cell lysate were analyzed on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose using a semidy electromophoretic transfer cell (15 V, 15 min). Nitrocellulose membranes were blocked (5% milk in PBS) and probed using a rat monoclonal antibody specific for an HA epitope (anti-HA clone 3F10; Roche Molecular Biochemicals) or rabbit antisera to the SV5 NP and P proteins (Parks et al., 2001). For measuring p53 levels, HT7 cells (Koumenis et al., 2001) were treated for 20 h with 0.5 μg/ml doxycycline in RPMI/2% FBS to induce synthesis of p53, and cell lysates were analyzed by Western blotting with mouse monoclonal DO-1 specific for human p53 (Santa Cruz Biotechnology). Proteins were visualized using HRP-conjugated goat secondary antibodies and ECL (Pierce Chemicals). For dot-blot analysis, the protein concentration of infected cell lysates was determined using the BCA assay (Pierce Chemicals). Equivalent amounts of protein were serially diluted in PBS and
applied to nitrocellulose using a vacuum manifold before analysis by Western blotting with antibodies specific for TK, NP, or P proteins.

Cell killing assays and time-lapse video microscopy

Between 1 and 5 × 10⁵ cells in six-well dishes were mock infected or infected at an m.o.i. of 50 with rSV5-GFP or rSV5-TK for 1 h. Cells were covered in media containing 2% FCS and various concentrations of ACV or GCV (Sigma Chemicals) as described in the figure legends. At the indicated times postinfection, cells were washed and trypsinized (for adherent cells), and viable cell numbers were determined by trypan blue exclusion. Data are expressed as a fold increase in cell number relative to the starting culture.

Time-lapse video microscopy was carried out using 25-cm² flasks of MDBK cells that were mock infected or infected with rSV5-GFP or rSV5-TK. Cells were cultured in the presence of 10 μg/ml ACV on a time-lapse microscopy system (37°C, 5% CO₂) using a Zeiss inverted Axiovert phase-contrast microscope with an attached video camera. Pictures were recorded at intervals on standard VHS tape. Digital images for Fig. 3 were derived from the VHS tape. The accumulation of rounded cells that did not undergo cell division was determined as a function of time and is expressed as a percentage of the total number of cells in the field.

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