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PRINCIPAL INVESTIGATOR: Lesia K. Dropulic, M.D.

CONTRACTING ORGANIZATION: Johns Hopkins University
School of Medicine
Baltimore, MD 21205-2196

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Lesia K. Dropulic, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Johns Hopkins University School of Medicine
Baltimore, MD 21205-2196

E-Mail: lesia@jhmi.edu

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13. ABSTRACT (Maximum 200 Words)

The purpose of the proposed research is to develop a propagation-competent (PC) alphavirus vector that is targeted specifically to receptors expressed on breast cancer cells or to receptors expressed on tumor-associated vasculature. This type of targeted vector would provide a very efficient means of specifically killing a large number of malignant cells. Two Sindbis virus (SV) vectors containing the epidermal-like growth factor domain of heregulin in place of a portion of its receptor-binding domains, were impaired in their ability to assemble and bud from transfected cells. However, transfection of cells with the heregulin-containing SV RNAs resulted in preferential killing of breast cancer cells. Using a sequence encoding a 13 amino acid NGR-containing peptide ligand, we were able to produce a replication-competent SV. This recombinant virus did not preferentially replicate and kill cells expressing the target CD13 receptor. Breast cancer tumors were produced in nude mice using MDA-MB-231 cells. Difficulties with production of PC heregulin containing virus and with production of sufficient quantities of viral RNA precluded performance of properly controlled animal studies. Future studies will focus on identifying other ligands, permissive sites of replacement versus insertion in the SV genome, and further development of the animal models.

14. SUBJECT TERMS

breast cancer, Sindbis virus, propagation-competent, targeted, erbB-2, heregulin, NGR-peptide, tumor associated vasculature

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Table of Contents

I. **Introduction.** Two main challenges of cancer gene therapy are the development of vectors targeted specifically to tumor cells and the efficient delivery of the therapeutic agent to all or to the majority of tumor cells. Addressing these two issues, we intend to develop Sindbis virus (SV), an alphavirus, into a novel vector for breast cancer gene therapy. The advantages of SV vectors include lack of serious disease caused by SV in humans, the ability of SV to infect nondividing and dividing cells, no risk of insertional mutagenesis because SV is an RNA virus, and the ability to produce high titer stocks and achieve high level of heterologous gene expression. Since SV kills cells by apoptosis, specific destruction of tumor cells will occur if the virus is targeted to tumor cells (1). Furthermore, use of a propagation-competent viral vector will provide a very efficient means of obtaining access to most or all of the tumor cell population. As a first step, erbB-2, a tumor-associated growth factor receptor over-expressed in 20-30 % of human breast carcinomas was used as a model target. The EGF-like domain of heregulin was cloned to replace portions of the receptor-binding domains of the E2 glycoprotein to target ErbB-2 overexpressing breast cancer cells. As an additional approach, an NGR-containing peptide motif that binds to the CD13 receptor on tumor associated vasculature was used to modify the receptor-binding domain of E2. The long-term goal of this proposal is to develop target-specific SV vectors for application to breast cancer therapy by modifying the SV E2 envelope glycoprotein with ligands that recognize specific cell surface receptors on breast cancer tumors.

II. **Body.**

Statement of Work (SOW) Technical Objective #1: Construct targeted Sindbis virus (SV) vectors.

A. SV vectors with the epidermal growth factor domain of heregulin replacing portions of the putative receptor-binding domain of the viral E2 glycoprotein were constructed. In clone 12, the E2 amino acid residues 60 to 111 have been replaced with the heregulin domain. In clone 15, amino acid residues 115 to 173 have been replaced.

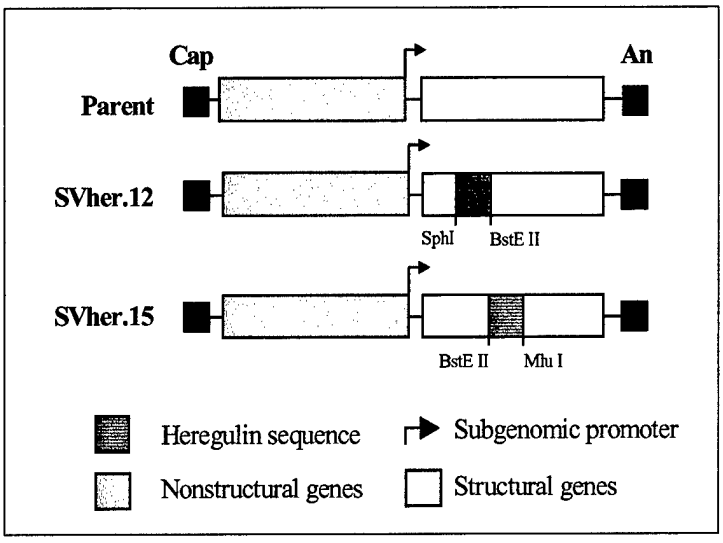

Figure 1. Schematic diagrams of the genome of the heregulin-containing SV clones. SV E2 amino acids 61 to 111 and 115 to 173 were replaced with the EGF-like domain of heregulin to create SV clones SVher.12 and SVher.15, respectively. The unique restriction enzyme sites used to replace these regions of the E2 receptor-binding domain are depicted. The parent genome contains the unique restriction enzyme sites with no other modifications from the wild-type SV sequence. The diagram is not drawn to scale.

Plus-strand genomic RNA was made by in vitro transcription using SP6 polymerase and SV vector cDNA as the template. BHK-21 cells and SK-BR3 human breast cancer cells were transfected with 2.5 μg of the plus-strand genomic RNA using Lipofectin or DMRIE-C, respectively. The transfection efficiency determined using an SV replicon expressing lacZ was 13 % in BHK-21 cells and 10 % in SK-BR3 cells (Fig. 2D and H). Twenty-four and forty-eight hours after transfection, the cells were observed for a cytopathic effect. With different preparations of SV RNA, we repeatedly detected a significant cytopathic effect only in the human breast cancer cells that were transfected with SVher.12 or SVher.15 viral RNA (Fig. 2G). Repeatedly, no cytopathic effect was observed in BHK-21 cells transfected with the modified viruses (Fig. 2C).

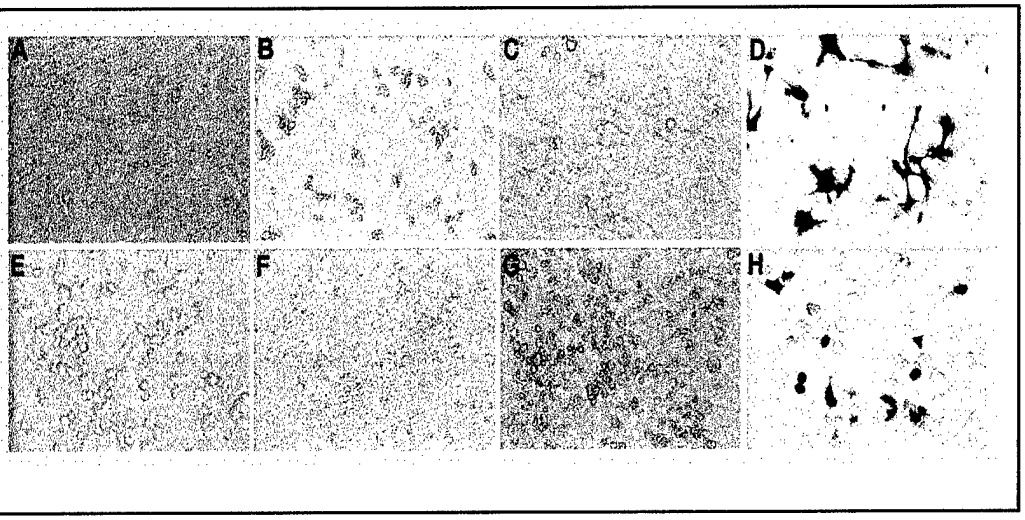

Figure 2. Cytopathic effect in BHK and SK-BR3 cells transfected with SVher.15 RNA (similar results were obtained with SVher.12). Forty-eight hours after transfection, the cells were visualized via phase contrast microscopy. Figures A, B, C, D, are BHK cells transfected with yeast, parent, SVher.15 or SV replicon-LacZ RNA, respectively. Figures E, F, G, H are SK-BR3 cells transfected with yeast, parent, SVher.15 or SV replicon-LacZ RNA, respectively. Similar results were obtained when the cells were transfected with SVher.12RNA (200X magnification).

The number of dead cells in the transfected cultures was quantified using the Trypan blue or propidium iodide exclusion assay (Fig. 3). The parent virus RNA killed both cell lines, but was less efficient in killing SK-BR3 cells than BHK-21 cells (Fig. 3A and 3B). At 48 hours after transfection, 88 ± 2.0 % of BHK-21 cells were killed by the parent virus RNA versus 20 ± 0.7 % of the SK-BR3 cells (Fig. 3A and 3B). Transfection of SK-BR3 cells with SVher.12 RNA killed 26 ± 1.0 % of the cells, and transfection with SVher.15 RNA killed 18 ± 0.8 % of the cells (Fig. 3B). Mock-transfected and SV replicon-transfected SK-BR3 cells exhibited 6 ± 1.1 % and 7 ± 1.4 % dead cells, respectively (Fig. 3B). Transfection of BHK-21 cells with SVher.12 RNA or SVher.15 RNA did not result in any significant killing of BHK-21 cells compared to mock-transfected or SV replicon-transfected BHK-21 cell cultures (Fig. 3A). Transfection of SK-BR3 cells with SVher.12 RNA resulted in enhanced killing of SK-BR3 cells compared to transfection with parent SV RNA, 26 ± 1.0 % dead cells versus 20 ± 0.7 % dead cells, respectively (Fig. 3B). These data demonstrate that we successfully modified the tropism of SV, such that transfection with the heregulin-containing viral RNAs induced death in the SK-BR3 human breast cancer cell line, but not in the BHK-21 cell line.

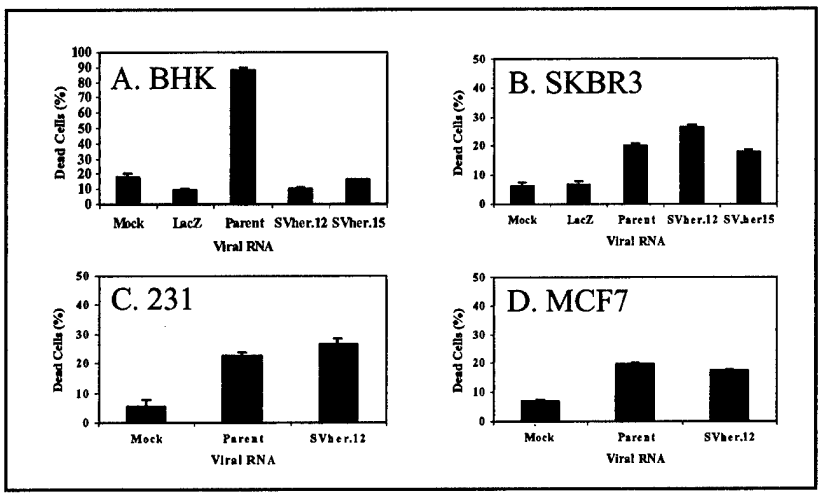

Figure 3. Quantification of cell death in transfected mammalian cell lines. Forty-eight hours after transfection, the cells were harvested and stained with Trypan blue. The number of dead (blue) cells was counted and indicated as a percentage of all cells in the sample on the y-axis. The y-error bars represent the standard deviations of the samples. A. BHK-21 cells. B. SK-BR3 cells. C. MDA-MB-231 cells. D. MCF-7 cells.

Other breast cancer cell lines, which express different levels of the erbB receptors, were also killed by the heregulin-containing viral RNAs. These included MDA-MB-231 cells, which, relative to the SK-BR3 cells, express low levels of erbB-2 and erbB-3 and MCF-7 cells which express low levels of erbB-2, but high levels of erbB-3 (2). SK-BR3 cells express very high levels of erbB-2 and levels of erbB-3 that are between those expressed in MDA-MB-231 and MCF-7 cells. The degree of cell death 48 hours after transfection of MDA-MB-231 cells with parent virus RNA was 23 ± 1.0 % (Fig. 3C). After transfection of MD-MB-231 cells with SVher.12 RNA the degree of cell death was 27 ± 1.8 % (Fig. 3C). The degree of cell death after transfection of MCF-7 cells with parent virus RNA was 20 ± 0.2 % and 17 ± 0.6 % after transfection with SVher.12 RNA (Fig. 3D). These results suggest that the ability of the heregulin-containing viral RNAs to kill breast cancer cells is not related to the level of expression of the erbB receptors on different breast cancer cell lines.

The E2-heregulin-containing viruses kill cancer cells by apoptosis: We investigated the mechanism of killing of breast cancer cells by the heregulin-containing SV RNAs. We noted that SK-BR3 cells transfected with SVher.12 or SVher.15 RNA exhibited extensive membrane blebbing, a characteristic of apoptosis (Fig. 2G). Given this feature and the fact that SV infection is known to kill cells via apoptosis (1), the transfected SK-BR3 cell cultures were assayed for DNA fragmentation, an hallmark of apoptosis, using the TUNEL reaction (Fig. 4). Dying cells in the supernatant fluids were harvested and deposited on slides using a cytospin. The SK-BR3 cultures transfected with SVher.12 and SVher.15 RNAs revealed numerous apoptotic cells compared to an occasional apoptotic cell in mock-transfected cultures (Fig. 4A, B, and C). SK-BR3 cells transfected with parent virus RNA were also apoptotic (Fig. 4D). To prove that the apoptotic cells were the cells that were transfected with the heregulin-containing SV RNAs, SK-BR3 cells were transfected with an heregulin-containing SV vector expressing the enhanced green fluorescent protein (EGFP), SVher.12EGFP. These results demonstrated that the cells that expressed EGFP were the cells that were dying an apoptotic death (Fig. 4E and F).

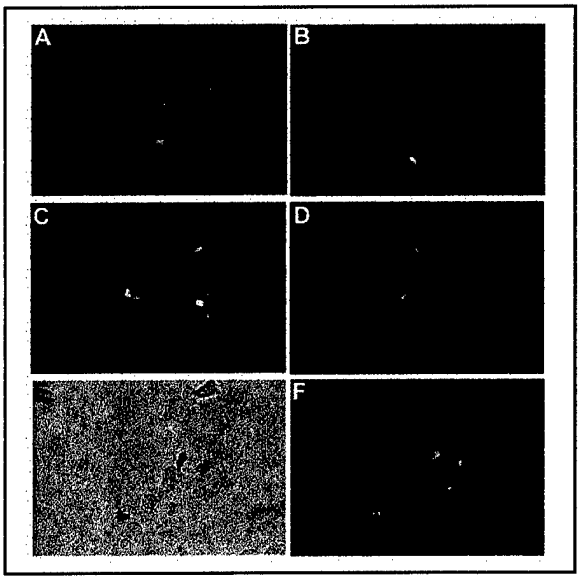

Figure 4. Detection of apoptosis. Forty-eight hours after transfection of SK-BR3 cells with A. SVher.15 RNA, B. yeast RNA, C. SVher.12 RNA or D. parent virus RNA, dying cells in supernatant fluids were harvested and deposited on slides using a cytospin. Apoptosis-induced nuclear DNA fragmentation was detected via TUNEL and fluorescence microscopy. The bright green and greenish-yellow appearing cells are positive for the TUNEL reaction. E. and F.: SK-BR3 cells were also transfected with an heregulin-containing SV vector expressing EGFP, dsSVher.12EGFP. E. Phase contrast microscopy of transfected cells, some of which exhibit membrane blebbling. F. Fluorescent microscopy of transfected cells.

These studies demonstrate that the tropism of SV was modified by replacing regions of the putative-receptor binding domain of the E2 glycoprotein with the EGF-like domain of heregulin. The SV transcripts expressing the heregulin-containing E2 glycoprotein were able to induce cell death of human breast cancer cells but not of BHK-21 cells, which are highly permissive to the cytolytic effects of SV.

Analysis of SV structural protein synthesis after transfection of SVher.12 and SVher.15 RNAs: To determine whether the presence of the EGF-like domain of heregulin in E2 affected the synthesis and processing of the E1 and E2 glycoproteins, we performed both Western blot and immunoprecipitation assays. BHK-21 cells were transfected with SV genomic RNA derived from the cDNA clones, SVher.12 or SVher.15. Cell lysates derived from the transfected cell cultures were immunoblotted with an anti-α-heregulin antibody that recognizes an epitope present in the EGF-like domain of α-heregulin. Synthesis of the chimeric heregulin-E2 glycoprotein was detected at the expected molecular weight in BHK-21 cells transfected with SVher.12 and SVher.15 RNAs (Fig.5A, lanes 12 and 15, respectively; arrow points to chimeric E2). The size of the heregulin-containing E2 approximated the size of wild-type E2, as expected [200 basepairs (bp) of SV E2 sequence were replaced with 200 bp of the heregulin sequence]. In contrast, no heregulin-containing E2 was detected in mock-transfected (Fig. 5A, lane M) or parent virus RNA-transfected BHK-21 cells (Fig. 5A, lane P). As a positive control for the antibody, the anti-α-heregulin antibody detected endogenous heregulin in MDA-MB-231 breast cancer cells (Fig. 5A, lane C, arrowhead; band is very faint).

To determine whether the presence of the EGF-like domain of heregulin in E2 affected the processing of the E1 and E2 glycoproteins, we performed an immunoprecipitation assay using radiolabelled cell lysates derived from BHK-21 cells transfected with SVher.12 and SVher.15 RNAs (Fig. 5B). Immunoprecipitation of these cell lysates with anti-SV rabbit serum and with two monoclonal antibodies (mAb), #2 and # 136, that recognize different epitopes of E2, revealed that the heregulin-containing precursor of E2, PE2, was processed to the mature chimeric E2 glycoprotein (Fig.5B). The chimeric PE2 and mature E2 were readily detected after immunoprecipitation with anti-SV rabbit serum and with anti-E2 mAb #2. Unlike mAb #2, mAb # 136 co-immunoprecipitates E2 with E1 (Fig. 5B, lower panel). In SVher.12 and SVher.15-transfected cultures, mAb #136 predominantly immunoprecipitated the heregulin-containing E2. E1 was barely detectable. These data suggest that the usual heterodimeric interaction between

the E1 and E2 glycoproteins was disrupted because of the presence of the EGF-like domain of heregulin in E2. However, despite this alteration in the heterodimerization of E1 and E2, the E2 precursor was processed into the mature chimeric E2 glycoprotein. The amount of E1 and chimeric E2 glycoproteins synthesized in the cell cultures transfected with the heregulin-containing viral RNAs was significantly less compared to the amount of glycoproteins synthesized in cell cultures transfected with the same amount of parent SV RNA (Fig. 5B). These results indicate that the replication of the heregulin-containing SV clone RNAs was attenuated because of the substitution of regions of the putative E2 receptor-binding domain with the EGF-like domain of heregulin.

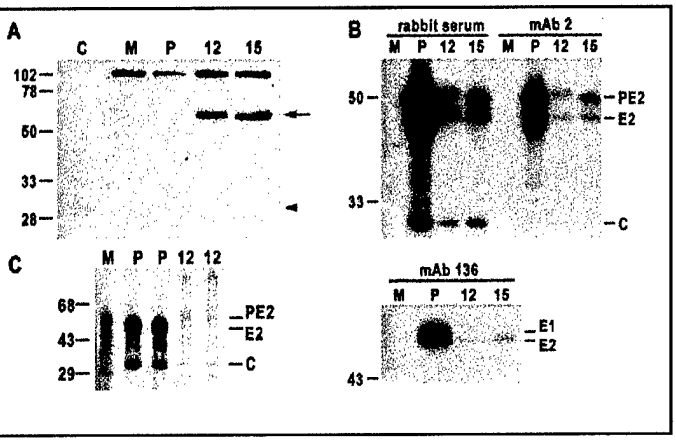

Figure 5. A. Immunoblot of transfected BHK cells using anti-heregulin antibody. Lane M: mock-transfected, Lane P: parent virus RNA-transfected, Lane 12: SVher.12 RNA-transfected, Lane 15: SVher.15 RNA-transfected, Lane C: non-transfected MDA-MB-231 cells. Arrow denotes heregulin-containing E2 glycoprotein. Arrowhead denotes mature form of heregulin that contains the EGF-like domain. PE2, E2, and E1 glycoproteins. C =capsid protein. **B.** Immunoprecipitation of radiolabelled cell lysates with anti-SV rabbit serum or with anti-E2 monoclonal antibodies, mAb 2 and mAb 136. Lanes as noted above. **C.** Pelleted radiolabelled heregulin-containing virions. M, mock-transfected supernatant. P, parent virus RNA-transfected supernatant. 12, SVher.12-transfected supernatant.

To determine whether the heregulin-containing E2 glycoprotein was transported to the cell membrane and expressed on the surface of the cell, an immunofluorescence assay was performed using the anti-SV rabbit serum and the anti-heregulin antibody. Flourescent staining with these antibodies was detected in the cytoplasm (data not shown) and on the surface of BHK-21 and SK-BR3 cells that had been transfected with SVher.12 and SVher.15 RNAs (figure 6). This assay confirmed that the structural proteins, including the heregulin-containing E2 glycoprotein, are synthesized in transfected SK-BR3 and BHK-21 cells and that the heregulin-containing E2 is expressed on the surface of these cells. These results suggest that the modified E2 glycoprotein traffics through the secretory pathway to the surface of the cell where it is inserted into the plasma membrane. Since SV derives its envelope from the plasma membrane of the cell, the heregulin-containing E2 glycoprotein would be expected to be incorporated into the envelope of the virion.

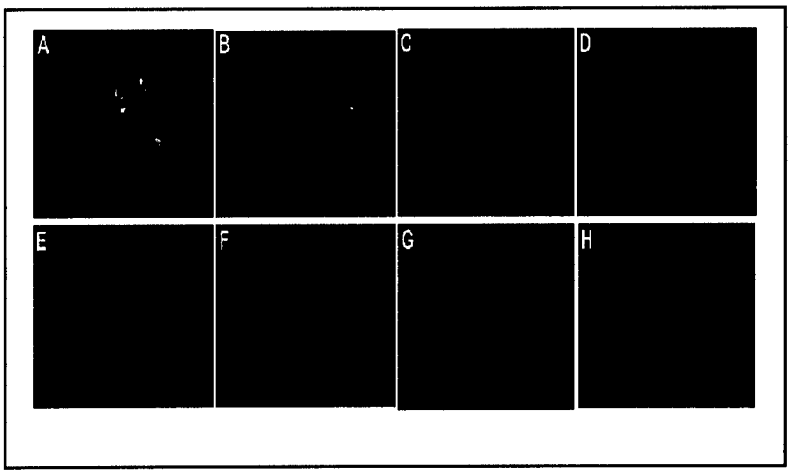

Figure 6. Immunofluorescence assay of BHK-21 and SK-BR3 cells. BHK-21 (A-D) and SK-BR3 (E-H) cells were transfected with SVher.15 RNA (A, C, E, G) or yeast RNA (B, D, F, H), fixed with 4 % paraformaldehyde, and then stained with anti-SV rabbit serum (A, B, E, F) or with anti-heregulin antibody (C, D, G, H). Similar results were obtained after transfection with SVher.12 RNA, however, the immunofluorescence staining was less intense.

We attempted to more definitively identify whether the E1 and chimeric E2 glycoproteins were expressed on the surface of the virion. BHK-21 cells were electroporated with either SVher.12 or SVher.15 RNA. Transfection efficiencies of up to 90% were achieved with electroporation. At 20 hours after electroporation, the cells were metabolically labeled with Tran^{35}Slabel for 3 hours. The supernatant fluids were harvested and clarified of cellular debris. Virions in the supernatant fluids were pelleted at 26,000 rpm for 2 hours at 4 ° C in a Beckman ultracentrifuge and analyzed on a polyacrylamide-SDS gel. Autoradiography was performed. These experiments revealed that a very small amount of virus was produced, suggesting that the heregulin-sequence most likely altered virus particle assembly and release (figure 5C). The pattern of proteins detected in the SVher.12 virions mimicked the pattern seen in cell lysates immunoprecipitated with anti-SV rabbit serum and with monoclonal antibody #2. The heregulin-containing PE2 was incorporated into the SVher.12 virions along with the mature heregulin-E2, suggesting that the precursor was not completely processed into the mature protein. A very small amount of the E1 glycoprotein was detected. The post-transfection supernatant fluids were used to infect BHK and SKBR3 cells and no cytopathic effect was observed. Titering the supernatant fluids on BHK cells resulted in no plaque formation.

These experiments with the heregulin-containing SV revealed that removing 200 bp of the SV sequence and replacing it with 200 bp of a heterologous sequence impaired the assembly and release of recombinant SV. However, the tropism of the virus was altered by replacement of the receptor-binding domain with the heterologous sequence. Hence, we pursued the same approach in a different model system using a smaller ligand.

B. Arap et al. demonstrated that bacteriophage expressing the 13 amino acid peptide, CNGRCVSGCAGRC, containing the NGR (Asn-Gly-Arg) motif home specifically to breast tumors in nude mice (3). This same group of investigators has discovered that this NGR-containing peptide binds to the CD13 (or aminopeptidase N) receptor expressed on tumor-associated endothelial cells (4). CD13 plays an important role in angiogenesis (4). These discoveries have provided us with another model system in which to develop and test the targeted SV vectors.

We replaced SV amino acids E3 58 to E2 6 (dsNGRE3/58-E2/6-EGFP) and also E2 61 to E2 73 (dsTE-NGR) with this NGR-containing peptide. NGR-containing SV vector RNAs that express EGFP were transfected into BHK-21 cells. Using a fluorescent microscope, inspection of monolayers after transfection with dsNGRE3/58-E2/6-EGFP, revealed single cells expressing the EGFP and lack of spread of the vectors in BHK-21 cells over time (data not shown).

An indirect immunofluorescence assay revealed that TE-NGR is able to replicate and spread in BHK cells and SLK cells, a human Kaposi's sarcoma cell line that expresses high levels of the CD13 receptor (figure 7). These data indicated that, despite the presence of the 13 amino acid peptide in the E2 glycoprotein of TE-NGR, the virus was able to assemble, bud, and infect BHK and SLK cells. SLK cells were infected with TE-NGR or the parent virus at a multiplicity of infection (MOI) of 5 PFU/cell to determine the ability of TE-NGR to kill SLK cells compared to parent virus (figure 8). This experiment revealed that the ability of each virus to kill SLK cells was quite low. However, TE-NGR did exhibit a tendency to kill SLK cells more efficiently than the parent virus. The experiment was performed at higher MOIs (10, 20) that did not reveal a significantly enhanced ability of TENGR to infect SLK cells. We attempted to obtain breast cancer cell lines overexpressing the CD13 receptor, however, we learned that the expression of the CD13 receptor is not stable in these cell lines and they need to be repeatedly sorted using a flow cytometer (R. Pasqualini, personal communication). This procedure was not within the capability of this project. Since the NGR motif preferentially binds to the "angiogenic" form of the receptor (R. Pasqualini, personal communication), the true ability of TE-NGR and other NGR-containing viruses to infect NGR-expressing cells via a ligand–receptor interaction will be need to be assayed in an in vivo tumor model.

These data with the TE-NGR virus revealed that replacement of smaller regions of the E2 receptor-binding domains of the SV E2 glycoprotein permits normal virus assembly and release. This strategy will enable the production of high titer recombinant SV vector stocks.

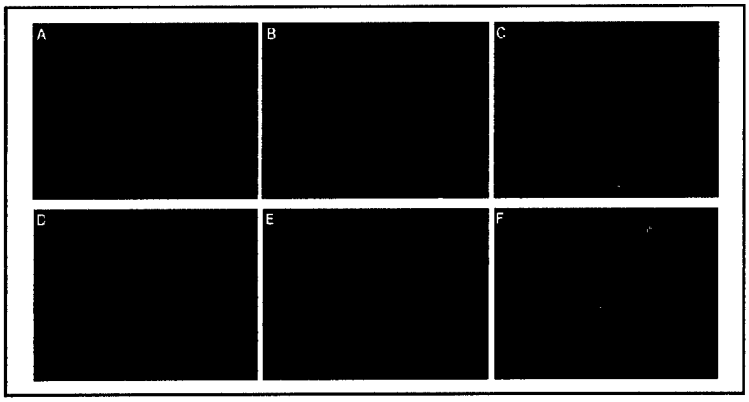

Figure 7. BHK (A, B, C) and SLK (D, E, F) cells were infected with the parent virus (B, E) or with TE-NGR (C, F) at an MOI of 5 PFU/cell. The cells in panels A and D were mock-infected. After fixing with paraformaldehyde, the cells were stained with anti-SV rabbit serum and with a secondary Ab conjugated to FITC and observed with a fluorescent microscope. Numerous BHK and SLK cells stained positively (green) for the SV structural proteins when infected with the parent virus or with TE-NGR.

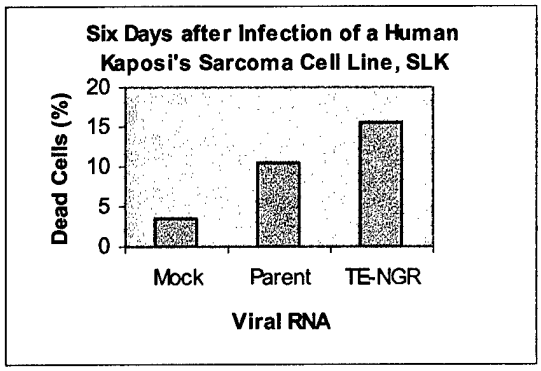

Figure 8. Infection of SLK cells with parent virus and TE-NGR virus at a MOI of 5 PFU/cell. Six days after infection, the cells were harvested, stained with propidium iodide and the percentage of dead cells was determined using a FACScan flow cytometer.

C. Over the past year, repeated difficulties were encountered with synthesizing SV RNA in sufficient quantities to perform additional experiments. We were forced to devise an alternative method for producing SV. SV can be produced by transfecting cells with SV genomic cDNA expressed from a pol II promoter. A strong pol II promoter, the CMV promoter, was cloned

upstream of the 5'untranslated region (UTR) of the SV plasmid dsTE-EGFP (figure 9). The CMV promoter was cloned into dsTE-EGFP so that transciption initiates as close as possible to the authentic 5' end of the 5'UTR. A fragment that contains an intron and poly A signal was inserted downstream of the poly A stretches of dsTE-EGFP to increase the transport efficiency of the viral RNA genome from the nucleus to the cytoplasm.

PCMV-dsTE-GFP-InA DNA was electroporated into BHK-21 cells. Forty-eight hours after transfection, the amount of viral vector in supernatant fluids was determined by plaque titration on BHK cells. Viral titers similar to those achieved after transfection of native SV RNA (10^8 to 10^9 pfu/ml) were obtained. This vector will be used in future studies of the development of targeted SV vectors and will obviate the need to produce genomic viral RNA which is more tedious and less reliable than production of cDNA for virus production. We need to try replacing other sites in the E2 glycoprotein, preferably with smaller ligands, to attempt to produce replication competent targeted virus.

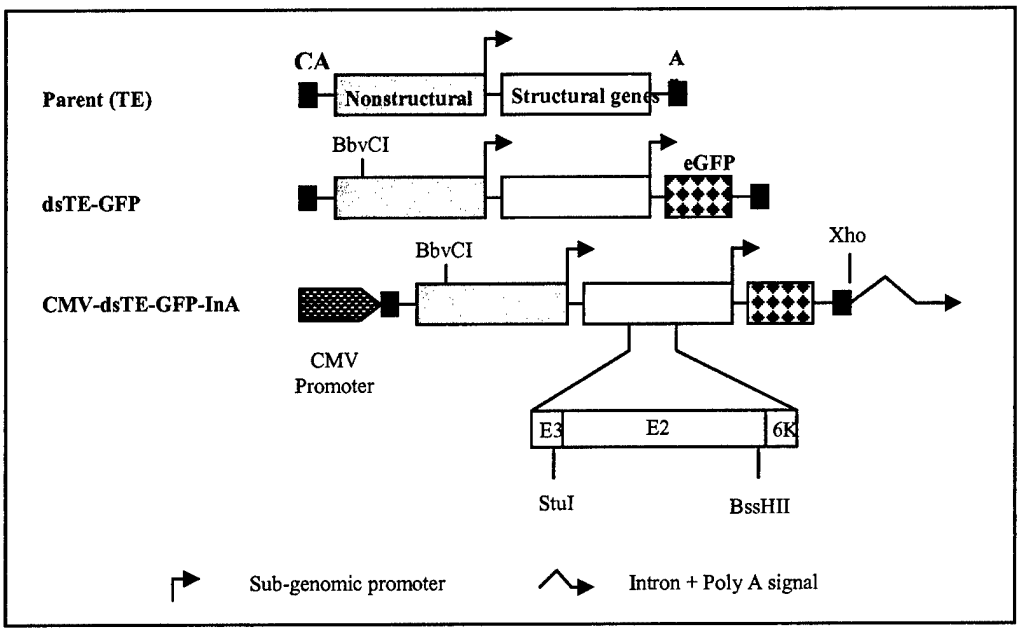

Figure 9. Schematic diagrams of the parental SV genome (TE), the genome containing eGFP expressed from a duplicated sub-genomic promoter (dsTE-GFP), and the cDNA construct for making SV (CMV-dsTE-GFP-InA). CMV-dsTE-GFP-InA contains the CMV IE promoter upstream of the 5' UTR and a fragment containing a synthetic intron and the late SV40 poly A signal at the 3' end of the genome. This diagram is not drawn to scale.

SOW Technical Objective #2: Test targeted viral vectors for specificity of binding and specific infectivity of erbB-2 over-expressing breast cancer cells.

1. After transfection of the heregulin-containing viral RNAs into BHK-21 cells and SKBR- 3 human breast cancer cell lines that express the appropriate receptors, the heregulin-containing RNAs, killed SKBR3 cells and not BHK-21 cells. BHK-21 is a cell line commonly used to prepare stocks of all strains of SV. To determine whether the cytopathic effect and cell death seen in SKBR3 breast cancer cells after transfection with viral RNA was the result of a ligand-receptor interaction, competition assays in the presence of a GST-α-heregulin fusion protein (fig. 10) and an anti-erbB-3 receptor-blocking antibody (fig. 11) were performed. No difference in the amount of cell death was detected in the presence of GST-α-heregulin or of the anti-erbB-3 receptor-blocking antibody. Optimally, these experiments should be performed with viral vector stocks rather than viral vector RNAs. But, as noted, we were not able to produce virus stocks.

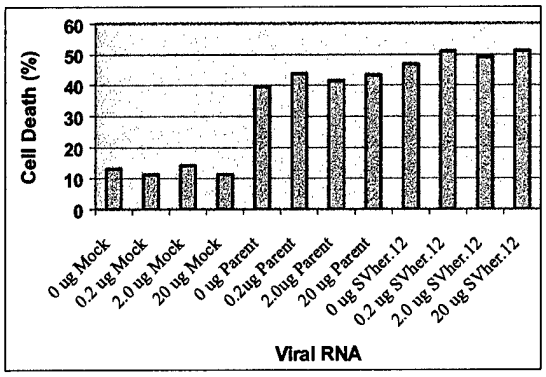

Figure 10. Cell death in the presence or absence of GST-α-heregulin 48 hours after transfection of SKBR3 cells. Zero, 0.2, 2.0 or 20 μg of heregulin was added to the cultures 4 hours after transfection. The amount of cell death in the cultures was quantified via a propidium iodide (PI) assay using a flow cytometer.

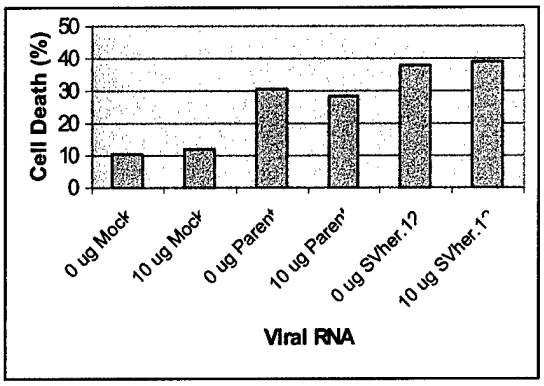

Figure 11. Cell death in the presence or absence of an anti-erbB-3 receptor-blocking antibody (Labvision) 48 hours after transfection of viral RNA. The amount of cell death in the cultures was quantified via a PI assay and flow cytometry.

SOW Technical Objective #3: Test the targeted SV vectors in a mouse model of breast cancer.

1. We attempted to establish tumors in Swiss athymic nude mice after injection of SKBR3 cells into the mammary fat pads or into the rumps of nude mice. We were not able to establish tumors in this manner, despite reports of the contrary in the literature.
2. We successfully established tumors using MDA-MB-231 and MDA-MB-231-HER-2-overexpressing cell lines.
3. We were not able to inject the tumors with the targeted vectors because we were not able to produce a sufficient amount of viral vector or viral vector RNA for injection into the tumors. Virus production was significantly decreased after modification of several E2 domains with the targeting heregulin sequence.
4. In future studies, we can attempt to establish Kaposi's sarcoma-like tumors in nude mice with the SLK cell line and determine whether the TE-NGR virus is able to destroy these tumors.
5. In the future, we will investigate the ability to produce tumors with other breast cancer cell lines.

III. Key Research Accomplishments.

- We have learned that replacement of the putative-receptor binding domains of SV with larger ligands results in significant impairment of SV production and release.
- We have demonstrated preferential killing of SKBR3 human breast cancer cells versus BHK-21 cells after transfection with viral vector RNAs that express the EGF-like domain of heregulin.
- We have demonstrated that it is possible to modify the receptor-binding domain of the E2-glycoprotein with a small peptide ligand (NGR-containing peptide) and generate propagation-competent Sindbis virus (TE-NGR).
- The SV vector clone expressing the NGR-containing 13 amino acid peptide (TENGR) did not preferentially infect SLK cells that express the target CD13 receptor.
- We established breast cancer tumors using MDA-MD-231 cells in a nude mouse model.

IV. Reportable Outcomes.

1. Abstract. Lesia K. Dropulic, J. Marie Hardwick, Jennifer L. Nargi. Preferential Killing of Breast Cancer Cells by a Heregulin-containing Sindbis Virus Vector. Era of Hope Department of Defense Breast Cancer Meeting, Atlanta, Georgia, June 8 – 11, 2000.
2. Abstract, DOD Era of Hope Meeting, Orlando, Florida, Sept. 2002.
   Lesia K. Dropulic, J. Marie Hardwick, Boro Dropulic, Holly L. Hammond.
   Incorporation of the EGF-like Domain of Heregulin into the E2 Glycoprotein Modifies the Tropism of Sindbis Virus.
3. Pilot Project Grant awarded by the Breast SPORE grant at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins.
   Cancer-specific replication-competent Sindbis virus.
4. The data obtained under this award have been used to apply for an R01 research grant from the National Institutes of Health.
5. The technician partially funded by this award received the necessary training and experience to obtain a higher level job in a lentiviral vector laboratory that also works on breast cancer.
6. One of the goals of the Career Development Award was for the principal investigator to gain experience in vector design, molecular cloning, and experimentation with animal models. This has been accomplished.

V. Conclusions.

In our attempts to modify the SV E2 glycoprotein with ligands to try to target SV to specific receptors on breast cancer cells, we have learned that this strategy can result in a significant decrease in virus production. This decrease in virus production has also been noted by other investigators working on a similar strategy using other viral vectors such as adenovirus. Working with the cDNA genome of SV for virus production, the issues encountered with RNA production and stability can be avoided. As new ligands and their specific receptors on breast cancer cells or on tumor associated vasculature are discovered, these can be applied to and tested in the SV vector system. Smaller ligands will be sought as they are less likely to disrupt the processing of the SV glycoproteins. A SV vector specifically targeted to breast cancer cells or the tumor associated vasculature can potentially become a very useful therapy for breast cancer.

VI. References.

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3. Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 1998; 279: 377-380.
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VII. Personnel receiving pay from the research effort:

Holly L. Hammond

Lesia K. Dropulic