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13. ABSTRACT *(Maximum 200 Words)*

The future progress of cancer gene therapy relies on the development of efficient and safe vectors that can deliver therapeutic genes specifically to tumor cells. Using a replication-competent viral vector targeted to tumor cells may be the most efficient way of specifically killing a large number of malignant cells. We intend to develop Sindbis virus (SV), an alphavirus, into a targeted replication-competent viral vector for breast cancer gene therapy. Since SV kills by apoptosis, specific destruction of tumor cells will occur if the virus is targeted to breast cancer cells. To target SV to breast cancer cells, the putative receptor-binding domain(s) of the SV E2 glycoprotein was replaced with the ligand, heregulin, or with an NGR-containing peptide motif that binds to the CD13 receptor expressed on tumor associated endothelial cells. We have demonstrated that a heregulin-containing SV preferentially kills a breast cancer cell line that expresses the appropriate epidermal growth factor receptors. Analysis of protein expression via metabolic labeling, protein immunoblotting or indirect immunofluorescence microscopy revealed synthesis of the viral structural proteins and the heregulin-containing E2 glycoprotein. SV vectors expressing the green fluorescent protein are being used to study the replication and spread of heregulin- and NGR-containing SV vectors in various cell lines.

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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. Furthermore, use of a replication-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 will be used as a model target. As an additional approach, an NGR-containing peptide motif that binds to the CD13 receptor on tumor vasculature will be used to modify the receptor-binding domain of the E2 glycoprotein. 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 tumor cells.

II. Accomplishments.

One of the goals of the Career Development Award is for the principal investigator to gain experience in vector design, molecular cloning, and experimentation with animal models. Over the past year, the principal investigator has designed and cloned several viral vectors. We have begun analyzing these vectors in vitro.

Task 1 of the Statement of Work. Construct targeted Sindbis virus vectors.

A. Cloning of ligands into the Sindbis virus E2 glycoprotein.

Sindbis viruses with the epidermal growth factor domain of heregulin replacing portions of the putative receptor-binding domain of the viral E2 glycoprotein have been constructed. In clone 12, the E2 amino acid residues 60 to 114 have been replaced with the heregulin domain. In clone 15, amino acid residues 114 to 175 have been replaced. We have discovered that when 200 basepairs of the E2 glycoprotein sequence are replaced with 200 basepairs of the heregulin sequence, the replication of the heregulin-containing Sindbis viruses is significantly attenuated. Because of this information, we decided not to clone the approximately 700bp long anti-erbB2 single chain antibody sequence into Sindbis virus (SV). This type of substitution of the E2 glycoprotein would most likely be lethal to the replication of this virus. Therefore, we identified another ligand that could potentially target the Sindbis virus to breast cancer tumors. This ligand, an NGR-containing peptide that is 13 amino acids in length, has been demonstrated to bind to the CD13 receptor expressed on tumor associated endothelial cells (Arap W. et al). Dr. Ruoslahti and colleagues have demonstrated that bacteriophage expressing this peptide home specifically to breast cancer tumors in nude mice. We have replaced Sindbis virus amino acids E358 to E26 and also amino acids E2 61 to E2 73 with this NGR-containing peptide. The heregulin domain and the NGR-containing peptide also have been cloned into SV vectors expressing EGFP (green fluorescent protein). The correct construction of the modified viruses and viral vectors was confirmed by restriction enzyme analysis and DNA sequencing.

B. Synthesis of viral RNA using the cDNA of the heregulin-E2-containing and NGR-E2-containing SV clones followed by transfection into BHK cells.

After production of linearized template from the above described plasmids, viral RNA was synthesized in vitro using an SP6 DNA-dependent RNA polymerase. The viral RNA was transfected into BHK cells (baby hamster kidney cells, the standard cell line used for SV production) via electroporation or using Lipofectin (Life Technologies, Inc.). A cytopathic effect was not observed after transfection of the heregulin-containing viral RNA, indicating that the tropism of the virus was successfully altered or that the replication of the virus was significantly attenuated. Given the inability of the heregulin-containing Sindbis viruses to replicate in BHK cells, it was not possible to generate high titer purified virus stocks for binding assays. Therefore, we proceeded to perform experiments with the viral RNA, given that it is positive-stranded and hence, infectious (task 2).

To observe the spread of the viral vectors in cell monolayers, we transfected the NGR-containing SV vectors that express EGFP into BHK cells. Transfection of BHK cells with the EGFP-expressing NGR-containing SV RNAS revealed single cells expressing the EGFP and lack of spread of the vectors in BHK cells over time (see appendix B, figures 1 to 4). We are currently performing these experiments in the CD-13-expressing Kaposi's sarcoma cell line. Similar experiments will be performed with the heregulin-containing SV-EGFP vectors in BHK and breast cancer cell lines.

We have cloned an NGR-containing SV that is not significantly attenuated and is able to infect BHK cells and also the human Kaposi's sarcoma cell line (see appendix B, figures 5 & 6). Experiments are underway to determine if the NGR-containing SV differentially infects and kills BHK cells versus the Kaposi's sarcoma cell line.

C. Evaluation of viral protein synthesis.

To determine whether the recombinant E2 glycoprotein was synthesized, BHK cells were transfected with heregulin-containing viral RNA. The transfected cultures were pulse-labeled with TranS^{35}label for 3 hours and cell lysates were prepared. The lysates were immunoprecipitated with rabbit serum against SV or with mAbs against the E2 glycoprotein (figure 7). Immunoprecipitation with these antibodies revealed that the precursor of E2 was processed to the mature E2 glycoprotein. These data also suggested that the interaction between the E1 and heregulin-containing E2 glycoproteins, which in their native states form a

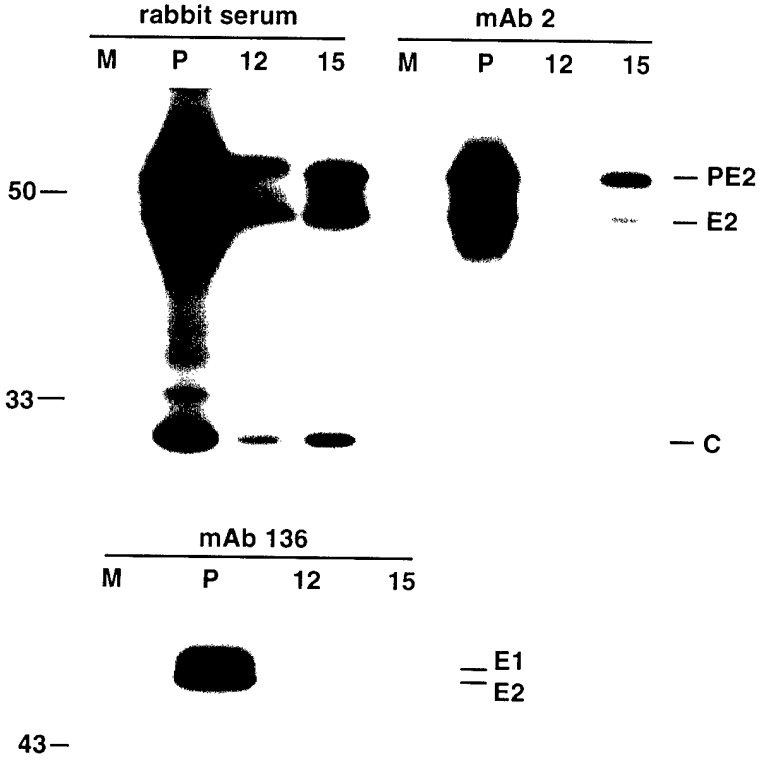

Figure 7. Immunoprecipitation of radiolabeled cell lysates derived from BHK cells transfected with RNAs of the parent virus, P, clone 12 and clone 15. The same number of counts per minute were immunoprecipitated. Rabbit serum = anit-SV rabbit serum; mAb 2 and mAb 136 are anti-E2 antibodies.

heterodimer in the endoplasmic reticulum, was altered given the lack of immunoprecipitation of this heterodimer with mAb 136. A protein immunoblot of transfected BHK cell lysates revealed

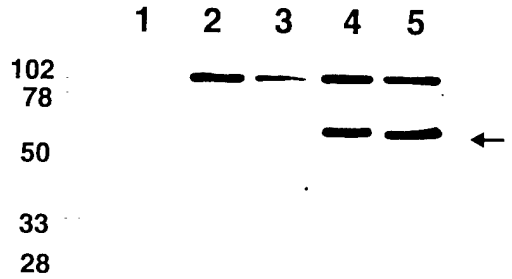

Figure 8. Western blot of transfected cell lysates using an anti-heregulin antibody. Lanes indicated on the top of the gel correspond to cell lysates derived from the following cells: 1. MDA-MB-231 cells (express wild-type heregulin), 2. Untransfected BHK cells, 3. BHK cells transfected with parent virus RNA, 4. BHK cells transfected with clone 12 RNA, 5. BHK cells transfected with clone 15 RNA. The arrow points to the heregulin-containing E2 glycoprotein.

the presence of the processed heregulin-containing E2 glycoprotein (figure 8). Viral structural protein expression and E2-heregulin expression were also assayed via indirect immunofluorescence mocroscopy (see appendix B, figures 9 & 10). We are attempting to demonstrate the presence of the E1 and E2 glycoproteins and the heregulin EGF domain on the surface of the Sindbis virion. The latter task is difficult because the conformation of the EGF domain may be altered such that this domain is no longer recognized by the available anti-heregulin antibodies.

Task 2 of the Statement of Work. Test the targeted SV vectors for specificity of binding to and specific infectivity of erbB-2-over-expressing breast cancer cell lines.

To determine the specificity of infection, the heregulin-containing viral RNAs were transfected into BHK cells or into cells that over-express the appropriate receptors (SKBR3 human breast cancer cell line). Forty eight hours after transfection of BHK and SKBR3 cells, the percentage of dead cells in the cultures was determined by the trypan blue exclusion assay (figures 11 &12). The parent virus replicated in both cell lines albeit less efficiently in SKBR3 cells. The heregulin-containing viruses killed SKBR3 cells and not BHK cells and exhibited cytopathic effect only in SKBR3 cells. These data demonstrate that it is possible to destroy the ability of SV to replicate in BHK cells, a cell line which is commonly used to prepare stocks of all strains of SV, but retain the ability to replicate in a breast cancer cell line that expresses the targeted receptors. Similar experiments are underway with the NGR-containing virus clones.

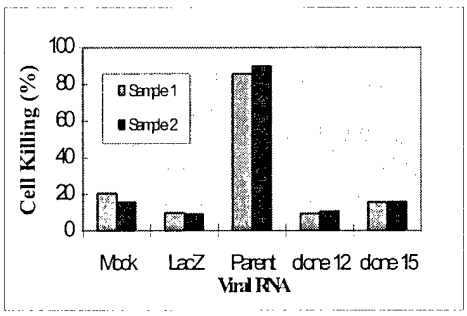

Figure 11. Cell killing 48 hours after transfection of BHK cells. Transfection efficiency was 5% as per β-galactosidase assay. Sample 1 and sample 2 represent duplicate wells.

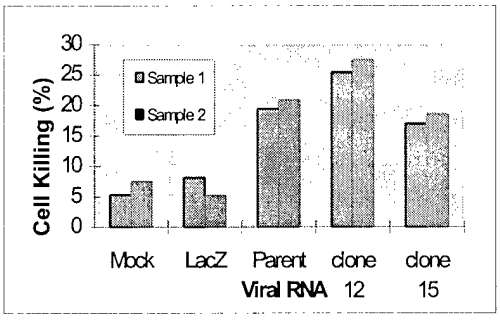

Figure 12. Cell killing 48 hours after transfection of SKBR3 cells. Transfection efficiency was 1% as per β-galactosidase assay. Sample 1 and sample 2 represent duplicate wells.

Appendix A.

I. Key Research Accomplishments.

- In terms of training, the principal investigator is acquiring the necessary skills for vector design and molecular cloning. The principal investigator was able to obtain a technician as a result of this award. She is being trained in molecular cloning and also has become a tissue culture expert and maintains the breast cancer cell lines in the laboratory.
- Constructed heregulin-containing and NGR-containing Sindbis viruses and Sindbis virus vectors.
- Analyzed expression of structural proteins, including modified heregulin-containing E2 glycoprotein.
- Initiated specific infectivity experiments.

II. Reportable Outcomes.

- 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.
- U.S. Patent Application. Use of Targeted Sindbis Virus Vectors to Treat Cancer, Submitted July, 1998.

Conclusions. Despite some of the difficulties encountered with the development of targeted SV vectors, several results indicate that these vectors may become useful therapeutic agents for breast cancer therapy. We have demonstrated preferential killing of breast cancer cells (SKBR3 cell line) by the heregulin-containing vectors. We are learning which sites of the putative E2 receptor-binding domains can be replaced with ligands. We have learned that larger ligands decrease viral replication to a significant extent. Preliminary results with vectors containing smaller ligands, such as the 13 amino acid NGR-containing peptide, indicate that replacement with smaller ligands is more favorable for SV replication. As new ligands and their specific receptors on breast cancer cells or on associated tumor vasculture are discovered, these can be applied to and tested in the SV vector system.

References

Arap W, Pasqualini R, Ruoslahti E. Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model. Science 1998; 279: 377-380.

Appendix B.

Transfection of BHK Cells with an NGR-containing SV-EGFP Vector

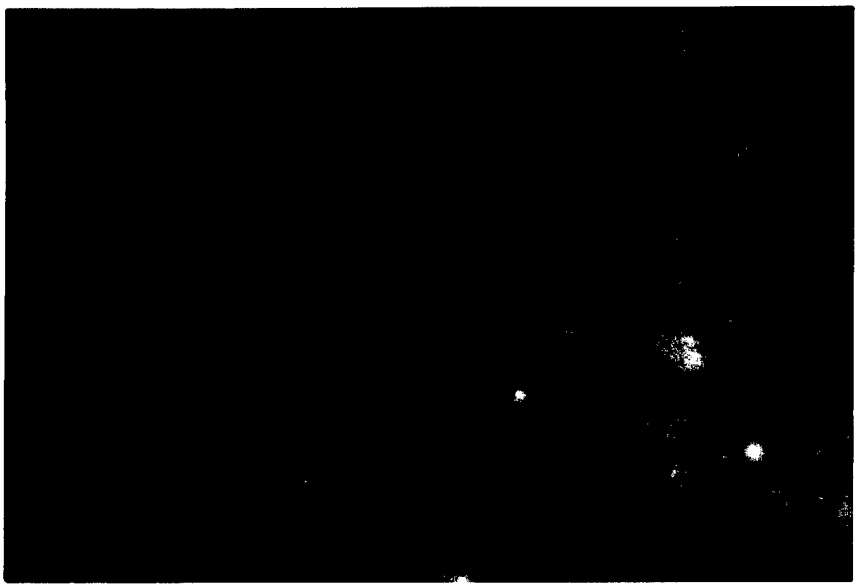

Figure 1. BHK cells 22 hours after mock transfection with lipofectin alone.

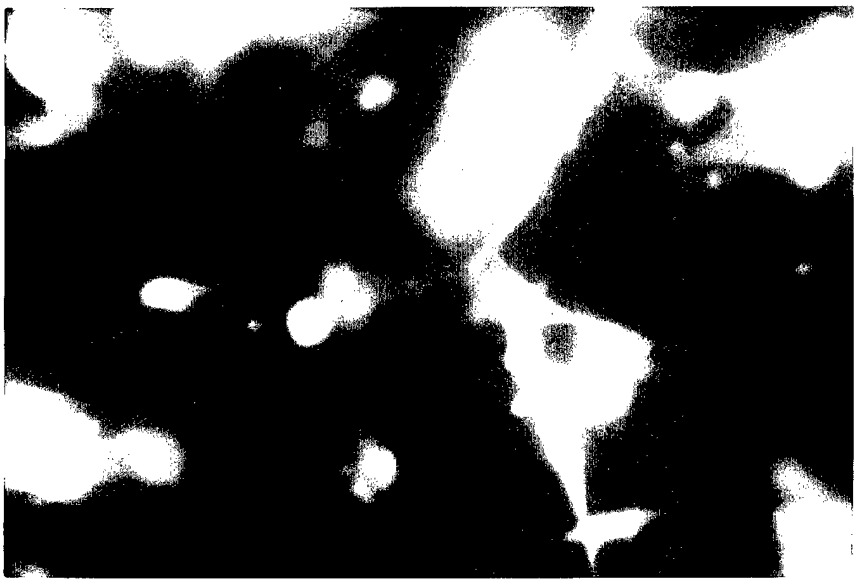

Figure 2. BHK cells 44 hours after transfection with dsTE-EGFP—the parent vector. At earlier timepoints after transfection, patches of cells are fluorescent. With time, all the cells become infected and express EGFP.

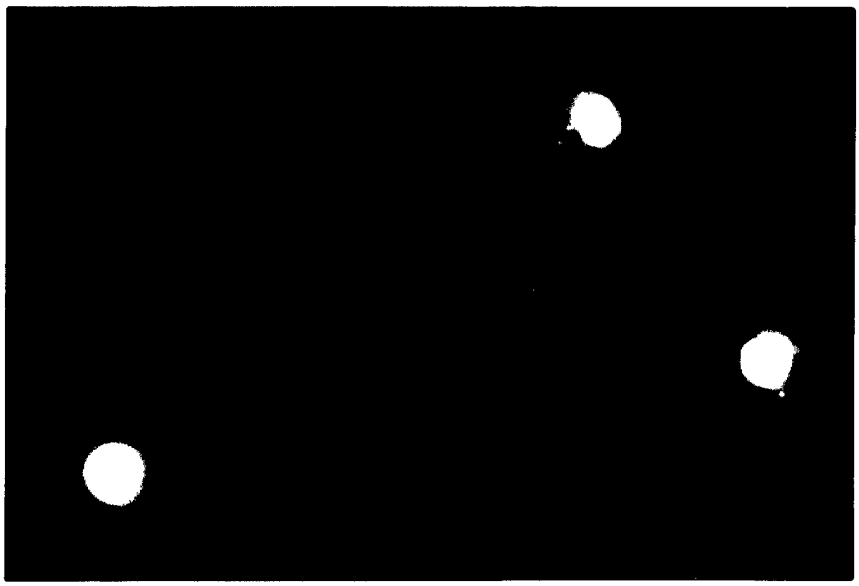

Figure 3. BHK cells 22 hours after transfection with dsNGRE3/58--E2/6--EGFP. Single cells are infected without evidence of spread of the vector.

Figure 4. BHK cells 30 hours after transfection with dsNGRE3/58–E2/6--EGFP. There is evidence of possible viral spread. Since the putative receptor-binding domain has been modified, we would not expect this vector to spread extensively in BHK cells.

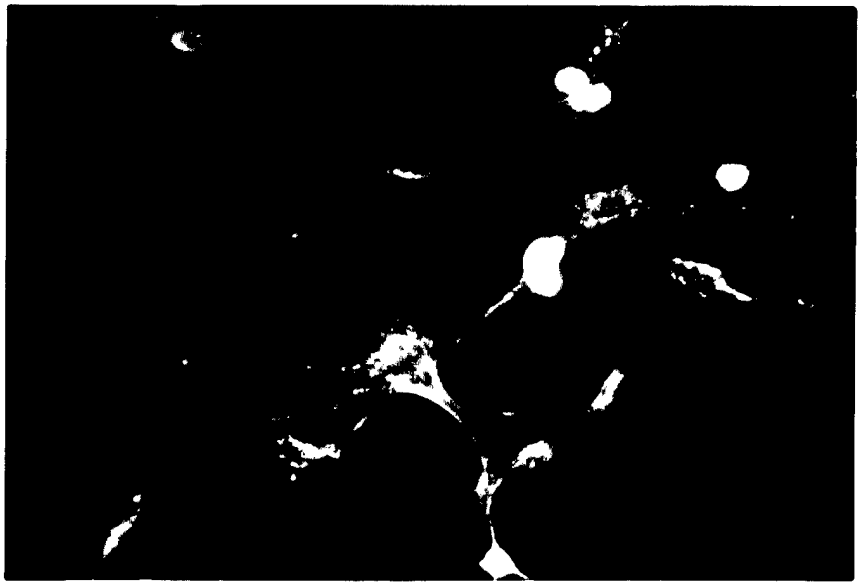

Figure 5. Indirect immunofluorescence microscopy of BHK cells infected with a TE-NGR virus that is not significantly attenuated. The cells were stained with anti-SV rabbit serum.

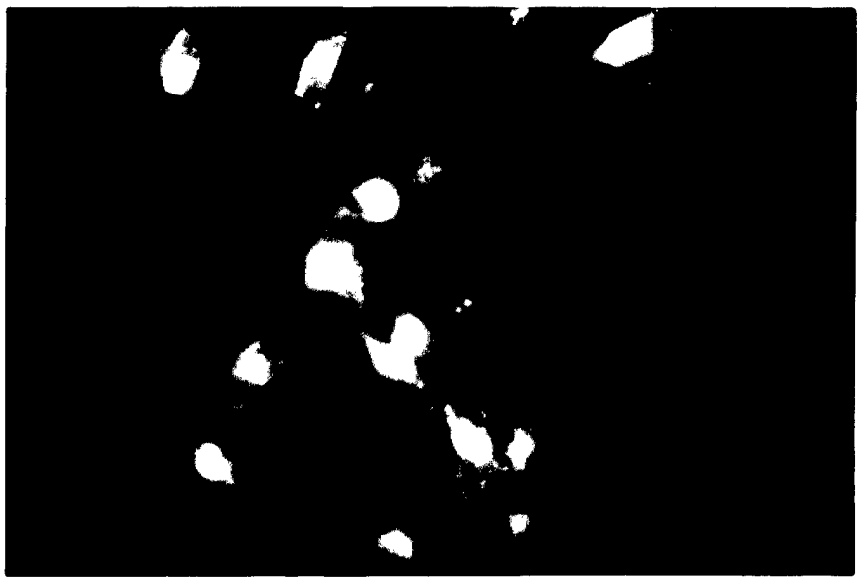

Figure 6. Indirect immunofluorescence microscopy of Kaposi's sarcoma SLK cells infected with the TE-NGR virus. The cells were stained with anti-SV rabbit serum.

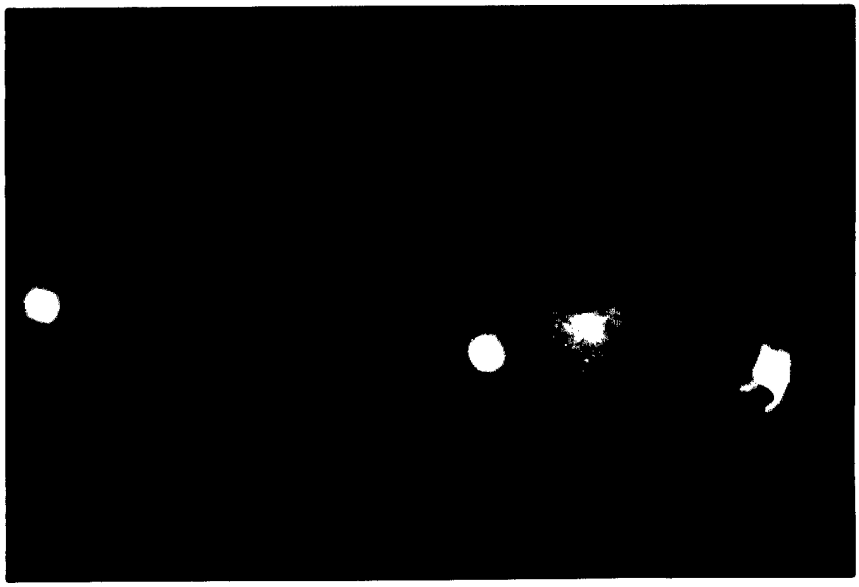

Figure 9. BHK cells transfected with clone 15, heregulin-containing SV RNA, and stained with anti-SV rabbit serum.

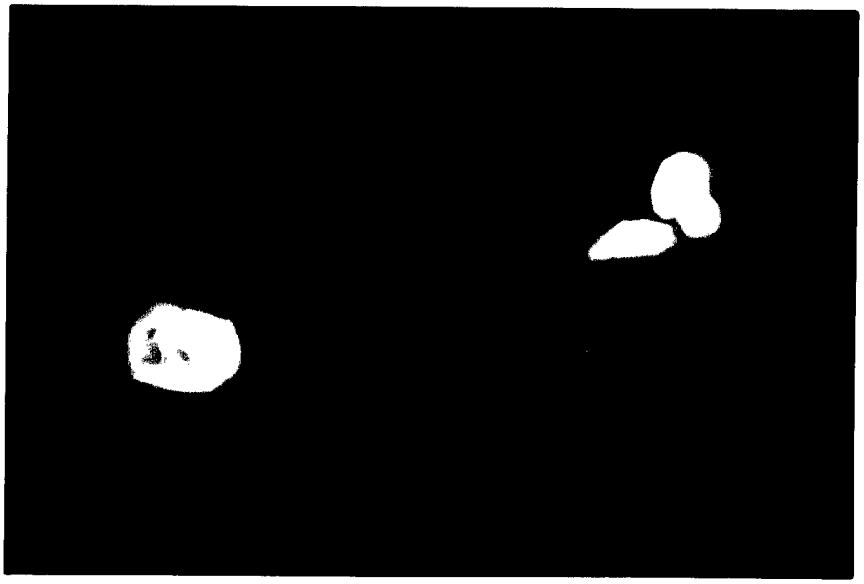

Figure 10. BHK cells transfected with clone 15 heregulin-containing SV RNA and stained with an anti-heregulin antibody.

PREFERENTIAL KILLING OF BREAST CANCER CELLS BY A HEREGULIN-CONTAINING SINDBIS VIRUS VECTOR

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The future progress of cancer gene therapy relies on the development of efficient and safe vectors that can deliver therapeutic genes specifically to tumor cells. Using a replication-competent viral vector targeted to tumor cells may be the most efficient way of specifically killing a large number of malignant cells. We intend to develop Sindbis virus (SV), an alphavirus with a positive sense RNA genome, into a targeted replication-competent viral vector for breast cancer gene therapy. Since SV kills cells by apoptosis, specific destruction of tumor cells will occur if the virus is targeted to breast cancer cells.

We are attempting to target Sindbis virus to breast cancer cells by replacing the putative receptor-binding domain of the E2 glycoprotein with the ligand, heregulin. Heregulin has affinity for breast cancer cells that express the human epidermal growth factor receptors, HER-2, HER-3, and HER-4. SKBR3 breast cancer cells and baby hamster kidney cells, BHK-21, were transfected with genomic SV RNAs containing the heregulin sequence in place of a portion of the putative receptor-binding domain of the E2. The percentage of dead cells in the cultures was determined by the trypan blue exclusion assay 48 hours after transfection. The parental SV was able to replicate in BHK (80% dead cells) and SKBR3 cells (20% dead cells), albeit less efficiently in SKBR3 cells. Transfection of the heregulin-containing viral RNAs caused significant cell death only in SKBR3 cells (25%) and not in BHK cells. Cytopathic effect was evident only in SKBR3 cells.

Preferential killing of the MCF-7 breast cancer cell line was observed with a heregulin-containing SV that was complemented with a defective SV helper RNA. MCF-7 breast cancer cells were infected with parent SV or with a complemented heregulin-containing virus. At 74 hours after infection, a propidium iodide assay using a flow cytometer was performed to determine the percentage of MCF-7 cells that was killed. The heregulin-containing SV killed more MCF-7 cells (42%) compared to parent virus (20%) and to mock-infected cultures (6% dead cells).

These data demonstrate that a recombinant SV can preferentially kill breast cancer cell lines expressing the human epidermal growth factor receptors. These data support the hypothesis that SV can be reengineered into a tumor-specific, replication-competent virus by replacing the receptor-binding domain with a ligand that recognizes a specific receptor on the surface of a tumor cell.

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