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Interleukin-12 Expression Enhances Vesicular Stomatitis Virus Oncolytic Therapy in Murine Squamous Cell Carcinoma

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Objectives: **Replication-competent, vesicular stomatitis virus (VSV) has been demonstrated to be an effective oncolytic agent in a variety of malignant tumors. Cytokine gene transfer has also been used as immunomodulatory therapy for cancer. To test the use of combining these two approaches, an oncolytic VSV vector (rVSV-IL12) was designed to express the murine interleukin 12 (IL12) gene. This cytokine-carrying oncolytic virus was compared with an analogous noncytokine-carrying fusogenic virus (rVSV-F) in the treatment of murine SCC VII squamous cell carcinoma (SCC).** ***Study Design and Setting:*** **The authors performed in vitro testing of recombinant VSV-F and recombinant VSV-IL12 in SCC cell lines. In vivo testing of multiple direct intratumoral injections of rVSV-F or rVSV-IL12 in an orthotopic floor of mouth murine model was performed. Each cell line was tested using rVSV-F or rVSV-IL12 at multiplicity of infection of 0.01. The ability of each virus to replicate was tested by real-time reverse transcriptase–polymerase chain reaction over 48 hours to determine viral copies of RNA. Cell survival was determined by MTT assay over 72 hours. IL12 expression by rVSV-IL12-treated cells was determined by enzyme-linked immunosorbent assay.** ***Results:*** **Both viruses demonstrated similar infection efficiency, viral replication, and cytotoxicity in vitro. In an SCC VII orthotopic floor of mouth model in immunocompetent C3H/HeJ mice, multiple intratumoral injections with each virus caused a significant reduction in tumor volume when compared with saline injections alone. The rVSV-IL12-treated tumors showed a striking reduction in tumor volume when compared with rVSV-F and saline-treated tumors ($P < .005$). This striking reduction in tumor volume translated into a substantial survival benefit in rVSV-IL12-treated animals. No treatment-related toxicity was observed in either group.** ***Conclusion/Significance:*** **rVSV-IL12 is a novel oncolytic vesicular stomatitis virus that effectively expresses IL12 and significantly enhances the treatment of head and neck murine carcinoma. Such combined oncolytic and immunomodulatory strategies hold promise in the treatment of head and neck cancers.** ***Key Words:*** **IL12, VSV, rVSV-IL12, interleukin, oncolytic virus, murine squamous cell carcinoma.**

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INTRODUCTION

The incidence of head and neck cancer in the United States is 6% per year with one-third resulting in death.[1] Despite aggressive initial management of the primary tumor, locoregional recurrence may occur in up to 60%.[2] Salvage surgery is effective in a subset of patients who have resectable recurrent disease, achieving a 5-year overall survival between 16% and 36%.[3] Patients present with inoperable disease have even more dismal survival. These unsatisfactory results highlight the need for new treatments for these patients.

Oncolytic viruses represent a novel class of therapeutic for the treatment of cancers.[4] By taking advantage of the inherent attenuated antiviral response of tumor cells, viruses can be developed into oncolytic agents with innate tumor specificity. Vesicular stomatitis virus (VSV) is a potent oncolytic virus as demonstrated in several preclinical tumor models, including glioma, hepatocellular carcinoma, breast carcinoma, and melanoma.[4] Ebert et al. recently generated a fusogenic VSV with enhanced oncolytic potential for the treatment of hepatocellular carcinoma.[5] In this modification, VSV is capable of inducing syncytia (multinucleated giant cells formed by fusion of tumor cells), which enhances its cytopathic effects.

Another novel antitumor strategy involves the use of viral vectors to deliver genes coding for immunomodulatory agents such as cytokines, chemokines, or costimula-

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tory molecules to sites of tumor growth.[6,7] This strategy seeks to recruit and activate immune cells (B cells, T cells, NK) that have antitumor effects and that may also provide specific lasting immunity.[8] Interleukin 12 (IL12) is a proinflammatory cytokine produced by the innate immune system that acts to initiate cellular responses to pathogens and to direct the subsequent adaptive immune responses mediated by T and B lymphocytes.[9] In addition to its role in infection, IL12 has been shown to possess potent antitumor effects.[10]

The goal of this study is to investigate the in vitro and in vivo efficacy of IL12 gene transfer combined with an oncolytic vesicular stomatitis virus (rVSV-IL12) in the treatment of head and neck squamous cell carcinoma. In addition, this study examines whether such combination therapy (rVSV-IL12) enhances tumor control and survival when compared with an analogous noncytokine-carrying fusogenic virus (rVSV-F) in the same model.

MATERIALS AND METHODS

Generation of Viruses

The recombinant virus was generated by the methods described in Ebert et al.[5] IL12 is a heterodimeric cytokine composed of the p35 and p40 subunits. A plasmid containing the fragment murine p40-internal ribosome entry site-p35 has previously been constructed and used in other adenoviral constructs.[11] This fragment was released and cloned 3′ of the G protein gene in pVSV. Sequencing confirmed the correct nucleotide sequence. Recombinant VSV-IL12 viruses were generated using the reverse genetic technique.[11]

Assessment of In Vitro Viral Infection rVSV-F or rVSV-IL12

For the in vitro studies, two human squamous cell carcinoma cell lines (SCC 09 originated from the head and neck and FaDu originated from the tongue obtained from American Type Culture Collection) and one murine squamous cell carcinoma cell line (SCC VII, gift of Dr. Richard Wong and Dr. Bert W. O'Malley, Jr.) were studied.

To evaluate the in vitro IL12 expression, all cells were plated in tissue culture treated 48-well plates at a density of 1×10^4 cells/200 μL media/well. The next day, the cells were infected at the appropriate multiplicity of infection (MOI; MOI of 0.01 is infection of one virus per 100 cells) of rVSV-IL12 as well as saline-infected cells serving as mock infection control. After 24- and 48-hour incubation, 100 μL of the cell supernatant was removed. Murine IL12 was quantified using the mouse IL12 p70 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions.

For the quantitative evaluation of viral replication, 1×10^6 cells were grown in six-well plates and, after 24 hours, infected with the recombinant virus at a MOI of 0.01 (one virus to 100 cells). Supernatants were harvested at multiple time points (4, 16, 24, 36, and 48 hours postinfection). Each time point was tested in duplicate. Total RNAs from the culture supernatants were prepared; these RNA samples were then analyzed for the presence and concentrations of genomic VSV RNA by real-time reverse transcriptase–polymerase chain reaction assays using specific primers. Experiments were performed on three separate occasions.

For viral cytotoxicity, 5×10^4 cells were grown in 24-well plates, and after 24 hours, cells were infected with VSV-F or VSV-IL12 at a MOI of 0.01. The cytotoxic effects on the cells of each virus were quantified by an MTT assay and expressed as a fraction of mock-infected cells at each time point. Each point was tested in duplicate, and the experiments were repeated in triplicate to ensure consistency of results. Using the Student *t* test, the cytotoxic effect of each virus on the same cell lines as well as the efficacy of each virus in different cell lines was compared.

Assessment of In Vivo Intratumoral Infection rVSV-F or rVSV-IL12 of Murine Squamous Cell Carcinoma VII Floor of Mouth Tumors

For the in vivo studies, we studied the floor of mouth tumor model in murine species C3H/HeJ. Six-week-old female C3H/HeJ mice were used in accordance with guidelines for animal use and care established by the Institutional Animal Care and Use Committee. This orthotopic model was chosen because it has been characterized to be aggressive and reflective of the human counterpart.[12] On day 0, syngeneic SCC VII cells (5×10^5) were transcutaneously implanted into the floor of mouth at the level of the mylohyoid. Tumor area was determined by the greatest tumor height and width. By day 5, tumors grew to an average diameter of 5 to 6 mm in all animals. Daily direct intratumoral injections were then administered for 5 consecutive days (days 5–9). Each tumor was injected with rVSV-F or rVSV-IL12 at 1×10^7 plaque-forming units in 15 μL of phosphate-buffered saline (PBS). Control animals were injected with PBS, and treated animals were injected with rVSV-F or rVSV-IL12. The end points examined were tumor size using Student *t* test to compare virus-injected tumors with the control group as well to compare rVSV-F with rVSV-IL12-treated tumors, and Kaplan-Meier survival curves were compared using log-rank test using the Graph Pad Prism 3.0 Program (GraphPad Software, San Diego, CA).

RESULTS

In Vitro Interleukin 12 Expression

Squamous cell carcinoma cell lines were infected with rVSV-IL12 at varying MOI to determine whether they can express IL12. After 24- and 48-hour incubation, murine IL12 was quantified. Human squamous cell carcinoma cell lines demonstrated a high level of expression of IL12 (1.00 E + 04 pg/mL) at MOI of 0.01 at 48 hours (Figs. 1, 2, and 3), whereas mock-infected controls of each tumor cell line

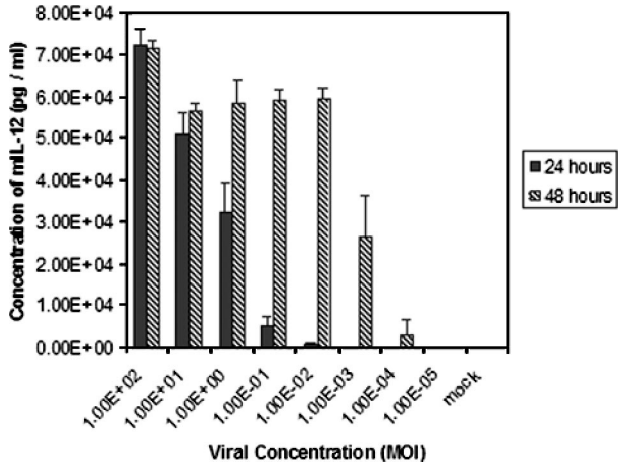

Fig. 1. Murine interleukin12 evaluated by enzyme-linked immunosorbent assay after infecting SCC09 with rVSV-IL12. IL12 is highly expressed at low multiplicity of infection (MOI) after 48 hours (1.00 E + 04 at 0.01 MOI). Mock controls failed to produce detectable levels of interleukin 12.

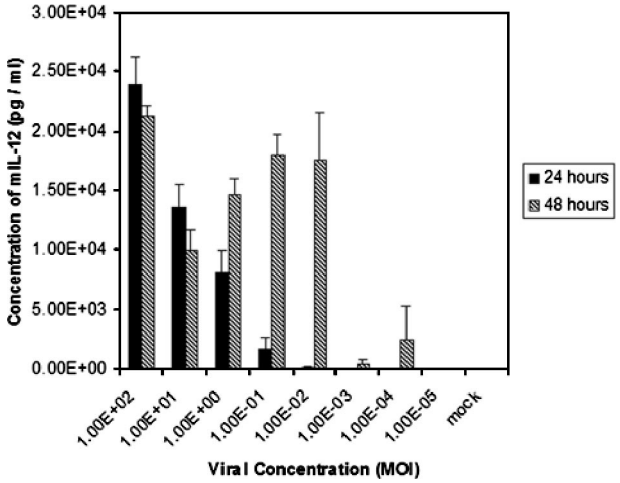

Fig. 2. Murine interleukin12 evaluated by enzyme-linked immunosorbent assay after infecting FaDu with rVSV-IL12. IL12 is highly expressed at low multiplicity of infection (MOI) after 48 hours (1.00 E + 04 at 0.01 MOI). Mock controls failed to produce detectable levels of interleukin 12.

produced no detectable levels of IL12. (P = 5.0 E-06 for mock vs. SCC VII, P = 4 E-05 for mock vs. FaDu, P = 1E-04 in mock vs. SCC09).

In Vitro Replication

Recombinant VSV-F and r VSV-IL12 replicate preferentially in human and murine squamous cell carcinoma cell lines (Figs. 4 and 5). In all tumor cell lines, replication approaches a plateau of 1×10^8 viral RNA copies within 48 hours and this number is 10,000 times more than that found in human keratinocytes (all P values $<.04$ for each virus in each of tumor cell line tested). Both viruses demonstrated similar infection efficiency and viral replication when tested in human and murine squamous cell carcinoma cells in vitro.

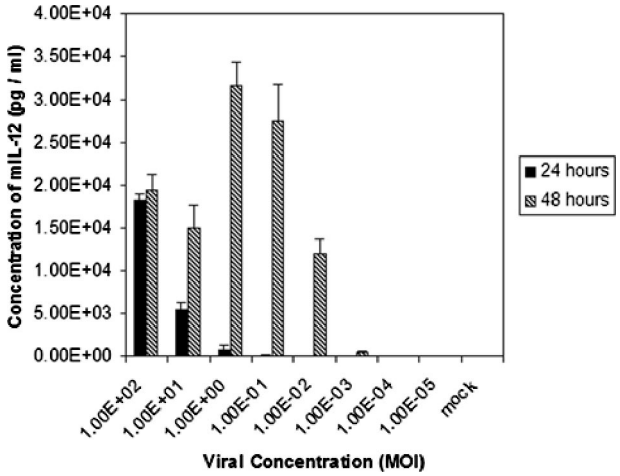

Fig. 3. Murine interleukin12 evaluated by enzyme-linked immunosorbent assay after infecting SCCVII with rVSV-IL12. IL12 is highly expressed at low multiplicity of infection (MOI) after 48 hours (1.00 E + 04 at 0.01 MOI). Mock controls failed to produce detectable levels of interleukin L12.

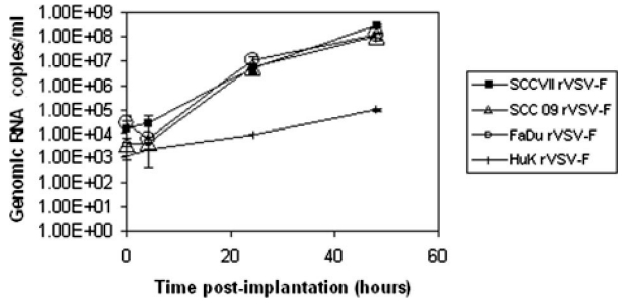

Fig. 4. Viral replication expressed as amount of RNA copies on a $\log_{10}$ scale. In all tumor cell lines, replication approaches a plateau of 1×10^8 within 48 hours and is 10,000 times that in human keratinocytes. At 48 hours, rVSV-F replicates to higher levels in tumor cells than in human keratinocytes (P value = .03 HuK vs. SCC VII, P = .005 HuK vs. SCC09, and P = .02 HuK vs. Fadu). rVSV-IL12 replicates to higher levels in tumor cells than in human keratinocytes (P value = .03 HuK vs. SCC VII, P = 7 E-05 HuK vs. SCC09 and FaDU). Error bars represent standard error of mean.

In Vitro Cytotoxicity

Recombinant VSV-F and rVSV-IL12 each preferentially and efficiently killed each human and murine squamous cell carcinoma cells when compared with normal human keratinocytes ($P < .001$ for each virus in each of tumor cell line tested) (Figs. 6 and 7). The human keratinocytes population was essentially unaffected by rVSV-F and rVSV-IL12. Both viruses demonstrated similar cytotoxicity when tested in human and murine squamous cell carcinoma cells at 72 hours.

In Vivo Tumor Size

Treatments with rVSV-F or rVSV-IL12 reduce tumor volume (Fig 8). Tumor area was found to be smaller in treated animals whether with rVSV-F or rVSV-IL12 (n = 19, n = 17, respectively) than the control PBS group (n = 19) (on day 15, rVSV-F vs. PBS-treated animals P value $<.03$; on day 15, rVSV-IL-12 vs. PBS P value $<.002$).

When comparing the rVSV-IL12 with the rVSV-F, the rVSV-IL12-treated tumors showed a striking reduc-

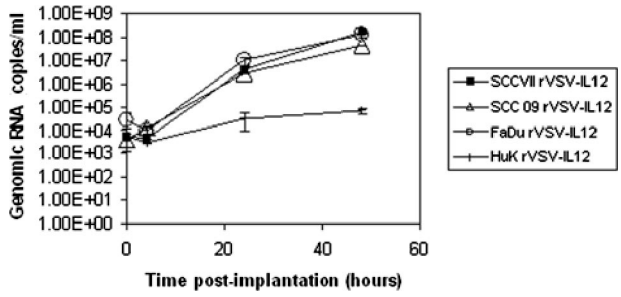

Fig. 5. Viral replication expressed as amount of RNA copies on a $\log_{10}$ scale. In all tumor cell lines, replication approaches a plateau of 1×10^8 within 48 hours and is 10,000 times that in human keratinocytes. At 48 hours, rVSV-F replicates to higher levels in tumor cells than in human keratinocytes (P value = .03 HuK vs. SCC VII, P = .005 HuK vs. SCC09 and P = .02 HuK vs. Fadu). rVSV-IL12 replicates to higher levels in tumor cells than in human keratinocytes (P value = .03 HuK vs. SCC VII, P = .7 E-05 HuK vs. SCC09 and FaDU). Error bars represent standard error of mean.

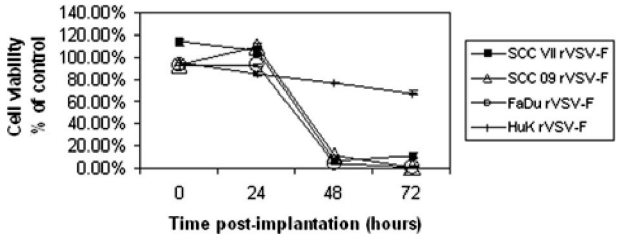

Fig. 6. Cell viability expressed as percentage of control. Cell viability decreases significantly over time in the tumor cell lines but not in human keratinocytes when treated with VSV-F. (At 72 hours for VSV-F, P = 8E-06 Huk vs. SCC VII, P = 2E-07 HuK vs. SCC09, P = 3E-07 Huk vs. Fadu. At 72 hours for rVSV-IL12, P = 1E-05 Huk vs. SCC VII, P = 2E-07 HuK vs. SCC09, P = 9E-07 Huk vs. Fadu.) Error bars represent standard error of mean.

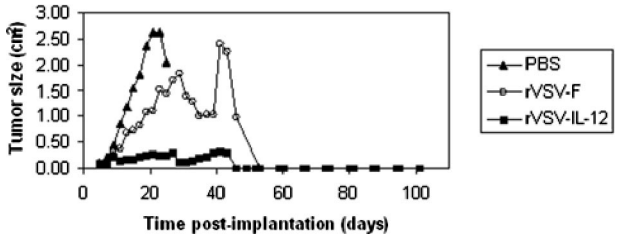

Fig. 8. Floor of mouth squamous cell carcinoma VII tumor area (greatest tumor height $\times$ width) after treatment with five daily intratumoral injections of rVSV-F (1×10^7 plaque-forming units), rVSV-IL12 (1×10^7 plaque-forming units), or phosphate-buffered saline. rVSV-F-treated animals had smaller tumors when compared with controls (days 15, 19, and 21 P values = 1 E-04, 8 E-04, 0.02, respectively). rVSV-IL-12-treated mice had markedly smaller tumors than rVSV-F-treated mice. (On days 15, 19, and 21, P values = 6 E-06, 6 E-04, 0.005, respectively.)

tion in tumor volume compared with rVSV-F (comparison on days 15, 19, 21; all P values $<.005$).

In Vivo Survival

Kaplan-Meier survival curves demonstrate that treated animals had better survival than control animals (Fig. 9). By day 30, none of the control PBS mice survived. In contrast, three animals in the rVSV-F group and 10 animals in the rVSV-IL12 group survived beyond day 30. By day 60, none of the treated animal by rVSV-F survived in contrast to 40% of the animals treated by rVSV-IL12. These animals are still alive and tumor-free at the last measurement (day 100).

The difference in the survival curves between the group treated with rVSV-F and the one treated with rVSV-IL12 was highly significant (P value $<.0001$) in favor of rVSV-IL12.

DISCUSSION

Tumor-targeted replication-competent viruses represent a class of novel agents for cancer therapy. In addition to adenovirus, herpes simplex virus, and influenza viruses that are molecularly engineered to replicate specifically in tumor cells, viruses with inherent tumor specificities are actively being developed as oncolytic agents for cancer treatment. Vesicular stomatitis virus is a negative-strand RNA virus with inherent specificity for replication in tumor cells resulting from the attenuation of their antiviral responses. Infections in humans are asymptomatic in most cases or result in a mild febrile illness.[4] VSV has now been shown to be efficacious against malignant glioma, melanoma, hepatocellular carcinoma, breast adenocarcinoma, and some selected leukemias.[13] A more effective VSV that replicates by the formation of multinucleated giant cells or syncytia was developed.[5] In this method, the infected cell fuses with adjacent tumor cells to spread infection. In addition to skipping steps of the viral life cycle (budding and endocytosis), the direct cell-to-cell fusion allows for more efficient spread. Also, these viral particles remain intracellular; thus, they may avoid humoral immune mechanisms that could neutralize viral particles. Such fusogenic oncolytic viruses have improved local control and survival in several tumor models.[5]

IL12 is one of the most promising cytokines in cancer treatment as a result of its multiple effects. It is produced by antigen-presenting cells and played an important role in regulating the immune response and enhancing the activity of T helper cells, natural killer cells, and cytotoxic T cells, which may have significant antitumoral activity.[14]

Other recent studies have also evaluated the use of cytokines in combination with oncolytic viral therapy in an attempt to improve tumor kill.[15] These studies showed that combining two different strategies for treatment of

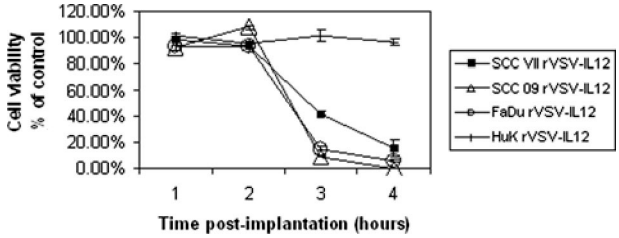

Fig. 7. Cell viability expressed as percentage of control. Cell viability decreases significantly over time in the tumor cell lines but not in human keratinocytes when treated with rVSV-IL-12. (At 72 hours for VSV-F, P = 8E-06 Huk vs. SCC VII, P = 2E-07 HuK vs. SCC09, P = 3E-07 Huk vs. Fadu. At 72 hours for rVSV-IL12, P = 1E-05 Huk vs. SCC VII, P = 2E-07 HuK vs. SCC09, P = 9E-07 Huk vs. Fadu.) Error bars represent standard error of mean.

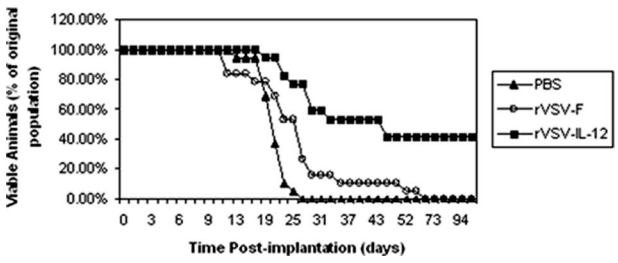

Fig. 9. Animal survival. Kaplan-Meier survival curves of mice with floor of mouth squamous cell carcinoma VII tumors after treatment with five daily intratumoral injections of rVSV-F (1×10^7 plaque-forming units), rVSV-IL12 (1×10^7 plaque-forming units), or phosphate-buffered saline starting on day 5 postimplantation of tumor cells (control n = 19, treated with rVSV-F n = 19, treated with rVSV-IL12 n = 17). rVSV-IL-12-treated animals had markedly improved survival over rVSV-F-treated animals ($P < .0001$).

murine head and neck carcinoma had a synergistic effect. The oncolytic virus had a direct cytotoxic effect on the cancerous cells, whereas IL12 produced immunostimulatory anticancer effects.

This study evaluated the use of a novel oncolytic virus carrying the gene for IL12 (rVSV-IL12) as treatment for murine squamous cell carcinoma of the head and neck. In addition, it also compared the efficacy of this novel virus with a known potent fusogenic oncolytic virus (rVSV-F). In vitro studies showed that tumor cells infected with rVSV-IL12 produced interleukin after 24 hours and continued to produce more interleukin after 48 hours. Mock-infected tumor cells do not produce detectable levels of interleukin 12 (Figs. 1–3). Both rVSVs were able to replicate more efficiently in and more effectively kill human and murine SCC cell lines than human keratinocytes. Even in vitro human keratinocytes likely retain innate antiviral responses suppressing replication and subsequent cytotoxicity (Figs. 4–7). When compared with rVSV-F, rVSV-IL12 demonstrated similar infection efficiency, viral replication, and cytotoxicity.

Recombinant VSV-IL12 also had an effect on tumor size and survival. In the SCC VII orthotopic floor of mouth model in immunocompetent C3H/HeJ mice, multiple intratumoral injections with each virus caused a significant reduction in tumor volume when compared with saline injections alone ($P < .005$). The rVSV-IL12-treated tumors showed a striking reduction in tumor volume when compared with rVSV-F and saline-treated tumors ($P < .005$). This striking reduction in tumor volume translated into a substantial survival benefit in rVSV-IL12-treated animals. Control animals survived only to 27 days, whereas treated animals with rVSV-F survived up to 59 days. However, animals treated with rVSV-IL12 had statistically better survival when compared with the control group and the rVSV-F group ($P < .0001$ for both). Forty percent of the rVSV-IL12-treated groups remain alive and tumor-free at day 100.

These current experiments demonstrated that combining an oncolytic virus with immune-based gene therapy is superior to an oncolytic virus alone. This novel therapy might be useful alone or in combination with other modalities like chemotherapy or radiation for treatment of recurrent nonoperative squamous cell carcinoma of the head and neck. This synergistic relationship between viral oncolysis and immunotherapy seems promising and deserves further investigation to delineate the underlying immune mechanisms involved in enhancing the antitumor effect. Once delineated, future studies may examine rVSV-IL12 in combination with other immunostimulatory agents (GM-CSF, 41BB, and so on) that have synergistic antitumor effects for better overall survival. rVSV-IL12 alone or in combination with other agents may have particular application in patients with metastatic disease.

CONCLUSION

Recombinant VSV-IL12 is a novel oncolytic vesicular stomatitis virus that effectively expresses IL12 and significantly enhances the treatment of head and neck carcinoma in a murine model. Such combined oncolytic and immunomodulatory strategies hold promise in the treatment of head and neck cancers.

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