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PRINCIPAL INVESTIGATOR: Andreas Kurtz, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, Massachusetts 02114-2554

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Andreas Kurtz, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts General Hospital
Boston, Massachusetts 02114-2554

E-Mail: kurtza@helix.mgh.harvard.edu

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13. ABSTRACT (Maximum 200 Words)

Peripheral nerve sheath tumors are the major tumors in neurofibromatosis type 1 (NF1). Malignant transformation of these tumors leads to extremely poor prognosis without therapy. The main goal of this project is to to determine the efficacy of recombinant oncolytic herpes simplex type 1 viruses (HSV) for the therapy of nerve sheath tumors. To that aim we will generate reliable tumor models for malignant peripheral nerve sheath tumors (MPNST). Several existing oncolytic HSV will then be tested on these models for therapeutic utility. In addition, oncolytic therapy will be combined with experimental anti-angiogenic therapy.

Here, we have derived mouse and human MPNST-derived cell lines and established their tumorigenic potential in mice. In addition, we have detected significant biochemical differences between distinct $NF^{-/-}/p53^{-/-}$ MPNST. We show that these differences modulate susceptibility to HSV. Furthermore, we have established that oncolytic HSV effictively target MPNST. Oncolytic HSVare cytotoxic against MPNST and inhibit tumor growth after local administration. Finally, we have shown that the anti-angiogenic factors dominant-negative fibroblast growth factor receptor (dnFGFR) and platelet factor 4 (PF4) inhibit angiogenesis and growth of MPNST in our mouse model. In addition, dnFGFR inhibits proliferation of tumor cells directly. Oncolytic viruses expressing dnFGFR have been constructed.

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Table of Contents

Introduction

The defining tumors in NF1 are discrete and plexiform neurofibromas and malignant peripheral nerve sheath tumors (MPNST). Much of the morbidity and mortality in NF1 patients is caused by these peripheral nerve tumors. Improvement of existing therapies and development of new therapies for these tumors is hindered by difficulties to systematically characterize tumor development and to control treatment strategies, and by the lack of appropriate animal models. The goal of the work funded by this grant is to develop appropriate animal models for testing of experimental therapies for MPNST. Oncolytic viruses will be used to explore their efficacy in MPNST therapy in these models.

Genetic and cell biological technologies have recently made available a number of mouse models in which peripheral nerve tumors and MPNSTs are formed. In this proposal we will use these and develop other models to determine the therapeutic efficacy of oncolytic herpes simplex type I viruses (HSV) alone and in combination with anti-tumor immune- and anti-angiogenesis treatment, respectively. Oncolytic HSVs have been shown to be effective treatments for a number of nervous system tumors in animal models, such as glioblastoma and medulloblastoma (1,2). These viruses have also been shown to safe for use in humans in a phase 1 clinical trial and are currently in phase 2 (3).

In the first aim, we planned to screen human and murine neurofibroma and MPNST derived cells for sensitivity to oncolytic HSVs. Sensitive cells will be used to generate orthotopic peripheral nerve tumors in mice.
In the second aim, we will treat the experimental peripheral nerve tumors with oncolytic HSV by local virus delivery and differentiate between direct oncolytic effect on tumors and tumor inhibition by induction of anti-tumor immune responses.

In the third aim, the effect of oncolytic HSV after systemic delivery in tumors derived from grafted cells and in *Nf1/Trp53* knockout mice will be determined.

In the final aim we will combine treatment of peripheral nerve tumors with oncolytic HSV and anti-angiogenesis therapy by targeting pathways that inhibit angiogenesis as well as proliferation of neurofibroma derived Schwann-like cells.

Taken together, we will establish whether oncolytic HSV are effective against MPNST in animal models, whether anti-tumor immunity can be induced against these tumors, and whether expression of anti-angiogenic factors from HSV-vectors provides additional benefit.

The present report will present data derived mainly from work on aim 1 and 2 as outlined in the statement of work.

Body

A. Testing of human and murine MPNST derived cells

To develop a useful cell-grafting model for MPNST it is necessary to obtain appropriate cell lines. These cells need to be tested for their sensitivity to different oncolytic viruses. This test provides a first test of the hypothesis – the use of oncolytic HSV for MPNST-therapy. Secondly, these cells need to be tested for their potential to form tumors in mice. A reproducible tumor growth profile needs to be established to be able to accurately monitor treatment.

Murine MPNST-derived cells:

We have originally obtained murine MPNST-derived cell lines from Dr. L. Parada (SW University, Texas). These cells were derived from MPNSTs that developed spontaneously in NF1/p53 double heterozygous mice (4,5). The cells have been characterized by Parada's laboratory and shown to be NF/p53 negative. Thus providing a genetically appropriate model for MPNST.

We have also obtained NF1/p53 double heterozygous mice in order to generate more MPNST-cell lines, and for subsequent direct treatment of spontaneous tumorigenesis in these mice. Using these mice, we have generated 5 additional MPNST-derived murine cell lines (not shown). All further experiments are performed with cell lines M1-M6.

Human MPNST-derived cells:

In collaboration with Dr. VF. Mautner and S. Frahm, we have generated in our laboratory 3 human MPNST-derived primary cell lines (S520, S462, S805). These cells have been characterized genetically and show loss of heterozygosity (LOH) for NF1, and in two of the cell lines LOH of p53 could be detected (S520, S462). Genotyping of the tumors of origin confirmed that these cells are indeed tumor cells.

Confirmation of the genetic identity of these cells make their use advantageous over most existing Schwannoma or neurofibrosarkoma derived cell lines (e.g. ST8814), and we decided to work with these newly established human MPNST cells.

In addition to MPNST cells, we also established primary cultures of human Schwann cells and fibroblasts derived from plexiform neurofibromas and normal nerve. These cells are used to generate data on in vitro virus cytotoxicity.

Table 1: Human and murine cell lines

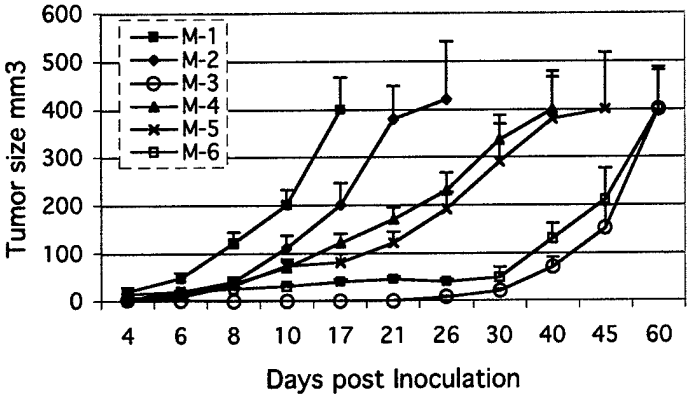
Cell line	Species	Origin	Genotype
M1	Mouse (B6/129)	MPNST	$NF^{-/-}/p53^{-/-}$
M2	Mouse (B6/129)	MPNST	$NF^{-/-}/p53^{-/-}$
M3	Mouse (B6/129)	MPNST	$NF^{-/-}/p53^{-/-}$
M4	Mouse (B6/129)	MPNST	$NF^{-/-}/p53^{-/-}$
M5	Mouse (B6/129)	MPNST	$NF^{-/-}/p53^{-/-}$
M6	Mouse (B6/129)	MPNST	$NF^{-/-}/p53^{-/-}$
S805	Human (NF1)	MPNST	$NF^{-/-}/p53^{-/+(?)}$
S520	Human (NF1)	MPNST	$NF^{-/-}/p53^{-/-}$
S462	Human (NF1)	MPNST	$NF^{-/-}/p53^{-/-}$
S909	Human (NF1)	Plexiform nf Schwann cells	$NF^{-/-}/p53^{+/+}$
F909	Human (NF1)	Plexiform nf Fibroblasts	$NF^{-/+}/p53^{+/+}$
S811	Human	Normal nerve Schwann cells	$NF^{+/+}/p53^{+/+}$

Tumorigenicity:

All MPNST-derived cells have been tested for their potential to form tumors in athymic nude mice and syngeneic mice (murine cells) (Figure 1).

Figure 1: Tumor growth of (A) murine MPNST cells and (B) human MPNST cell lines in athymic nude mice. 10^6 murine cells and cells 10^7 human cells, respectively, were inoculated subcutaneously and tumor growth monitored biweekly.

(A)



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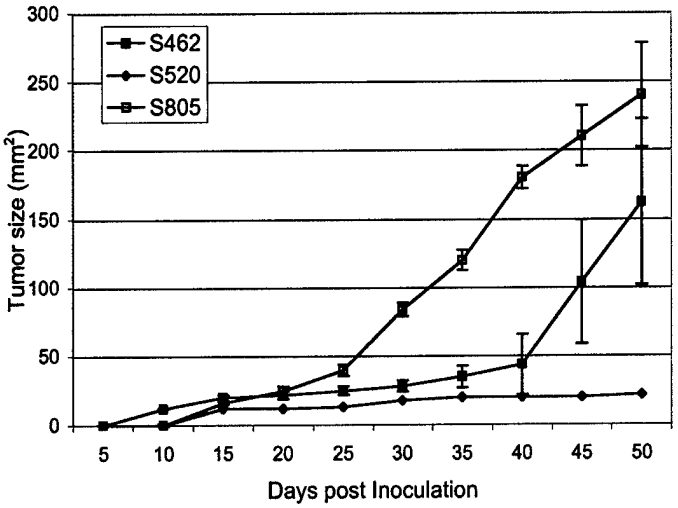


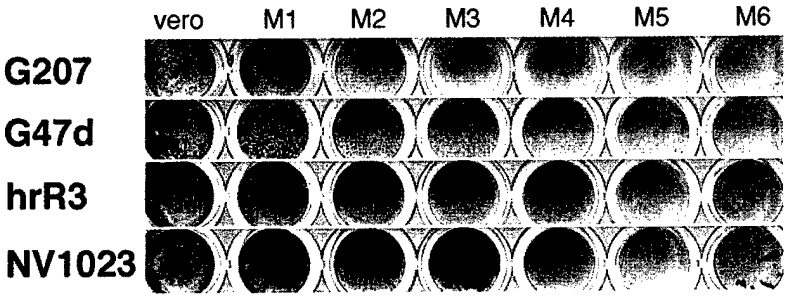
All murine MPNST-derived cells form tumors in athymic nude mice. Tumor growth rates are different and correlate with basic P-Erk activity (see also Figure 3). All murine MPNST cells except M-5 form tumors in syngeneic mice with similar relative growth rates. Of the human MPNST-cell lines, only line S805 formed tumors reproducibly in nude mice. Cell line 520 formed tumors at a less than 100% take rate.

B. Sensitivity of MPNST-derived cell lines to oncolytic HSV

HSV growth assays:
All cells have been assayed for the cytotoxic effects of oncolytic HSV, and for the growth potential of oncolytic HSV (Figure 2).

Figure 2: Susceptibility of recombinant oncolytic HSV on murine and human MPNST-derived cells.
(A) Cytotoxicity effects of HSV-constructs indicated on the (A) murine (MOI 0.1) and (B) human cell lines as indicated. Cells were plated in 48 well plates and virus was added at a multiplicity of infection (MOI) of 1.0, 0.1 and 0.01. Cell infection (X-gal staining) and survival (neutral red staining) was monitored at days1, 2 and 3. Shown is day 1.

(A)



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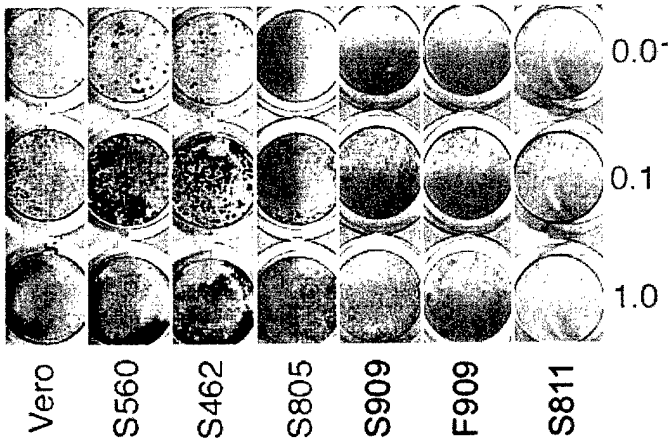
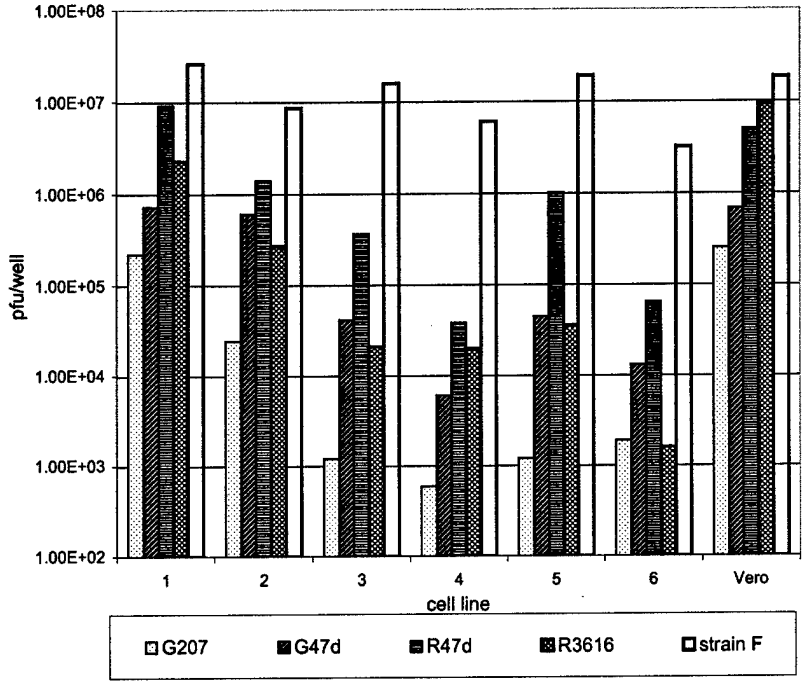
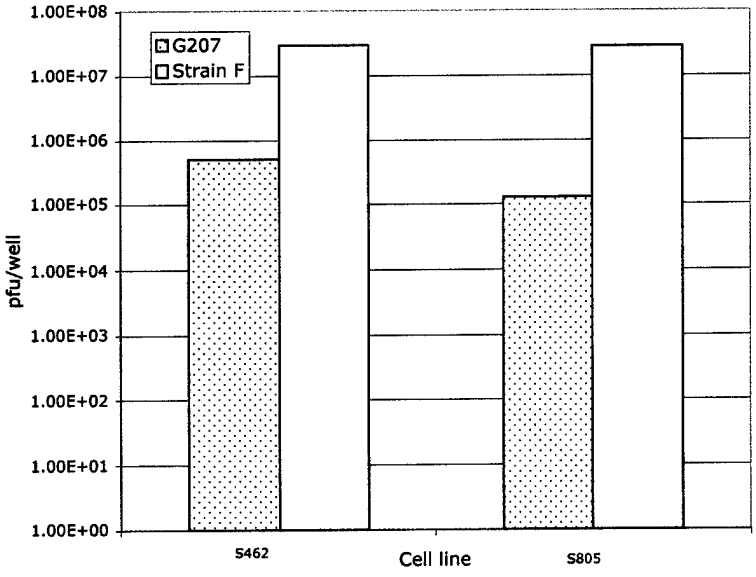


(C) Single step growth assay of different HSV-constructs on the murine MPNST-derived cells as indicated. Strain F was used as a wild-type HSV strain for control. Cells were plated to confluency and infected with an MOI of 2.0. Virus production was determined at 20 hours post infection.



(D) Single step growth assay of G207 on human MPNST-derived cells. The experiment was performed as in (C).

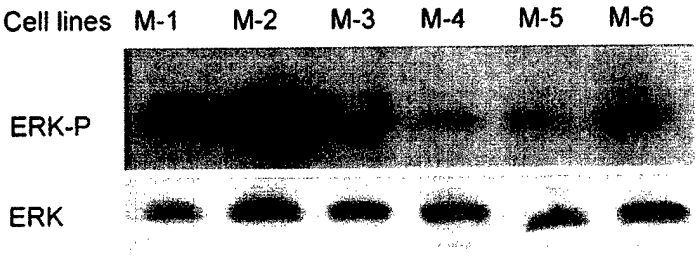


Signaling pathways and HSV susceptibility:

We could show that although all cells are effectively killed by several different recombinant oncolytic HSV, there is a distinct pattern of variability, which is most obvious in the murine MPNST cells but also detectable in human cells. This is unexpected for the genetic uniformity of all cell lines with regard to NF and p53. further testing revealed that the differential growth of oncolytic HSV correlates with activation of Map-kinase (Erk). By using specific inhibitors of the Ras-Erk-pathway we deduced that its activation critically influences HSV-replication (Figure 3) and tumorigenesis (see below).

Figure 3: Dependence of viral production on P-erk activity.
(A)Basic P-Erk activity in different murine MPNST-derived cells. Lysates from the different cell lines grown in complete medium were probed by Western analysis using an anti-P-Erk(42/44) antibody.
(B) Cells with different P-Erk levels were exposed to inhibitors of the Ras-P-Erk (Farnesyl-tyransferase inhibitors FTI-277, JNK inhibitor SP600125, Erk-inhibitor PD98059, EGF-receptor inhibitor AG1478, pan-Tyrosine-phosphorylation inhibitor Genistine, PI3Kinase inhibitor Wortmannin) pathway and infected with oncolytic HSV G207. 15 hours later, the cells were lysed and viral protein production detected by Western analysis.

(A)



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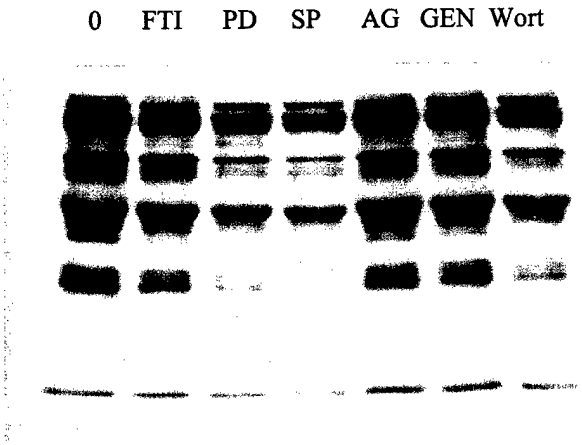
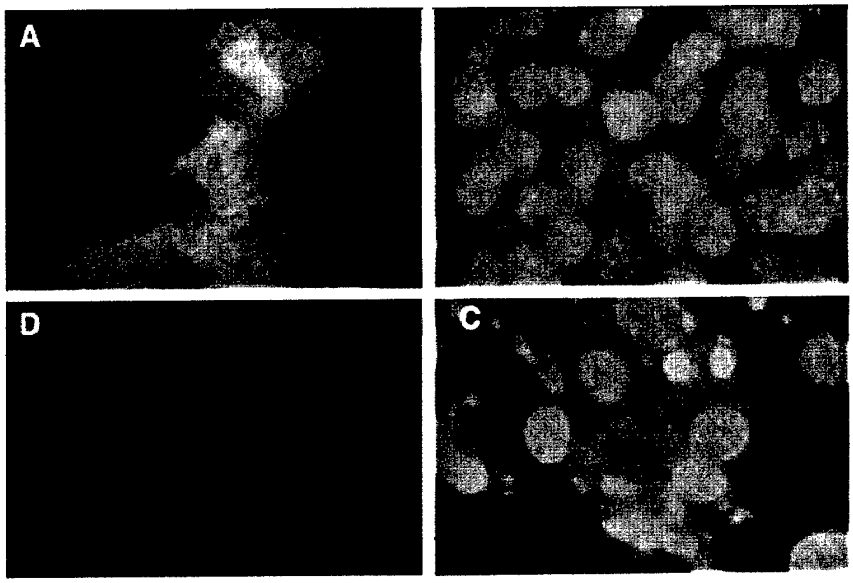


HSV susceptibility and induction of apoptosis:

In addition to Ras-pathway activation, induction of apoptosis by HSV may inhibit growth and spread of the virus and reduce its therapeutic value (Figure 4). The induction of apoptosis in dependency of viral susceptibility was confirmed by FACS-analysis (not shown). Thus, we detected a duality between Ras activation and sensitivity to apoptotic signals, which both have modulate significantly replication of oncolytic HSV in MPNST-derived cells.

Figure 4: Induction of apoptosis by HSV infection prevents virus production and spread in MPNST cells. Nuclei are stained by DAPI (blue fluorescence); virus proteins are detected by immonocytochemistry for GP4 (green fluorescence).
(A) The highly sensitive cell line M-1 shows production of virus protein and (B) intact nuclei after HSV infection.
(C) The less sensitive cell line M-4 shows almost no virus protein production and (D) fractionated nuclei typical for apoptotic cells.



Conclusions:

In summary, our results confirm that MPNST derived cells are killed by oncolytic HSV, while normal Schwann cells and plexiform neurofibroma derived fibroblasts and Schwann cells are significantly less sensitive. Between different MPNST cell lines, the efficacy of HSV-killing depends on Ras-pathway activation and sensitivity to apoptotic signals.

B. Virus production

Recombinant viruses and immune-stimulating viruses:

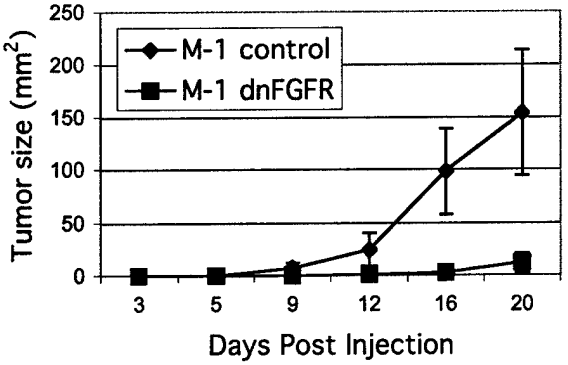
Stocks of recombinant oncolytic HSV G207, G47delta, NV10223 and NV1042 have been generated and are available in the laboratory for the experiments proposed. Titers vary between 108 and 109 plaque forming units (pfu) /ml, a typical and sufficient virus concentration.

Anti-angiogenesis viruses: testing of anti-angiogenesis factors:

We have tested the effects of the anti-angiogenesis factors dominant negative FGF-receptor (dnFGFR) and platelet factor 4 (PF4) on MPNST-tumor growth. To this end, we have transfected MPNST-derived cells with dnFGFR and PF4 expression vectors. The cells were subcutaneously inoculated into mice and tumor growth of dnFGFR and PF4 expressing cells and control cells was measured. PF4 and dnFGFR expression resulted in reduced tumor growth (Figure 5).

Figure 5: Tumor growth of (A) dnFGFR, (B) PF4 and control vector expressing cell line M-1.

(A)



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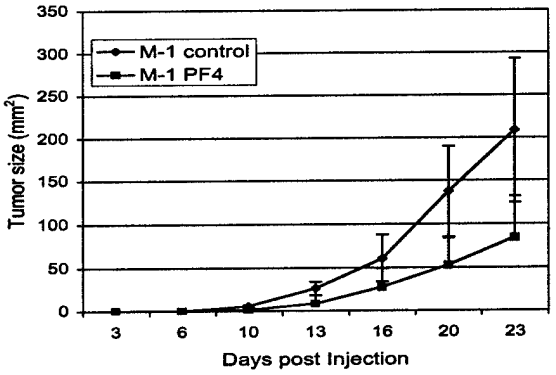
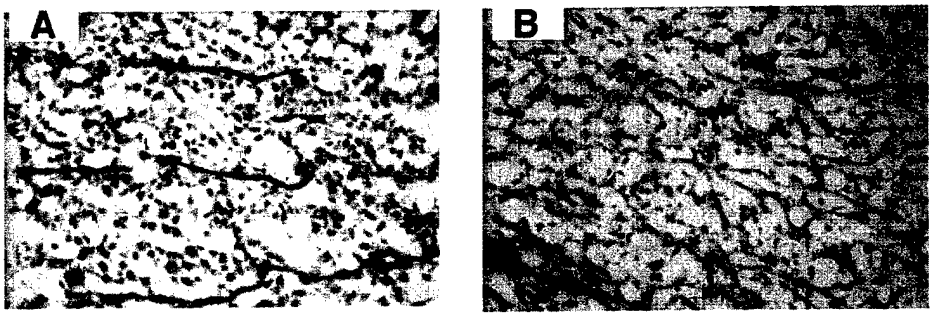
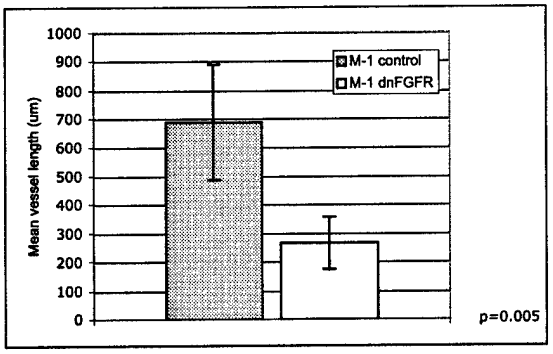


Expression of dnFGFR results in paracrine inhibition of tumor angiogenesis (figure 6), and reduced tumor cell proliferation by neutralizing FGF-2 produced by tumor cells (not shown).

Figure 6: Inhibition of angiogenesis by dnFGFR in M-1 tumors and cells.
(A) In vivo reduction of blood vessel integrity in control tumors and (B) dnFGFR tumors. Endothelial cells are detected by immunohistochemistry using anti-CD-31 antibodies.
(C,D) Quantification of blood vessel length and number of CD31 positive structures.



(C)



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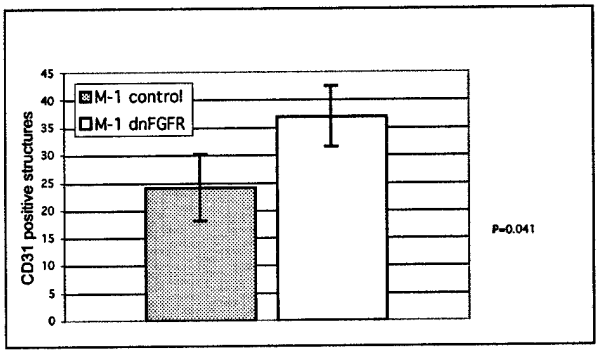
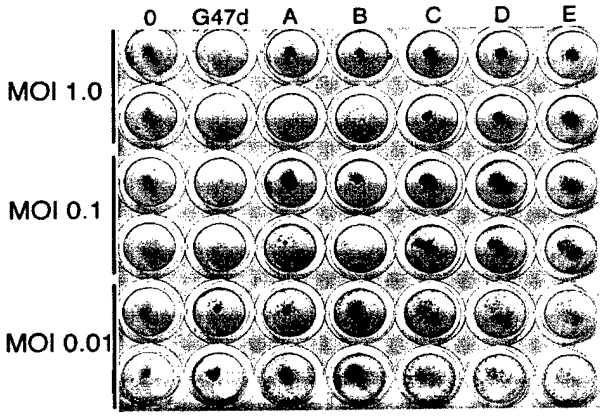


Generation of anti-angiogenesis HSV:

We have started to produce G47delta for the expression of the anti-angiogenic factors dnFGFR and PF4. Here, we are using a newly developed Bac-based recombination system (G47deltaBac). Using a shuttle vector system, the transgene is inserted into the viral ICP-6 gene, knocking it out and inducing tumor specificity. We have generated 5 different G47deltaBac-dnFGFR isolates and have tested their cytotoxicity, which is slightly reduced when compared to the parental G47delta virus (Figure 7).

Figure 7: Cytotoxicity assay of G47delta and G47deltaBac-dnFGFR viruses (A-E). Reporter cells were infected at a MOI of 1.0, 0.1 and 0.01. X-gal staining indicates HSV-infection. Neutral red staining indicates vital cells.



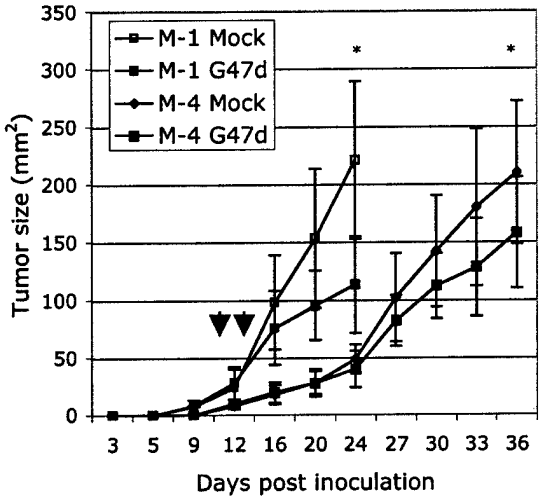
Conclusion:

We have shown that dnFGFR and PF4 both inhibit growth of tumors derived from MPNST-cells. DnFGFR acts by inhibiting angiogenesis as well as tumor cell proliferation. We have generated oncolytic G47delta-dnFGFR using a Bac-system and tested its cytotoxicity in vitro.

C. Therapy of MPNST-cell derived tumors using G207

We have subcutaneously injected cell line M-1 and M-4 into mice and treated tumors by local injections of G47delta (Figure 8). We found that G47delta inhibits growth of M-1 tumors more effectively than M-4 tumors, corroborating our in vitro data. We are currently testing the effects of local administration of G47delta on tumors derived from human MPNST cell line S805.

Figure 8: Tumor growth inhibition by G47delta. M-1 and M-4 cells were injected subcutaneously into nude mice. Tumors were injected with HSV when they reached a size of 4x4x4 mm (arrowheads).10^6 pfu G47delta or inactivated virus were injected 3 times per week until the end of the experiment. The differences between Mock and control groups are significant at $P<0.02$ (*, t-test).



Conclusions:

The recombinant oncolytic HSV G47delta I effective in reducing tumor growth of murine MPNST-tumors in a subcutaneous model. G47delta appears to be more efficient in fast growing tumors with high levels of activated Ras.

Key Research Accomplishments

- Establishment and tumorigenicity testing of human and murine MPNST-derived cell lines.
- 6 murine and 2 human MPNST-derived tumorigenic cell lines were identified
- Significant differences in Ras-activation between genotypically identical ($NF1^{-/-}$ /$p53^{-/-}$) MPNST cells were detected
- Tumor models for MPNST were established
- A correlation between Ras activation and tumor growth rate was detected

- Establishment of the cytotoxicity and susceptibility of oncolytic HSV on MPNST-derived cells
- Detection of a positive association between Ras activation and HSV-susceptibility
- Detection of a negative association between apoptotic pathway activation and HSV-susceptibility
- Conventional oncolytic HSV therapy may require pre-treatment screening of NF-tumors (MPNST) for Ras-pathway activation to utilize appropriate therapeutic means

- The oncolytic recombinant HSV G47delta was selected for generating an anti-angiogenesis targeting HSV, using a novel Bac-based system
- cDNA coding for the anti-angiogenic factors dominant-negative FGF-receptor (dnFGFR) and platelet factor 4 (PF4) was introduced into G47delta
- G47delta-dnFGFR shows slightly reduced cytotoxicity (compared to G47delta)
- DnFGFR and PF4 both inhibit growth of MPNST-derived tumors in the established mouse model
- DnFGFR inhibits tumor cell growth and angiogenesis in MPNST tumors.
- PF4 inhibits angiogenesis in MPNST tumors

Reportable Outcomes

Abstract: AACR meeting (2003), Washington, DC: *MPNST and Ras Pathway: The Cross-Road Between Signalling Mechanisms and Therapeutic Approach.* Faris Farassati, Susan Henke, Camille M. O'Donnell, Robert L. Martuza, Andreas Kurtz.

Abstract: ACGT meeting (2002), Boston, MA: *Experimental Therapy of Peripheral Nerve Sheath Tumors Using Replicating Herpes Simplex Viruses.* Susann Henke, Laura J. Klesse, Luis Parada, Samuel D. Rabkin, Andreas Kurtz.

Abstract: International conference on oncolytic viruses for Tumor Therapy (2003), Banff, Canada: *Ras pathway and NF1: Where signaling mechanisms meet therapeutic approach.* Faris Farassati, Susan Henke, Camille M. O'Donnell, Robert L. Martuza, Samuel D. Rabkin, Andreas Kurtz.

Conclusions

We were able to show that it is possible to develop standardized models for MPNST in mice. By utilizing cell lines derived from mouse MPNST and human MPNST, we have established and standardized tumor growth patterns and started to treat these tumors with oncolytic HSV.

We have selected the oncolytic HSV for further studies for its effectiveness against human as well as murine MPNST cells. In addition, G47delta is a derivative of G207, which is in clinical trial for glioblastoma. Finally, G47delta can be comparatively easily manipulated to express novel transgenes. We have already generated G47delta expressing anti-angiogenic dnFGFR.

We have uncovered an important mechanism modulating viral susceptibility. Activation of Ras significantly increases HSV susceptibility and anti-tumor efficacy. Furthermore, our results indicate that Ras is not necessarily activate in MPNST cells, despite loss of the Ras-GAP neurofibromin.

We have developed data, which are in agreement with the original hypothesis - that peripheral nerve sheath tumors can be effectively inhibited by oncolytic HSV. The work has been concluded as anticipated in the 'statement of work' of the grant. In particular, the goals set in aim 1 and partially in aim two have been successfully addressed. We are optimistic to continue this study successfully.

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