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   The goal of this project is to establish an animal model of plexiform neurofibroma which then can be used to test emerging anticancer therapies for the management of tumor growth in NF1 patients. The first specific aim is to establish a syngraft model of NF1 tumorigenesis. Progress toward this aim includes: a) established and characterized numerous Schwann cell cultures from NF1 knockout mouse embryos; b) effectively transfected NF1-/- Schwann cell cultures with AAV-GFP; c) implanted GFP-labeled NF1-/- cells in mouse nerve and detected them with GFP immunohistochemistry; d) test alternate methods to achieve tumor growth and explain the failure of tumor growth in the syngraft model. The second aim is to establish a xenograft model to examine the growth of human neurofibroma tumor cells. Progress toward this aim includes: a) established a breeding colony of NF1-/- scid mice to be used as hosts for tumor grafts; b) achieved sizeable tumors by engrafting human neurofibroma-derived Schwann cells in mouse nerves; c) achieved rapid tumor growth by engraftment of human NF1 MPNST-derived cell lines; d) examined tumor growth by MRI and correlate with histological data and; e) established and applied methods to assess tumorigenic growth and neovascularization by engrafted NF1 tumor cells.

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INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common genetic disease characterized by a high incidence of neurofibroma, a nerve tumor composed predominantly of Schwann cells. The NF1 condition is characterized by a germline defect in one NF1 allele (NF1/+) whereas a homozygous NF1 deficiency (NF1−/−) is lethal in utero. It is generally believed that neurofibromas arise due to a constitutional mutation of the second NF1 allele that imparts abnormal growth of (NF1+/−) Schwann cells. The goal of this project is to establish an animal model of plexiform neurofibroma which then can be used to test emerging anticancer therapies for the management of tumor growth in NF1 patients. The first specific aim is to establish an allograft mouse model of NF1 tumorigenesis. To mimic the conditions believed to underlie neurofibroma formation, Schwann cell cultures will be isolated from embryonic Nf1+/− mice (prior to lethality). Nf1+/− Schwann cell cultures will be prelabeled with a tracer protein and then implanted into the nerves of young Nf1+/− mice. The grafts will be examined to see if neurofibroma-like tumors are formed. These experiments will test the hypothesis that Nf1+/− Schwann cells are the cause of neurofibromas and will determine if heterozygous nerve cells (with the Nf1+/− background) might also contribute to the formation of these tumors. The second aim is to establish a xenograft mouse model to examine the tumorigenic growth of human neurofibroma-derived Schwann cells (some of which have additional genetic defects). Schwann cell cultures established from human plexiform neurofibromas will be transplanted into the nerves of immunodeficient Nf1−− mice (to preclude immunorejection). Tumor formation will be assessed histologically and key features compared to naturally-occurring neurofibroma, including cell proliferation, nerve invasion and formation of new blood vessels. These experiments will test the hypothesis that human plexiform neurofibroma-derived Schwann cells are tumorigenic and that the transplantation model represents a valid model to study NF1 tumor development and biology. This work will also investigate and optimize methods to monitor neurofibroma growth in the living animal by magnetic resonance imaging (MRI). These efforts will increase the usefulness and clinical relevance of the NF1 mouse nerve model and should provide an effective means to examine plexiform tumor growth and response to therapies in the living animal.

BODY

Technical Objective 1: ESTABLISH A VALID SYNGRAFT MOUSE MODEL OF NF1 TUMORIGENESIS.

Task 1: Establish and label embryonic Nf1 knockout mouse Schwann cell cultures:

Progress:

We have tested numerous approaches to the culture of Schwann cells from day 12.5 embryos. The best differentiation and expansion of Nf1 Schwann cell cultures was accomplished by co-culture with ganglion neurons and later treatment with GGF2. These methods yielded highly enriched cultures of Schwann-like cells which express the Schwann cell antigens p75 and S-100 (see Annual Report 2000-01). We next compared the tumorigenic properties of Nf1+/−, Nf1+/+, and Nf1−− Schwann cells using in vitro assays for proliferation and apoptosis. Results showed that proliferation by Nf1+/− Schwann cells is nearly double that of heterozygous and wild-type cells. Also, proliferation by Nf1+/− Schwann cells was growth factor independent and was not further increased by elevated serum or addition of glial growth factor-2. On the other hand, proliferation by Nf1+/− and Nf1+/+ Schwann cells was increased significantly by elevated serum and glial growth factor-2, but even with mitogen stimulation neither genotype achieved the mitotic rates of the Nf1+/− Schwann cells. TUNEL assays showed that apoptosis was fairly
low (6-8% of cells) in all the cultures grown in standard culture conditions and was not altered
significantly by high serum or glial growth factor. Based on these in vitro data, we predicted that Nft−/
Schwann cells may have a greater tumorigenic potential.

Our next goal was to examine the tumorigenic growth of Nft Schwann cells in vivo. It was
necessary to prelabel the mouse Nft Schwann cell cultures to later identify them when implanted in the
host mouse nerves. We succeeded in labeling the mouse Schwann cells cultures in vitro with a green
fluorescent protein (GFP) adeno-associated virus expression vector (see Annual Report 2000-01). This
vector was developed by the University of Florida Vector Core. We have optimized labeling embryonic
Schwann cells and routinely achieve transduction efficiencies greater than 90%. After transplantation
into host nerves, GFP-expressing cells were labeled using anti-GFP immunohistochemistry, providing an
effect means to track the growth and migration of the (prelabeled) cells after transplantation into the host
nerve.

Task 2: Engraft Nft Schwann cells in Nft+/+ and Nft+/− host nerves:

Progress:
Thus far we have engrafted 52 nerves with GFP-labeled Nft or wild-type Schwann cell cultures.
Arious conditions were tested and grafts were examined after periods ranging from 6-84 days. Nft−/− and
Nft−/− Schwann cells were engrafted in wild-type mice and mice with a heterozygous Nft background.
GFP-labeled cells were identified in nearly ever engrafted nerve. However, cell numbers were general
quite low, well below the number originally injected (usually 10⁵). Quantitative estimates based on serial
sections indicated that less than 1,000 cells were present in any given nerve and more than one-half of
the nerve grafts contained less than 100 GFP-labeled cells. This was true for both Nft−/− and Nft−/−
Schwann cells implants. There was some indication that cell numbers were higher when implants were
made into mice with an Nft−/− background. Nevertheless, the overwhelming conclusion was that the
syngrafted cells did not grow well and no tumor-like formations were observed in any of the nerves, even
after 12 weeks.

In search of an explanation for the failure of the syngraft to grow, we examined the proliferation
of cells in the mouse nerves. After engraftment, mice were given systemic injections of the DNA analog
bromodeoxyuridine (BrdU). Several days later, the nerves were removed and then immunostained for
BrdU-DNA and GFP. In all cases we observed proliferating (BrdU-positive) cells amongst the GFP
labeled cells, indicating that at least some of the implanted cells were multiplying. This suggests that the
cells were viable and capable of proliferating in the nerve. Nevertheless, it was apparent that the number
of transplanted cells did not increase and, instead, cell loss was prevalent. Two explanations seemed
most likely; either the cells were dying or their expression of the GFP was falling off. Evidence does not
support that GFP expression per cell is falling off. First, in culture, the Schwann cells continue to
express GFP for many months. Also, in other projects we have seen that in vivo AAV-GFP
transduction in neurons can persist for well over six months and in many of the nerves where only a few
engrafted cells are seen, these show a strong GFP signal. Lastly, the same outcome was confirmed by
routine histology and other prelabeling techniques. The other possibility is that the AAV-GFP
transfected Schwann cells are evoking an immune response and are being actively rejected by the host.
This could be due to subtle differences in the Nf1 and wild-type B6 mouse strains or perhaps the
expression of AAV proteins as a result of prelabeling the cells. To circumvent this problem we engrafted
the Nft−/− and Nft−/− Schwann cells into immunodeficient scid mice, with and without an Nft background,
and obtained similar results indicating that embryonic Nf1 Schwann cell cultures did not show tumorigenic growth in the mouse nerve model.

**Task 3:** Test neonatal engraftment and nerve injury paradigms:

**Progress:**

We investigated various neonatal injection methods and found the use of very young mice to be impractical. There were real difficulties ensuring survival after surgery. A variable problem involved the lack of nurturing response by the mother. More importantly, the surgical procedure per se was very challenging because of the small size and delicacy of the nerve. Our beveled needles were too large and ruptured the nerve and microbore glass needles did not allow the delivery of adequate cell numbers. These limitations, combined with the poor growth of injected cells precluded reliable quantitation of outcomes using of neonatal hosts.

Another strategy to hasten the growth of implanted cells was to induce a proliferative response by nerve injury. We discovered that the injection procedure per se, which involves impaling the nerve with a 0.2 mm diameter needle, resulted in nerve damage and evoked a significant injury response in the surrounding nerve. This was confirmed by immunolabeling for GAP-43 (growth-associated protein) indicating axonal regeneration occurred after injection of cells. This is the same marker used to indicate the regenerative response after nerve crush injury. Based on these observations, we concluded that nerve injury did not improve the growth of the implanted cells. Moreover, there is some reason to suspect that injury to the nerve may actually evoke an inflammatory response that might hamper the growth of the injected cells. Also, our findings that Nf1-/- cells show growth independent growth suggests that the intended purpose for nerve injury (inducing the release of Schwann cell mitogens) might not be effective for these cells.

**Task 4:** Examine nerve grafts for tumorigenic growth and statistically analyze data:

**Progress:**

As described above, we have examined the growth (or lack thereof) of Nf1-/- cells prelabeled with GFP. Cell numbers were estimated by counting GFP-labeled cells in serial sections of the engrafted nerves. The data were categorized into 4 groups (no cells, 1-10, 11-100, more than 100 cells). No grafts contained more than 1,000 cells, indicating that in all grafts less than 1% of the injected cells were present 1-12 after injection. We concluded that there was a rapid initial dying off of the injected cells. Data indicated that on average 0.1% of the cells survived for up to 12 weeks, however, there was little indication that the surviving cells continued to expand in number. Thus, tumor formation has not yet been achieved and, if possible, will require very long periods of growth.

**Task 5:** Examine growth of syngrafted Schwann cells by *in vivo* MRI.

**Progress:**

MRI imaging was not performed on the syngrafts because of the lack of tumor formation. Our efforts focused exclusively on the MRI of xenografts (see below).
Technical Objective 2: Determine the tumorigenic properties of human plexiform neurofibroma-derived Schwann cells engrafted in mouse nerve.

Task 1: Breed mouse strain crossing Nfl knockout mice and immunodeficient scid mice:

Progress:

Protocols for rapid and reliable genotyping were established for Nfl knockout and scid (immunodeficient) genotypes. Breeding colonies of these strains are stable and maintenance breeding is routine. Cross-breeding was successful and a stable population of Nfl+/−/scid breeding pairs is maintained and litters achieved as needed. Adequate numbers of Nfl−/−/scid animals are available for grafting experiments.

Task 2: Engraft human plexiform tumor Schwann cells in Nfl+/+ and Nfl−/− host nerves:

Progress:

The tumorigenic growth of selected human neurofibroma Schwann cell (NF1-SC) cultures was first examined as xenografts in the sciatic nerves of adult immunodeficient scid mice (with no Nfl background). Each NF1-SC culture was engrafted into 4 nerves and 6 nerves were engrafted with an equal number of normal human SC. Engrafted nerves were examined after 8 weeks by immunostaining with an antibody specific to human glutathione s-transferase (GST) (see Annual Report 2000-01). These results demonstrate sustained tumor growth by neurofibroma-derived human SC implanted in the mouse nerve (Muir et al., 2001). Our general conclusions are that tumor development for all cell lines was very slow and that more than 8 weeks would be required to achieve sizeable tumor masses.

Because of the need for such long tumor growth periods, we also examined the in vivo growth of Schwann-like cell lines from two malignant peripheral nerve sheath tumors (MPNST) established previously in our lab. It is notable that both MPNST lines share many properties with the more transformed plexiform Schwann cell lines. One of the MPNST lines consistently developed sizeable tumors within 8 weeks of engraftment into the nerves of scid mice. These tumors were very reminiscent of plexiform neurofibromas and, despite being MPNST-derived, the tumors caused no observable neurological deficits and did not metastasize. Importantly, detailed examination of these tumors with vascular and angiogenic markers revealed significant neovascularization. On the other hand, the second MPNST line remained static and tumor expansion was not observed (n=8).

Both of the MPNST lines show highly transformed characteristics in vitro, including rapid proliferation and immortality. We expected extensive tumor growth by both when engrafted in the mouse nerve. However, the vastly different growth observed in vivo provided the opportunity to test the hypothesis that the Nfl background plays an important role in the tumorigenic growth by these NF1 cell lines. We have now engrafted 28 nerves (in both scid and Nfl−/−/scid) with these cell line for periods ranging from 2 to 24 weeks. Both MPNST were engrafted into mice with a Nfl background (Nfl−/−/scid) and tumors growth for each cell line was examined after 2 weeks (n=4), 8 weeks (n=4) and 24 weeks (n=4) and compared to tumor growth in mice without the Nfl background (Nfl+/+/scid). It is clear that, similar to our observations in the Nfl+/+/scid mice, one MPNST line grows very rapidly while the other is quite slow in the Nfl−/−/scid mice as well. Quantitative analysis of these tumors is in progress and no firm conclusions about the effect of Nfl background have been possible thus far.
Task 3: Test neonatal engraftment and nerve injury paradigms:

Progress:

As stated in Technical Objective 1, Task 3, we investigated various neonatal injection methods and found the use of very young mice to be impractical. Our other strategy to hasten the growth of implanted cells was to induce a proliferative response by nerve injury. We discovered that the injection procedure per se, which involves impaling the nerve with a 0.2 mm diameter needle, resulted in nerve damage and evoked a significant injury response in the surrounding nerve. In addition, nerve crush injury was performed at the time of NF1-SC engraftment. These caused a rapid degeneration of the nerve and proliferation of endogenous Schwann cells. However, examination of the nerves 2 weeks after engraftment indicated the engrafted tumor cells became highly dispersed in the injured nerve. At later time points, cell numbers were markedly decreased compared to uninjured nerve grafts. Based on these observations, we concluded that nerve injury did not improve the growth of the implanted cells. We speculate that the dispersal of the cells hampered their ability to cluster, which improves their growth properties. Also, injury to the nerve may actually evoke an inflammatory response that might antagonize the growth of the injected cells. Based on these findings we conclude that nerve injury is not an effective strategy to hasten the growth of the tumor grafts. Moreover, our efforts to improve the practical aspects of the xenograft model by decreasing the time required for tumors to become well established has been achieved by the use of the rapidly growing NF1MPNST cell lines.

Task 4: Examine growth of human tumor grafts by in vivo MRI:

Progress:

To examine the growth of tumors grafts in vivo by MRI it was necessary first to examine the imaging and contrast parameters of normal and pathological nerve. These experiments led to significant progress to enhance the imaging of nerve components (see Annual Report 2000-01). We have now performed MRI on 10 engrafted nerves, with excellent results. The contrast imaging of tumor growing within the nerve has been performed using T1, T2, and diffusion-weighted methods. Serial slices through the nerves provided a full representation of the extent of tumor growth. Thereafter, the nerves were examined histologically and we confirmed the MRI profiles were indeed representative of the distribution and density of the engrafted tumor cells. For volumetric quantitation we are creating 3D renderings of the MRI data. In parallel, we are creating 3D renderings of the histological images as well. This alleviates the any concerns about differences in imaging alignment (the imaging plane) between the MRI sections and the histology sections. It also provides the ability to reconstruct the entire volume of tumor growth for quantitative correlations between the two imaging methods. Furthermore, volumetric data will be the method of choice for assessing the extent of tumor growth and regression in this model when applied to testing of anti-tumor therapies. Most of the MRI data was obtained using ex vivo nerves. We have performed several in vivo imaging runs and are presently optimizing the parameters for repeated MRI in living animals. These efforts include the use of contrast enhancement agents for examining the vascular elements and blood flow within the tumor grafts. Our results in these efforts have been very encouraging and we are confident that MRI imaging can be successfully applied to this mouse tumor model.
Task 5: End-point histology assessment of tumor grafts and statistically analyze data:

Progress:

All tumor grafts, regardless of the amount of tumor growth, have been examined by routine histology and immunohistochemical staining. Human NF1 tumor cells have been clearly identified using the human-specific antibody to GST (a constitutively expressed marker protein). This labeling has provided a reliable assessment of neurofibroma growth and cell invasion. Tumors have also been labeled with a human-specific antibody to Ki-67, a proliferating cell marker, to estimate the proliferation of engrafted cells. As expected from overall tumor size, most of the plexiform tumors contain few Ki-67-positive cells, confirming that the GST-positive cells are surviving in the mouse nerve but proliferate very slowly. On the other hand, the MPNST-derived Schwann cell grafts show a much higher level of Ki-67 labeling and thus mitotic index. These labeling techniques will be exploited in all subsequent experiments, particularly those to examine methods to increase tumor growth (e.g., in neonatal implantation and nerve injury models).

An important measure of tumor development is its ability to attract blood vessels, or neovascularize. We have made excellent progress in examining the tumors for microvessel formation and key molecular components in this response. All well-developed neurofibroma grafts contained CD-31/von Willebrand factor (factor VIII-related antigen)-positive capillaries. These tumors also labeled intensely for both VEGF and the VEGF receptor, suggesting active recruitment of endothelial cells and vessel formation. These findings will be important for the assessment of tumor growth and in correlations with naturally occurring neurofibromas. In addition, our long-term goal is to use this mouse model to test anti-angiogenic therapies for neurofibroma.

Administrative Note:

This project has been approved for a 1 year, no cost extension. We originally applied for 3 years of funding, but the grant was awarded for 2 years. The project involves complex in vivo tumor grafting and the establishment of unique genetic mouse strains through cross-breeding. As described initially in the First Year Progress Report, (Objective 1, Task 1) it was unexpectedly necessary to create a new backcrossed mouse strain to obtain viable NF1+/− embryos. Also, we required scid mice to create another unique cross-bred mouse strain (Objective 2, Task 1). Obtaining healthy scid mice from the supplier was delayed several months (because of a viral infection in their colony), which caused cumulative delays in establishing the cross-breeds. Consequently, several aims of this project were not initiated as expected and our progress was delayed. An extension was approved to complete the Proposed Work.

Key Research Accomplishments

1) Established and characterized numerous Schwann cell cultures from Nf1 knockout mouse embryos.
2) Documented growth factor-independence and high proliferation by NF1+/− Schwann cells in vitro.
3) Effectively transfected NF1+/− Schwann cell cultures with AAV-GFP.
4) Implanted NF1+/− cells in mouse nerve and detected them with GFP immunohistochemistry.
5) Documented that NF1+− Schwann cells do not form tumors efficiently after syngraft to mouse nerve.
6) Reported findings (1-6) AT 2 national meetings in abstract/poster presentations.
7) Established a breeding colony of NF1+/−/scid mice (a unique strain).
8) Achieved sizeable tumors by engrafting human NF1 Schwann cells in mouse nerves.
9) Published a thorough characterization of human NF1 Schwann cells cultures and their tumorigenic properties.
10) Developed and applied MRI equipment and methods resulting in high resolution imaging of tumor xenografts.
11) Developed and applied methods to assess tumorigenic growth and neovascularization by engrafted NF1 tumor cells.

REPORTABLE OUTCOMES

Manuscripts

Abstracts


Animal Resources
New mouse immunodeficient mouse strain with the Nf1 background, Nf1<sup>+/−</sup>/scid.
CONCLUSIONS

Excellent progress was made in establishing Nf1−/− Schwann cell cultures. We documented heightened proliferative properties in these cells suggesting that Nf1-deficiency increases their tumorigenic potential. Nf1−/− Schwann cell cultures were effectively prelabeled with AAV-GFP which has proven to be a useful marker for cells implanted in the mouse nerve. Despite the tumorigenic properties observed in vitro, significant tumor formation by Nf1−/− implants has not been observed. We tested several methods to enhance tumor growth but none were successful. We examined several possibilities to circumvent potential pitfalls underlying their inability to growth as syngrafts, however, none of these resulted in tumor growth. Based on these data, we conclude that the in vivo tumorigenic potential of embryonic Nf1−/− Schwann cell is low. We can not exclude the possibility that the engraftment model per se is somehow non-permissive for tumor growth by these cells.

A thorough characterization of neurofibromin-deficient plexiform tumor-derived cell lines was published. This article included the first report of a xenograft model showing tumorigenic growth by human neurofibroma Schwann cells transplanted in the mouse nerve. Although most of the neurofibroma xenografts develop very slowly we also developed a more rapidly growing model of neurofibroma using a Schwann cell line derived from a MPNST. Sizeable tumors were grown in the mouse nerve and these were examined for tumorigenic growth, neovascularization and several key molecular components in this process. MRI has proven to be an effective means to assess tumor growth and may be used for volumetric quantitation to examine the effects of anti-cancer therapies.

A one-year, no cost extension was approved for the completion of the Proposed Work.

REFERENCES


APPENDICES

None.