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TITLE: Angiogenesis and Therapeutic Approaches to NF1 Tumors

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The main goal of this project is to specify how anti-angiogenic approaches can be effectively applied to NF1 tumors. To this end, we will first determine whether NF1 heterozygosity alters the responsiveness of endothelial cells to angiogenic regulators. We will test if Nfl-/+ endothelial cells are particularly responsive to pro-angiogenic factors produced by NF1 tumor cells and, perhaps even more importantly, which anti-angiogenic factors are most effective in abrogating the angiogenic response evoked by NF1 tumors. In particular, endostatin will be thoroughly examined as a potential anti-angiogenic therapy for NF1 tumors. Gene therapy for NF1 tumors has not been tested due to the lack of an appropriate NF1 tumor model. We have established a working xenograft model of neurofibroma in the mouse in which the efficacy of endostatin gene therapy will be assessed. This model involves the initiation of neurofibromas by implantation of human NF1 tumor-derived, neurofibromin-null Schwann cells into the nerves of mice with an Nfl-/+ background. Tumor progression and vascularity will be assessed in vivo by MRI non-invasive imaging. MRI data will be corroborated by endpoint histology using precise labeling of tumor and host cell components.
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INTRODUCTION

Neurofibromatosis type 1 (NF1) is a common genetic disease with a wide variety of features which primarily involve the nervous system and related tissues. NF1 is characterized by abnormal cell growth and a high incidence of neurofibroma, a nerve tumor composed predominantly of Schwann cells. Plexiform neurofibromas often grow very large and are debilitating or fatal to NF1 patients. Thus, there is a serious need for better therapies to manage NF1 tumor growth. To this end, we have developed and exploited two animal models of NF1. The first involves a strain of mice in which the Nfl gene was functionally deleted. These Nfl knockout mice are a valuable model for examining the biology of Nfl tissues both in vivo and in vitro. Secondly, we have cultured tumor cells from human NF1 tumors. These human cell lines form neurofibroma-like tumors when implanted into the mouse nerve. Using these resources and animal models we can examine the formation of NF1 tumors under controlled conditions. The Aims of this proposal are to determine how NF1 tumors induce the formation of new blood vessels and to test therapies to inhibit this process as an means to stop tumor growth.

There is considerable heterogeneity in the vasculature found in different tissue and tumor types. The first Aim of this work is to determine whether blood vessel formation might be altered in NF1 patients. For this we will use the Nfl knockout mouse. Endothelial cells will be cultured from wild-type and Nfl-/+ mouse tissues. The ability of these cells to form blood vessels in response to pro-angiogenic and anti-angiogenic factors will be tested in tissue culture assays. Important differences in the responsiveness of Nfl endothelial cells will be confirmed using in vivo assays conducted in wild-type and Nfl knockout mice.

We have established and characterized numerous cell cultures from human NF1 tumors, many of which have been grown as tumor grafts in the nerves of Nfl mice. We will test the hypothesis that the rate of growth by these NF1 tumor xenografts is associated with the degree of newly formed vasculature. Also, comparisons will be made between xenografts implanted in normal mice and Nfl mice. In vivo tumor growth and vascularity will be correlated with the expression of angiogenic regulators by the implanted cell lines. These experiments will test the hypothesis that tumor growth and invasion is dependent on the responsiveness of Nfl endothelial cells and other reactive cells in the nerve that contribute to tumor formation.

There are several anti-angiogenic factors that show excellent promise as potent inhibitors tumor growth. In this aim we will test endostatin as an anti-tumor treatment for peripheral nerve tumors in NF1. This Aim will be expanded to include other anti-angiogenic therapies based on discoveries made in the Aims described above. Gene therapy using endogenous angiogenic inhibitors, like endostatin, is considered by many to be the most promising approach to bring the anti-angiogenic therapy into the clinic. As a simplified experimental model, we will examine the growth and vascularity of tumor xenografts that are engineered to produce endostatin. Second, using a strategy more relevant to clinic treatment, we will apply an endostatin-viral vector (AAV-endostatin) to NF1 tumors already growing in the mouse. In both treatment models, growth and regression of tumor and neovasculature will be monitored in vivo by non-invasive magnetic resonance imaging (MRI) followed by autopsy examination of the tumor tissues. Our overall goal is to discover effective therapies for the treatment of plexiform neurofibromas by blocking the ability of these aggressive tumors to recruit the blood vessels required for their growth.
BODY

Technical Objective 1: EXAMINE THE RESPONSE OF NF1 ENDOTHELIAL CELLS TO ANGIOGENIC REGULATORS.

Task 1: Perform in vitro assays of Nfl +/- endothelial cell responses to pro-angiogenic factors:

Progress: This Task has been completed. In this aim we are testing the hypothesis that the in vitro response of Nfl +/- endothelial cells to pro-angiogenic factors differs from that of wild-type endothelial cells. We established cultures of endothelial cells from Nfl +/- knockout and wild-type mice. These cultures were characterized by immunocytochemistry for endothelial cell markers and for tube (vessel-like) formation in 3-dimensional culture. Endothelial cell cultures were established from microvessels isolated from Nfl +/- and wild-type littermates. In a base medium (containing serum but no endothelial cell mitogen supplements) approximately 5% of the wild-type endothelial cells had BrdU-positive nuclei compared to 7% of their Nfl +/- counterparts (Fig. 1). Treatment with endothelial cell growth supplement (a pituitary extract rich in mitogens) increased the BrdU-DNA to 13% for wild-type and 27% for Nfl +/- endothelial cultures. VEGF treatment caused a similar but less pronounced differential response. bFGF was a potent mitogen and more than doubled the proliferation of wild-type endothelial cells over that seen in the base medium alone. The response to bFGF by Nfl +/- endothelial cells was 3.6-fold greater than in base medium and nearly equaled that to the pituitary growth supplement. Overall, the response of Nfl +/- endothelial cells to mitogens was approximately double that exhibited by wild-type cells. These findings indicate that Nf1 heterozygous endothelial cells have an exaggerated mitogenic response.

Figure 1.

Mouse brain endothelial cell response to mitogens

Task 2: Perform in vitro assays of Nfl +/- endothelial cell responses to anti-angiogenic factors:

Progress: We have established the cultures required for this Task. The main aim is to test the response of Nfl +/- endothelial cells to endostatin. We have obtained an endostatin vector for use in subsequent in vivo testing. Cell cultures have been transfected with this vector and were found to produce endostatin. We are presently testing the biological activity of the endostatin product. Once this characterization is complete, this endostatin protein will be used to examine the response of Nfl +/- endothelial cells. Other anti-angiogenic agents will be obtained from commercial sources.
**Task 3:** Perform in vivo assays for angiogenesis in Nfl+/- knockout mice:

**Progress:** In this aim we are testing the hypothesis that the angiogenic responses of mice with an Nfl background differ from wild-type mice. The main goal of our in vivo angiogenesis assays has been accomplished. Developing, executing and analyzing in vivo assays for angiogenesis has been challenging and labor intensive. First, we established an in vivo angiogenesis assay that involves exposing newborn mice to an elevated oxygen atmosphere for 1 week followed by a return to normal atmosphere. The latter evokes a hypoxic response, including retinal neovascularization. New vessel formation was assessed by histological examination of sectioned retinas. Results showed that neovascularization in retinas from Nfl+/- mice was 66% greater than in wild-type litter mates (p<0.008). These data indicate that Nfl heterozygosity may, in general, exaggerate the angiogenic response. Results are shown in Fig. 2.

**Figure 2.**

To corroborate these findings, a mouse corneal neovascularization model was developed. In this model, the avascularity of the cornea highly facilitates the quantification of neovascularure induced by growth factors. Also, intrastromal implantation of a sucralfate bFGF pellet can induce a reproducible angiogenic response in a dose-responsive manner without inflammation. We conducted an initial test to confirm that Nfl+/- scid mice do not have an increased inflammatory response to the sucralfate pellet and could showed reliable corneal NV response to a growth factor. All eyes implanted with a pellet containing bFGF (90 ng) (n =20) showed abundant new blood vessel growth extending from limbal vessels and advancing toward the pellet (Fig 3A). In contrast, eyes implanted with placebo (n =10), like normal cornea, showed no vessel formation. For a quantitative assay, we prepared corneal flat-mounts stained with CD31 to reveal new blood vessels. Confocal fluorescence microscopy of CD31 immunostain showed greater new blood vessels in corneas implanted with 96 ng bFGF (Fig 3B) compared to 31 ng pellets (Fig 3C). Measurements of maximum new vessel length and circumference showed a positive correlation between the level of corneal NV and the concentration of bFGF. Increasing bFGF concentration from 31 ng to 96 ng per pellet resulted in 71% (P = 0.003, n=3) increase in maximum vessel length and one fold increase in vessel circumference (P = 0.0006, n=3). These data confirmed that the corneal NV induced by bFGF in Nfl+/-/scid mice is reliably reproducible in a dose-responsive fashion, and thus may provide a sensitive and reliable means to assess the effects of Nfl heterozygosity on angiogenesis. This finding indicates that heightened angiogenesis may play an important role in tumor development in NF1 patients and provides a foundation and justification for exploring anti-angiogenic therapies for neurofibroma.
To verify the effect of Nfl heterozygosity on angiogenic response, corneal NV induced by bFGF micro-implantation was compared between Nfl<sup>+/−</sup>/scid and Nfl<sup>+/+</sup>/scid mice. CD31-stained corneas at 6 days post-implantation (90 ng bFGF) showed an apparent increase of vascularity in Nfl heterozygous mice comparing to wild-type little mates (Figs. 4A and B). The maximum new blood vessel length measured from corneas of Nfl heterozygous mice (n=13) was 67% greater than that of wild-type controls (n=13) (P < 0.00002), while there was no significant difference between the circumferences in Nfl heterozygous and wild-type mice (P > 0.6) (Fig. 4D). Although corneal NV was apparent 4 days after bFGF implantation, the new blood vessel length examined at this time point showed no significant difference between Nfl heterozygous (n=9) and wild-type (n=9) mice (P>0.1), indicating a cumulative temporal effect of Nfl heterozygosity on angiogenesis. Taken together, these findings provide convincing in vivo evidence that Nfl heterozygosity significantly increases angiogenic response to both hypoxia and bFGF.
Task 4: Determine the angiotrophic potential of human tumor cell lines:

Progress: This task involves collecting extracts from NF1 tumor cultures and normal Schwann cell control cultures for testing in angiogenic assays. As stated for Task 1, our in vitro assays using endothelial cell cultures are established. On the other hand, we have collected and stored extracts from numerous NF1 tumor cultures and controls, but have not yet determined the angiotrophic potential of human NF1 cell lines.

Technical Objective 2: EXAMINE THE VASCULARITY AND ANGIOGENIC PROPERTIES OF NF1 TUMOR XENOGRAFTS.

Task 1: Develop MRI imaging of tumor growth and vascularity:

Progress: Our goal in this aim is to establish methods and parameters for MRI imaging of tumor growth and recession in a xenograft model of neurofibroma. Schwann cell cultures from NF1 patient tumors were implanted in the nerves of mice with an Nfl background. We have established and imaged numerous xenografts using various MRI parameters including T1 and T2-weighting. Also, the vascular properties of tumor xenografts were imaged using gadolinium enhancement. Xenografted tumors appeared as hyperintense regions on in vivo T2-weighted MRI. Figure 5 shows T2-weighted images from a representative xenografted mouse over time. A slight hyperintensity is seen two weeks after xenograft of sNF96.2 cells at the site of tumor cell injection (Fig. 5A). By five weeks, the tumor is easily visible (Fig. 5B) and is shown to increased in size by week eight (Fig 9C). In this and other experiments, the hyperintense tumor regions were shown to increase in size as the tumor developed and grew over time and were subsequently verified as xenografted sNF96.2 cells by huGST immunostaining. ThusT2-weighted, in vivo MRI is a useful tool for use in monitoring tumor growth over time and can subsequently be used to test the effectiveness of therapeutic agents in vivo. We have made excellent progress in imaging tumor xenografts by MRI and are ready to apply these techniques to monitor tumor growth and, in particular, tumor regression in response to anti-angiogenic treatments as required for subsequent aims.

Figure 5.

Task 2: Develop volumetric MRI and histology methods for tumor quantitation:

Progress: We have made excellent progress in the development of volumetric methods for quantitation of tumor growth by MRI and histology. As stated above, MRI imaging is now established and the data sets have been and continued to be studied by post-hoc image analysis. We made 3D renderings of many tumor image sets (from consecutive slices) and have performed volumetrics to indicate tumor size. Methods and software refinements were tested and volumes obtained. We are still learning about and setting criteria for defining tumor margins that specify areas of established tumor, growing margins and even regression. This is an ongoing aspect of this work that evolves along with improved imaging and image interpretation. In summary, we have learned how to calculate tumor volume from 3D composites.
and established initial criteria for volume calculations that correlate well between MRI and histology assessments. Although applying this to subtle aspects of tumor growth is an evolving skill, our methods are in place to assess tumor volume for conservative the quantitative scoring required in subsequent aims.

**Task 3:** Quantify the growth and neovascularity of NF1 tumor xenografts:

**Progress:** The development of MRI and histology imaging methods involve a continuing effort to improve and refine image quality, discrimination and quantitation. Our goal is to analyze and compare the growth and vascularity of various xenografts with different growth properties. Thus far, we developed two distinct xenograft models for NF1 tumors by engraftment of Schwann cell lines with varying tumorigenic properties, one representing plexiform neurofibroma and the other malignant peripheral nerve sheath tumor. Tumor growth has also been examined in animals by MRI at 2-week intervals, and tumor progression clearly delineated. Vascularity assessed by in vivo gadolinium enhancement has been established and quantified. To demonstrate increased vascular permeability, an assessment of angiogenesis, dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) was also performed 8 weeks after xenograft. DCE-MRI showed a hyperintense region in the xenografted area of the nerve, shown later by human GST immunostaining to be xenografted tumor, while a contralateral, uninjected sciatic nerve showed only a slight rise in contrast intensity. Approximately 17 minutes after contrast injection, when the level of contrast enhancement peaked, (Fig 6), the xenografted tumor displayed an average contrast enhancement 7.9 fold higher than the surrounding muscle while the normal, uninjected nerve showed only an average 2.1 fold increase over the surrounding muscle over the next 15 minutes. These results suggest an increased vascular permeability in the xenografted tumor, which correlate with our histological findings of tumor-induced angiogenesis. In summary, we have made excellent progress in the quantitation of tumor growth and vascularity by in vivo MRI imaging and this part of our work is complete.

**Figure 6.**

![Figure 6](image-url)
Task 4: Determine the effect of the Nfl +/- background on NF1 tumor xenografts:

Progress: This aim involves examining the growth of human cell line xenografts in wild-type and mice with an Nfl background. Our aim is to determine if the host Nfl background has an effect on tumor growth and if Nfl +/- cells might promote tumor growth compared to wild-type cells. Thus far, we examined the growth of two human NF1 tumor xenografts in Nfl +/- and wild-type mice. Tumors (n=6) were examined for extent of growth (size), proliferation, vascularity and infiltration of mast cells. Xenografts of sNF96.2 Schwann cells consistently proliferated rapidly and expanded quickly to large MPNST-like tumors. The resulting large tumors were firm, gray-tan in color, rapidly growing, and were similar in appearance to human NF1 MPNSTs. Figure 7 shows the gross morphology of a normal mouse sciatic nerve (Fig 7A) and a representative large 8 week-old sNF96.2 xenograft (Fig 7B). Nerves with large sNF96.2 MPNST-like tumors were up to five times the size of normal mouse sciatic nerves. Tumors in scid mouse sciatic nerves weighed 3.1 times more per millimeter (1.17 mg/mm +/- 0.293, n=5) than tumors from scid/Nfl +/- mouse sciatic nerves (0.38 mg/mm +/- 0.070, n=4) (Fig. 7C). Similar findings, though somewhat less dramatic were obtained for one other xenograft tumor type. Therefore, we conclude that Nfl +/- background does not enhance xenograft tumor growth. This findings was confirmed using other cell lines and contradicts recent findings by other labs using transgenic mouse models. We believe our xenograft model (using tumor cells with a more complex and representative genotype) is more predictive of tumorigenic growth in NF1 patients. Although this Task is mainly complete, we will verify these important findings in additional xenografts.

Figure 7.

![Image of Figure 7]

Task 5: Examine the angiogenic properties of Nfl +/- host cells within the NF1 tumor xenograft:

Progress: In this aim we will examine the possible tumorigenic and angiotrophic contributions of other Nfl +/- host cell types. We have already examined the distribution of host mast cells associated with the xenografts and found, while these reactive cells accumulate around the tumors, they rarely infiltrate the tumor cell mass. Next, we plan to examine mast cell association with neovascular elements at the tumor margins. We have not yet performed any immunocytochemical labeling to determine the expression of angiotrophic factors by reactive mast cells. In summary, only preliminary work on this aim has been performed.
Technical Objective 3: EXAMINE THE EFFECTS OF ANGIogenic INHIBITORS ON THE GROWTH AND NEOVASCULARIZATION OF NF1 TUMOR XENOGRaFTS IN NF1+/- MICE.

Task 1: Transduce endostatin in NF1 tumor cultures:
Progress: We have started transfection of AAV-endostatin into NF1 tumor cell lines. Transfection efficiency thus far is low, but we have been able to select for transfected cells and are growing stably transfected clones. This work is in its formative stages.

Task 2: Determine the effect of endostatin in vitro transduction on NF1 tumor xenografts:
Progress: No work has been done on this aim.

Task 3: Develop in vivo delivery of AAV-endostatin:
Progress: No work has been done on this aim.

Task 4: Assess the effect of endostatin delivery to established NF1 tumor xenografts:
Progress: No work has been done on this aim.
KEY RESEARCH ACCOMPLISHMENTS

1) Developed methods to culture brain microvessel endothelial cells from Nfl and wild-type mice.
2) Found that Nfl+/- endothelial cells have an exaggerated proliferative response to pro-angiogenic factors in vitro and in vivo.
3) Determined that the in vivo neoangiogenic response to hypoxia is heightened in retinas of Nfl+/- mice compared to wild-type mice.
4) Determined that the in vivo neoangiogenic response to FGF-2 is heightened in the corneas of Nfl+/- mice compared to wild-type mice.
5) Established and documented two valid xenograft models of NF1 plexiform neurofibroma and malignant peripheral nerve sheath tumors.
6) Established methods and assessed tumor growth and vascularity of NF1 tumor xenografts.
7) Quantified tumor growth by two xenografted NF1 tumors.
8) Imaged and quantified vascularity of xenografted tumors using MRI, gadolinium permeability and dynamic contrast enhancement.
9) Transfected NF1 tumor line with AAV-endostatin.

REPORTABLE OUTCOMES

Manuscripts: None published, 1 submitted, 3 nearing completion

Abstracts:

Animal Resources: None

CONCLUSIONS

Work on this research project has been conducted in a timely fashion with very good progress. In vivo and in vitro models were used to firmly conclude that NF1 haploinsufficiency in endothelial cells results in exaggerated proliferation and angiogenesis. Specifically, results indicate that the in vivo neoangiogenic response to hypoxia is heightened in retinas of Nfl+/- mice compared to wild-type mice. This finding was corroborated in a second in vivo model and we found that the in vivo neoangiogenic response to FGF-2 is heightened in the corneas of Nfl+/- mice compared to wild-type mice.

We have made excellent progress in aims to establish reliable procedures for xenografting of human NF1 cell lines in the mouse. Thus, far, we established and documented two valid xenograft models of NF1 plexiform neurofibroma and malignant peripheral nerve sheath tumors. Using these models, tumor growth and vascularity of NF1 tumor xenografts has been quantified by advanced MRI, gadolinium permeability and dynamic contrast enhancement. Corroborative histological measures are nearly complete and thus far show very high correlations, indicating the validity of the in vivo imaging approach.

In summary, the work and aims of this project are mainly proceeding on schedule. Definitive findings were made in the in vivo models of neovascularization and MRI assessment of tumor growth and vascularity. Work on other aims is progressing well. We are actively assembling our findings into reports for publication.
REFERENCES
None

APPENDICES
None