

Angiogenic and Invasive Properties of Neurofibroma Schwann Cells

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Abstract. Neurofibromas are benign tumors from patients with von Recklinghausen Neurofibromatosis (NF1) that are comprised primarily of Schwann cells. These Schwann cells are found both in association with axons and in the extracellular matrix that is prevalent in neurofibromas, and in which fibroblasts are also abundant. An unresolved question has been whether cells in neurofibromas are normal cells or are intrinsically abnormal. We have tested the hypothesis that cells in neurofibromas are abnormal and have shown that neurofibroma Schwann cells, unlike normal Schwann cells, promote angiogenesis in the chick

chorioallantoic membrane model system, and invade basement membranes in this system. In contrast, neurofibroma fibroblasts neither promote angiogenic reactions nor invade basement membranes. When injected into nude mice, neurofibroma Schwann cells do not form progressive tumors. These results suggest that NF1 Schwann cells differ from normal Schwann cells, that they are preneoplastic, and that genetic and/or epigenetic changes in Schwann cells may be required for development of peripheral nerve tumors in NF1.

A single gene defect causes 1 in 4,000 humans to develop a syndrome known as type 1 neurofibromatosis, which is inherited in an autosomal dominant manner (6, 8). von Recklinghausen type 1 neurofibromatosis (NF1)¹ is characterized by enormous variability in presentation; the number of different symptoms that can be expressed by patients with NF1 has made it difficult to assess which cell type, or types, are defective as a result of the NF1 mutation (33).

A common abnormality expressed in NF1 is the formation of peripheral nerve tumors called neurofibromas, which are composed of 60–85% Schwann cells and 10–20% fibroblasts, with lesser numbers of pericytes, perineurial cells, mast cells, endothelial cells, and smooth muscle cells, all of which are enveloped by extensive extracellular matrix (24). Several observations suggest that Schwann cells may be the primary target of factors stimulating neurofibroma formation (29). Schwann cells in normal nerves are found only in association with axons (50), whereas in neurofibromas they are present both associated with bundles of axons and free in the extracellular matrix (17, 27). In addition, Schwann cells are the major cell type amplified in neurofibromas (25, 42, 48), and malignant tumors (Schwannomas, neurofibrosarcomas) arising from neurofibromas are Schwann cell tumors (10, 15, 47). Strikingly, a rare form of neurofibroma termed “onion-bulb” neurofibromas are composed solely of concentric rings of Schwann cells surrounding axons (23).

However, it is not known whether neurofibroma Schwann cells are normal cells that behave aberrantly in the NF1

milieu or whether they are intrinsically abnormal. Some evidence suggests that neurofibroma Schwann cells might retain normal growth properties. We have recently shown that neurofibromas contain growth factor(s) that stimulate the proliferation of normal Schwann cells, and might stimulate Schwann cell division within tumors (29). In addition, when neurofibroma Schwann cells are placed into tissue culture, they do not proliferate. Instead, like normal Schwann cells, neurofibroma Schwann cells can be stimulated to divide when exposed to specific Schwann cell mitogens (19, 30, 39, 40).

In this study, we have determined the extent to which neurofibroma cells behave like normal or transformed cells by using several assays. The growth of tumors is dependent upon stimulation of angiogenesis (11–13), since solid tumors do not make their own blood vessels but induce them from the host (22, 49). We have previously shown that neurofibromas contain basic fibroblast growth factor (bFGF), a mitogen for mesenchymal cells (29) which is also a potent angiogenesis-promoting factor. Therefore, we have compared the ability of neurofibroma cells and normal Schwann cells to stimulate blood vessel formation, which can occur during transition of cells from hyperplastic to neoplastic state (13). We have also tested whether NF1 cells are able to invade basement membranes, since the ability to degrade matrix components is thought to be important in the development of the transformed phenotype (9, 37).

Angiogenic and invasive assays used the chorioallantoic membrane (CAM) of developing chick embryos, which provides a substrate for cells for long (up to 13 d) periods of time and enables observation of both angiogenic and invasive

1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; NF1, Von Recklinghausen type 1 Neurofibromatosis.

responses in the same experiment (1, 35). This model is useful since the chick embryo is immune incompetent, so that the behavior of tumor cells can be studied in the absence of a host immune response. In this assay, normal cells remain on the top of the CAM whereas transformed cells invade the ectodermal and mesodermal layers of the membrane.

To further analyze properties of NF1 cells, we examined their capacity to form tumors in nude mice, since transformed cells can form tumors in these immunocompromised hosts (36).

The data presented in this report indicates that NF1 Schwann cells differ from normal Schwann cells. Unlike their normal counterparts, NF1 Schwann cells manifest several, but not all, characteristics of transformed cells. In contrast, neurofibroma fibroblasts do not display any characteristics consistent with a transformed phenotype.

Materials and Methods

Human Tissue Specimens

NF1 tumor samples were obtained incidental to therapeutic surgery from 15 patients ranging in age from 9–74 yr, of both sexes, meeting the diagnostic criteria for NF1 (6). Two subcutaneous neurofibromas, four cutaneous neurofibromas, five plexiform neurofibromas, and four undesignated neurofibromas were studied. Specimens were collected into sterile tissue culture medium and used within 36 h for CAM assay or cell culture. Two non-NF1 human peripheral nerves (sural) were obtained. One nerve was obtained from a patient (age and sex data not available) with brachial plexus palsy whose normal sural nerve was removed for transplantation with the shoulder girdle. Another was obtained from a 14-yr-old female with osteosarcoma whose leg was amputated due to tumor growth; the sural nerve was distant from the tumor, which was limited to the bone. This patient manifested no clinical peripheral neuropathy. Sural nerves were collected into tissue culture medium within 30 min of biopsy and stored for no more than 20 h at 4°C. For addition to the CAM, human sural nerves were stripped of epineurium, and minced into 1-mm³ pieces. No attempt was made to purify cells from these control nerves since the sural nerves were not large enough to generate sufficient numbers of cells for CAM assays.

Preparation of Neurofibroma Tissue Fragments and Neurofibroma Cells

Cell-rich nodules were dissected from neurofibroma matrix and minced into 1-mm pieces in Leibowitz's L15 medium. In some experiments cells were dissociated from these nodules. Tumor nodules (1–5 g) were thoroughly minced and washed in L-15 medium, passed through cotton gauze to remove large pieces, and suspended in L-15 medium containing 1.25 U/ml Dispase (crude; Boehringer Mannheim GmbH, Mannheim, Federal Republic of Germany), 0.05% (wt/vol) CLS 1 collagenase (Worthington Biochemicals, Freehold, NJ), 0.1% (wt/vol) hyaluronidase (Sigma Chemical Co., St. Louis, MO) (10 ml/g tumor) as described by Pleasure et al. (26). Digestion was carried out on a rotating shaker (60 revolutions per minute) at 37°C for 16–24 h. Dissociated cells were washed twice in DME containing 10% FCS followed by two washes in serum-free DME, then suspended in DME such that 10 μ l contained 5×10^5 cells for placement onto the CAM.

To enrich neurofibroma cells for Schwann cells, 8.3×10^6 dissociated cells were allowed to attach to 100-mm² tissue culture plastic dishes for 5 min in DME + 10% FCS; nonadherent cells were subjected to a second round of differential adhesion. Alternatively, after differential adhesion, neurofibroma cells were plated onto 100-mm dishes coated with air-dried rat tail collagen in DME containing 10% FCS for 14 d; medium was replenished every 3 d, then cells were subjected to two additional rounds of differential adhesion. The remaining nonadherent cells (using either protocol) were 85–90% S100⁺, as analyzed by immunostaining after plating onto glass Lab-Tek slides, indicating that the cultures were enriched in Schwann cells.

To enrich for neurofibroma fibroblasts, the attached cells from the differential adhesion step 5×10^5 were plated into a T-75 flask, expanded to confluence in DME with 10% FCS, and split 1:3 as necessary. Cells were

removed from the substrate by trypsinization, washed as described above, and added to the CAM 7 or 14 d after initial plating. These cultures contained cells that were 95% fibronectin⁺, 5% factor VIII⁺, and smooth muscle actin⁻, indicating that 95% of the cells were fibroblasts (and/or perineurial cells), and 5% were endothelial cells.

Additional Cell Culture

Normal rat Schwann cells were purified from neonatal rat sciatic nerve as described previously (5). Normal peripheral nerve fibroblasts were purified from the same sciatic nerve cultures by plating cells at low density, expanding them to confluence, and replating after trypsinization using a 1:10 split. Two to three subcultures were carried out before the addition of cells to CAM. RN22 Schwannoma cells and neurofibrosarcoma cells were grown in DME with 10% FCS. Neurofibrosarcoma cell lines were obtained from R. Martuza (Massachusetts General Hospital, Boston, MA), and T. Glover (University of Michigan, Ann Arbor, MI). These cells were maintained in DME with 10% horse serum and 10% FCS.

Chicken Chorioallantoic Membrane Assay for Angiogenesis

The surface of 6-d postfertilization chicken eggs (White Leghorn; Spafas, Inc., Roanoke, IL) were sterilized with betadine and the CAM exposed by cutting a window (1 cm²) on one side of the egg using the false air sac technique (1). After 24–48 h, 1-mm³ tissue fragments (one to three pieces) or cells (5×10^5 , washed twice in DME) were placed on the exposed CAM, the windows sealed with Scotch tape, and the eggs incubated in a humid incubator at 35°C. Eggs were removed from the incubator at intervals for observation using a stereoscopic dissection microscope and assessed for angiogenesis; more than five loops of host blood vessels, delineating the added tissue or cells, was scored as a positive angiogenic response. In some experiments, 0.1–1 ng bFGF (Collaborative Research, Bedford, MA) was adsorbed to $1 \times 1 \times 2$ -mm pieces of Gelfoam (Upjohn Co., Kalamazoo, MI) and placed onto the CAM (44).

Invasiveness Assays

The portion of the CAM containing cells or fragments was excised, fixed in 10% neutral buffered formalin, paraffin embedded, and 6- μ m sections cut through the entire tissue block. For histological analysis, the sections were stained with hematoxylin and eosin and each section analyzed for the presence of tumors within the CAM.

Polystyrene Bead Experiments

11.9- μ m polystyrene beads (Ernest F. Fullam, Inc., Latham, NY) were washed in DME + 10% FCS twice by centrifugation, washed twice in DME, counted in a hemocytometer, and 5×10^5 added to the CAM in 10 μ l DME. Alternatively, beads were mixed with equal numbers of neurofibroma Schwann cells or neurofibroma derived fibroblasts, then harvested by centrifugation and added to the CAM.

Nude Mice Assays

Neurofibromas were obtained immediately after biopsy, minced in L-15 medium, the medium aspirated, and the resulting fragments suspended in 1 ml L-15 and injected into the subcutaneous tissue on both sides of the flank (0.5 ml/side) of 10-wk-old BALB/c-derived nude mice (Harlan Sprague Dawley, Indianapolis, IN) using a trocar needle and syringe (13-gauge). The inoculated mice were observed at weekly intervals and tumor growth was measured in three dimensions using a millimeter ruler. Animals were observed for up to 15 wk. Six animals showing palpable tumor were killed after 2–3 mo, the tumors removed and fixed in 10% neutral buffered formalin, cryoprotected, and frozen. 10- μ m cryostat sections were stained with hematoxylin and eosin or immunostained.

Immunohistochemistry

To identify cells in paraffin sections, sections were dried onto glass slides, deparaffinized, and then rehydrated. Sections were washed in phosphate-buffered saline and endogenous peroxidase activity blocked by a 15-min incubation in 0.3% hydrogen peroxide in methanol, washed in PBS, and treated with 0.1% trypsin (1:250; Gibco Laboratories, Grand Island, NY)

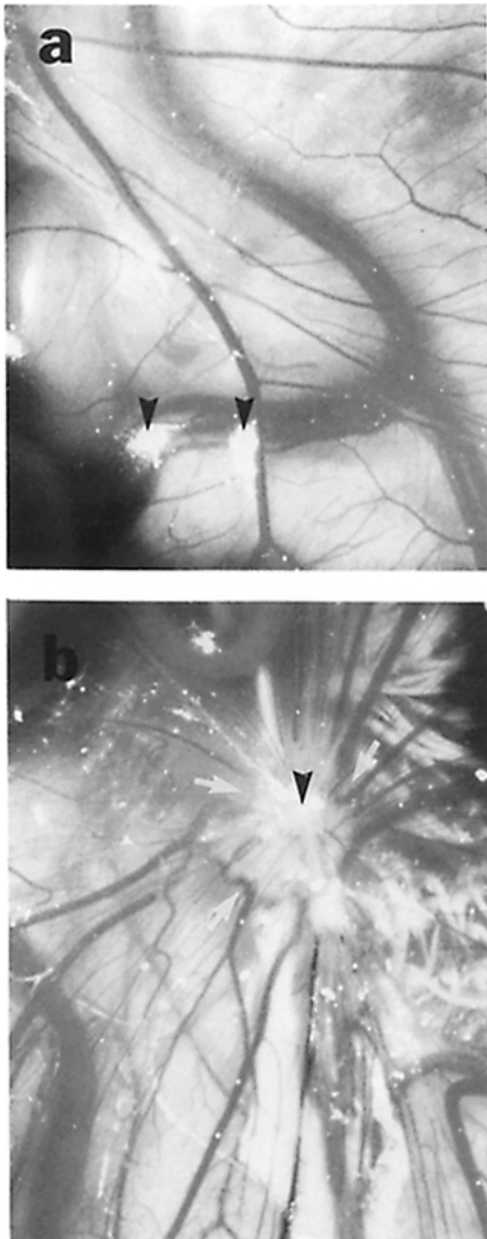


Figure 1. Angiogenic response of the chicken CAM to neurofibroma fragments. In *a*, fragments of non-NF1 human sural nerve were placed onto 7-d-old embryonic CAM. Blood vessels show a random arrangement with respect to the tissue fragments (arrowheads). In *b*, neurofibroma fragments were placed onto the CAM and stimulated a vigorous angiogenic response; many loops of host blood vessels (white arrows) delineate one fragment (arrowhead). Both specimens were photographed 10 d after addition of human tissue to the CAM.

and 0.1% calcium chloride, pH 7.6 for 15 min at 37°C. Sections were blocked in 2% normal goat serum diluted in PBS containing 1% BSA. The sections were then incubated with antibody overnight at 4°C or at room temperature for 30 min. Biotinylated second antibody (Vector Laboratories, Inc., Burlingame, CA) was visualized as directed by the manufacturer, using the ELITE kit. Staining for S100 (1:300) and Factor VIII (1:400) (Dako Corp., Santa Barbara, CA), fibronectin (1:100) (Biogenex Laboratories, Dublin, CA), and nerve growth factor receptor (1:2) (31) was carried out under identical conditions, using appropriate second antibodies. In some experiments anti-S100 was incubated with S100 protein (a gift of Dr. B.

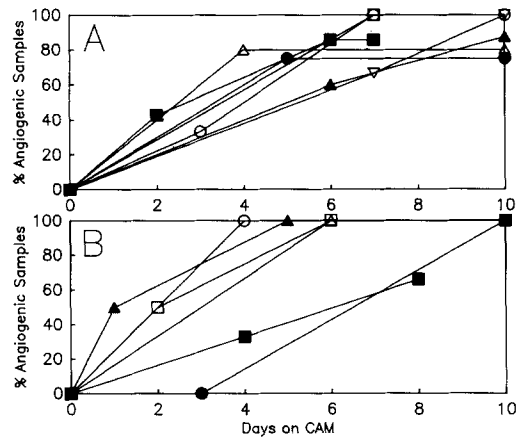


Figure 2. Angiogenic response of the chicken CAM to neurofibroma fragments or cells is time dependent. Neurofibroma fragments (A) or cells dissociated from neurofibromas (B) were placed on 6-7-d-old embryonic CAM and scored for angiogenesis at various intervals thereafter. Each symbol represents the percent of angiogenic reactions in four to six eggs incubated with cells or fragments from a different neurofibroma.

Moore, Washington University, St. Louis, MO) for 1 h before addition to sections; in other experiments the negative control was nonimmune rabbit serum; in either case, no staining was observed. S100 and nerve growth factor antibodies have been previously shown to label neurofibroma Schwann cells (41, 42). In some experiments sections were counterstained with hematoxylin and eosin. Cryostat sections were stained using the same procedure, except that deparaffinization and trypsinization were omitted and sections were blocked in 10% normal goat serum. Cultured cells were fixed in -20°C 100% methanol for 10 min, washed, and stained as described beginning with the blocking step.

Results

Induction of Angiogenesis

Normal rat Schwann cells, normal rat peripheral nerve fibroblasts, and fragments of non-NF1 adult human nerve

Table I. Neurofibroma Fragments and Cells from Neurofibromas Invade the Chick CAM

Sample	Invasion	Number of samples	Number of experiments
	%		
Non-NF1 human nerve fragments	0	12	2
Normal rat Schwann cells	0	16	3
Normal rat fibroblasts	0	11	2
Neurofibroma fragments	46	41	7
Neurofibroma cells	36	22	8
Rat Schwannoma, RN22	100	11	2
Human neurofibrosarcoma	100	6	2

Normal human sural nerve fragments (1 mm³), neurofibroma fragments (1 mm³), or dissociated cells (5 × 10⁵) were placed onto the chick CAM for 7-13 d. The CAM was then excised and fixed, and embedded in paraffin. Invasiveness was scored by microscopic inspection of hematoxylin and eosin stained paraffin sections. A sample was counted positive for invasion if cells had invaded only the ectoderm or both the ectoderm and mesoderm. Invasion of the ectoderm without mesodermal invasion occurred in 2 out of 63 samples of neurofibromas and never in other samples. Results obtained for invasion of neurofibroma fragments from different tumors are: % invasion (number of samples): 60 (5), 80 (5), 40 (5), 50 (6), 11 (9), 38 (8), 50 (6). The results for neurofibroma cells are 50 (2), 33 (3), 25 (4), 0 (1), 33 (6), 50 (6).

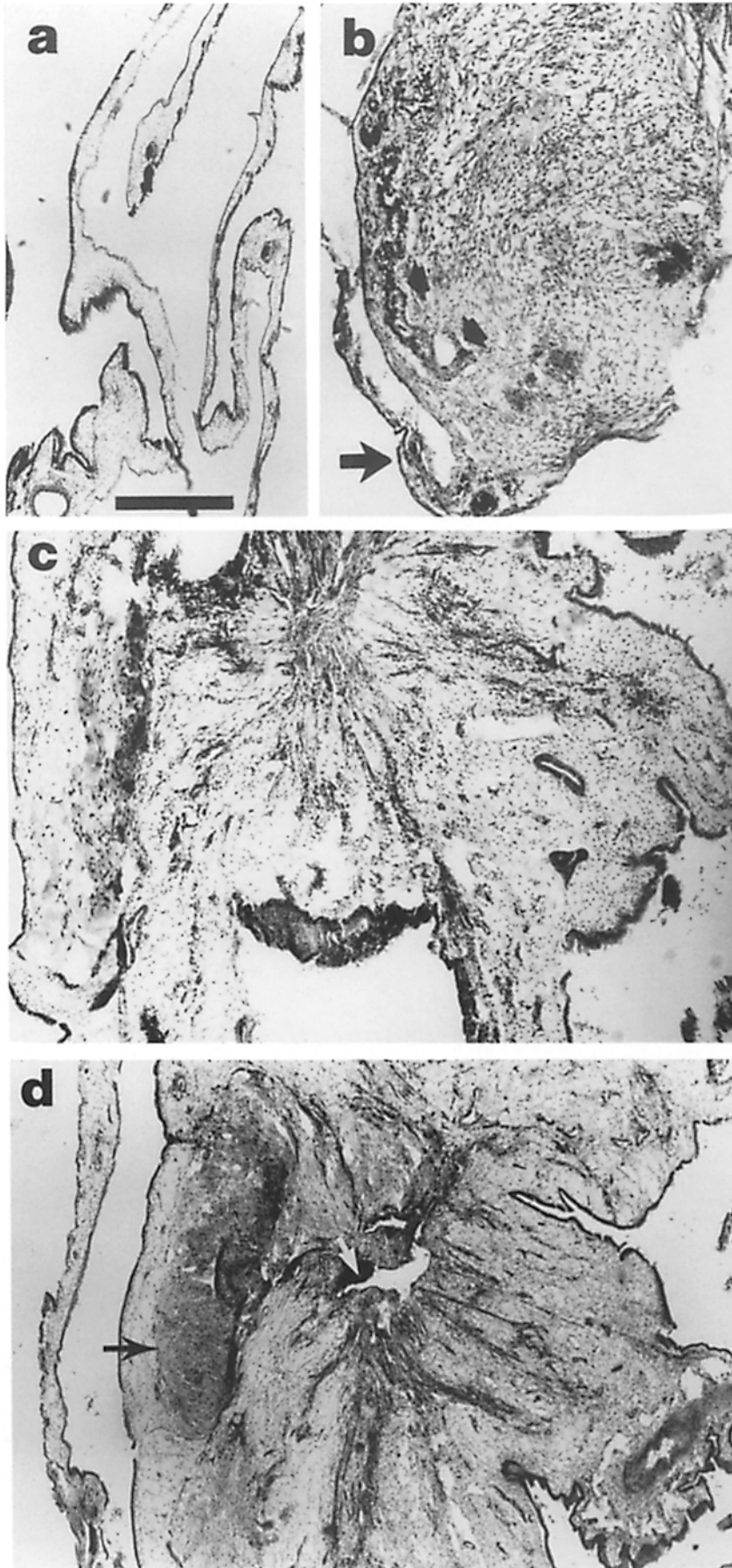


Figure 3. Histological sections of chicken CAM show the invasiveness of neurofibroma fragments and cells. Normal non-NF1 human sural nerve fragments, neurofibroma fragments, or cells dissociated from neurofibromas were placed onto the CAM for 10 d. The CAM was fixed, embedded in paraffin, and 10- μ m sections stained with hematoxylin and eosin. Normal nerve fragments (*a*) failed to invade CAM, leaving the CAM layers intact; NF1 fragments (*c* and *d*) or cells (*b*) caused the invasion of many cells into the mesoderm with enormous enlargement of the CAM. In *b*, small arrows show a large blood vessel, and the large arrow shows a region of normal CAM at the edge of the enlarged CAM tumor. In *d*, the white arrow points toward a large area of necrosis, while the black arrow points towards a tumor nodule containing tightly packed cells. Bar, 500 μ m.

failed to induce an angiogenic response on the CAM. In contrast, NF tumor fragments from eight patients stimulated angiogenesis, which was easily detected by visual inspection; loops of blood vessels delineated the fragments (Fig. 1). Angiogenesis was detectable in 25% of the samples after 2 d. By 10 d, 80% of eggs to which neurofibroma fragments had been added showed an angiogenic response (Fig. 2 A). In contrast, the angiogenic factor bFGF (0.1 ng) placed onto the CAM stimulated angiogenesis within 48 h in six out of six samples. The more gradual increase in angiogenesis observed after addition of tumor fragments to the CAM suggested that angiogenic factor(s) might be synthesized in neurofibromas, and not simply synthesized elsewhere and accumulated in the tumor matrix.

To distinguish these alternatives, cells were dissociated from neurofibromas, washed, and placed onto the CAM. Cells from six tumors were analyzed in this manner; as in the case of fragments, neovascularization increased with time. 95% of the samples tested showed blood vessel formation by 10 d on the CAM, suggesting that neurofibroma cells themselves produce angiogenic factor(s) (Fig. 2 B).

To determine whether either or both of the two major cell types in neurofibromas, Schwann cells, or fibroblasts was responsible for neovascularization we prepared cultures enriched for each of these cell types and applied them individually to the CAM. Cultures containing >95% fibroblasts did not stimulate angiogenesis (0 out of 12 samples stimulated angiogenesis), while Schwann cell-enriched cultures (85–90% S100+ cells) were highly angiogenic (6 out of 6 samples showed angiogenesis), suggesting that Schwann cells are responsible for the angiogenic response. This phenotype was retained after Schwann cells were removed from the tumor environment, since Schwann cell-enriched cultures, maintained *in vitro* for 2 wk before addition to the CAM, retained their ability to stimulate angiogenesis in six out of six samples.

Invasiveness of Neurofibroma Schwann Cells

Normal rat Schwann cells, normal rat fibroblasts, fragments of non-NF1 human nerve, and neurofibroma fibroblasts did not invade the CAM (Table I). In contrast, neurofibroma fragments and dissociated cells from nine different tumors applied to the CAM were invasive (Table I and Fig. 3). Invasive tumor cells infiltrated the CAM ectoderm and expanded into loosely arranged stroma of the mesoderm. Large tumors frequently induced areas of necrosis within the CAM mesoderm. Of neurofibroma fragments added to the CAM, 46% (19 out of 41) were invasive (Table I). Similarly, 36.4% (8 out of 22) of dissociated cell samples from neurofibromas were invasive. Similar percentages were observed at 5, 7, 10, or 13 d of incubation on the CAM; data was therefore pooled.

To identify the cell type(s) with invasive properties, paraffin sections were immunostained with antibodies that recognize S100 or nerve growth factor receptor (to mark Schwann cells), factor VIII (to mark endothelial cells), or fibronectin (to mark fibroblasts). Sections of CAM incubated with fragments of non-NF1 human nerve did not show any staining for S100 or nerve growth factor receptor within the ectoderm or mesoderm; stained cells were present only on top of the ectodermal layer (not shown). In contrast, when neurofibroma cells were added to the CAM, sections showed cells within the CAM mesoderm which were stained by the Schwann cell markers (Fig. 4, *b* and *c*). Lesser numbers of cells within the CAM mesoderm were labeled by using anti-fibronectin (Fig. 4 *a*). Factor VIII-specific antisera stained a few cells in some sections, and none in other sections (not shown). At the concentrations used, anti-fibronectin and anti-factor VIII antisera did not stain chicken epitopes within the CAM mesoderm. However, factor VIII antibodies did stain cells associated with chicken blood vessels.

As further confirmation that the Schwann cell is the major

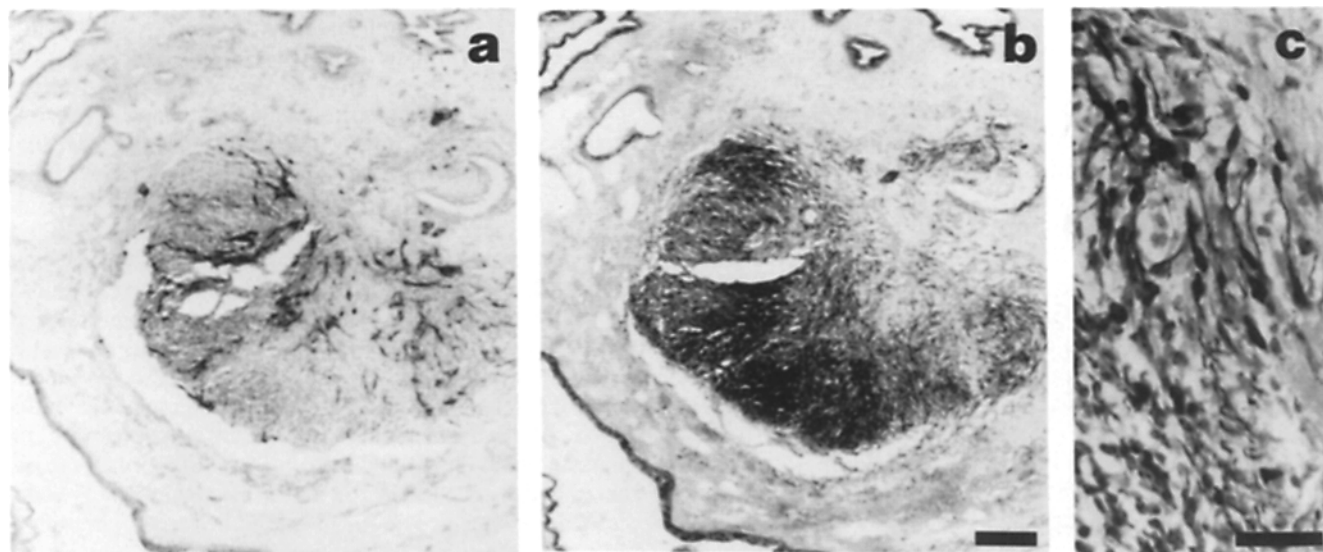


Figure 4. Immunohistochemical analysis of neurofibroma cells within the CAM. Neurofibroma fragments were placed onto the CAM as in Fig. 3, and serial 6- μ m paraffin sections stained with anti-fibronectin (*a*) to identify fibroblasts or anti-S100 to label Schwann cells (*b*). Sections were counterstained with hematoxylin before photography. In *c*, an enlargement of *b* showing the typical spindle-shaped morphology of cells stained by the S100 antibody, indicative of Schwann cells. Bars: (*a* and *b*) 100 μ m; (*c*) 200 μ m.

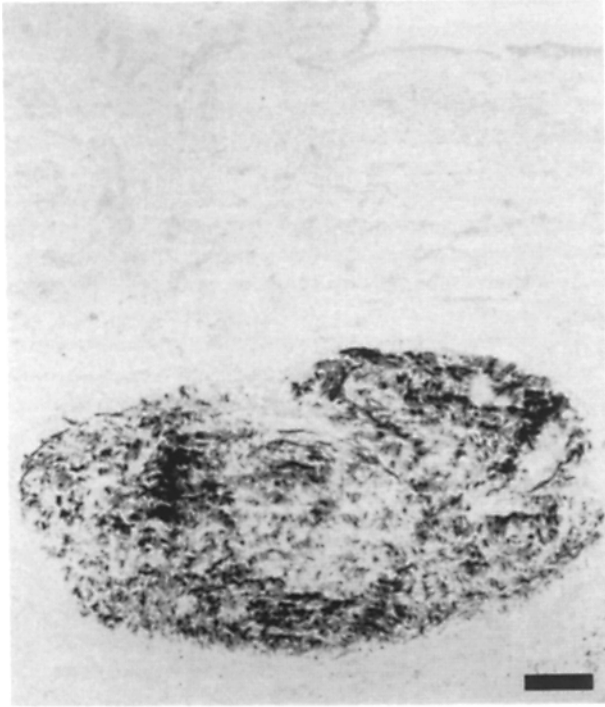


Figure 5. Neurofibroma Schwann cells invade the CAM. Schwann cells were enriched from neurofibroma cell cultures by differential adhesion. After 14 d in vitro, 5×10^5 cells were added to the CAM. 14 d later the CAM was excised, fixed, embedded in paraffin, and 6- μm sections were immunostained using a monoclonal antibody directed against human nerve growth factor receptor. No counterstain was used. A large number of cells within a tumor nodule are NGFR⁺ human Schwann cells. Bar, 400 μm .

invasive cell in neurofibromas, enriched cultures of Schwann cells or fibroblasts were prepared and separately added to the CAM. After 13 d, the CAMs were fixed. Histological sections from experiments in which Schwann cells were added to the CAM (six out of six) showed mesodermal tumors. In another experiment, Schwann cell-enriched cultures from different patients were maintained in vitro for 14 d before addition to the CAM. All of the samples (six out of six) showed invasion of the CAM. In contrast, neurofibroma fibroblasts from the same tumors failed to invade the CAM, further supporting the contention that Schwann cells in neurofibromas are responsible for the invasive behavior of neurofibromas. These results were confirmed by immunocytochemistry.

When paraffin sections of CAM to which Schwann cell-enriched cultures had been added were immunostained, large numbers of cells within the CAM were stained by S100 or nerve growth factor receptor antibodies (Fig. 5) whereas little, if any, specific cellular staining was observed using anti-fibronectin (not shown). Staining with anti-nerve growth factor receptor antibody is especially significant because this monoclonal antibody recognizes human, but not chicken Schwann cells, confirming that Schwann cells within the CAM are of neurofibroma origin. Human neurofibrosarcoma and rat Schwannoma cells were also invasive in this system (Table I), corroborating the idea that transformed Schwann cells are invasive.

Neurofibroma Schwann Cells Facilitate Entry of Beads into the CAM

We were puzzled by the observation that when mixed populations of neurofibroma cells were added to the CAM, not only Schwann cells but also fibroblasts and endothelial cells were detected in the CAM mesoderm. One possible explanation for this finding is that disruption of the ectodermal layer by neurofibroma Schwann cells allows nonspecific invasion of all tumor cells into the CAM mesoderm. To test this possibility, polystyrene beads were applied to the CAM alone or together with neurofibroma cells, the samples were fixed, embedded in paraffin, and sections analyzed. In samples containing beads alone or beads together with neurofibroma fibroblasts, the beads remained on top of the CAM ectoderm (Fig. 6, *a* and *b*). However, in the presence of neurofibroma Schwann cells, beads were observed not only on the surface of the CAM but also within the mesodermal layer (Fig. 6 *c*). These experiments suggest that while neurofibroma Schwann cells are likely to be sufficient for invasion of the CAM, other cells (or beads) can use breaches in the ectoderm formed by Schwann cells to enter the CAM mesoderm.

Dissociation of Angiogenic and Invasive Responses

The data in Fig. 1 and Table I indicate that 85% of samples containing tumor fragments or dissociated neurofibroma cells stimulated angiogenesis on the chicken CAM within 10 d. Invasion, however, was observed in only 55% of samples within the same time frame. This result indicates that angiogenic signals from neurofibroma cells were not sufficient to promote invasiveness. In addition, 2% of samples analyzed were invasive in the absence of angiogenesis, suggesting that invasiveness and angiogenesis are separable events in this system.

Growth of Neurofibromas in Nude Mice

Neurofibromas did not produce progressively growing tumors after injection into nude mice. Fig. 7 shows measurements of tumor volume from 20 samples (from eight patients) injected into nude mice. While none of these samples showed progressive growth, many transiently manifested small increases in volume over several weeks. By 10–13-wk post injection all tumors had become stationary or had regressed completely.

Cryostat sections of seven tumors were analyzed 8–12 wk after injection into nude mice by hematoxylin and eosin staining. In no case were areas of necrosis observed. Rather, a loose meshwork of extracellular matrix was observed, in which spindle shaped cells were embedded; nerve bundles were also observed (Fig. 8 *a*). A perineurial sheath around nerve bundles was not observed. To corroborate the identification of Schwann cells in these samples, sections were immunostained with antibodies to S100. S100⁺ cells were observed both within the matrix and in the apparent nerve bundles (Fig. 8 *b*). Since neuronal cell bodies are not present in most neurofibromas, the axons in these nerve bundles are probably of mouse origin, suggesting that neurofibroma Schwann cells form interactions with mouse axons and survive in nude mice for at least several months.

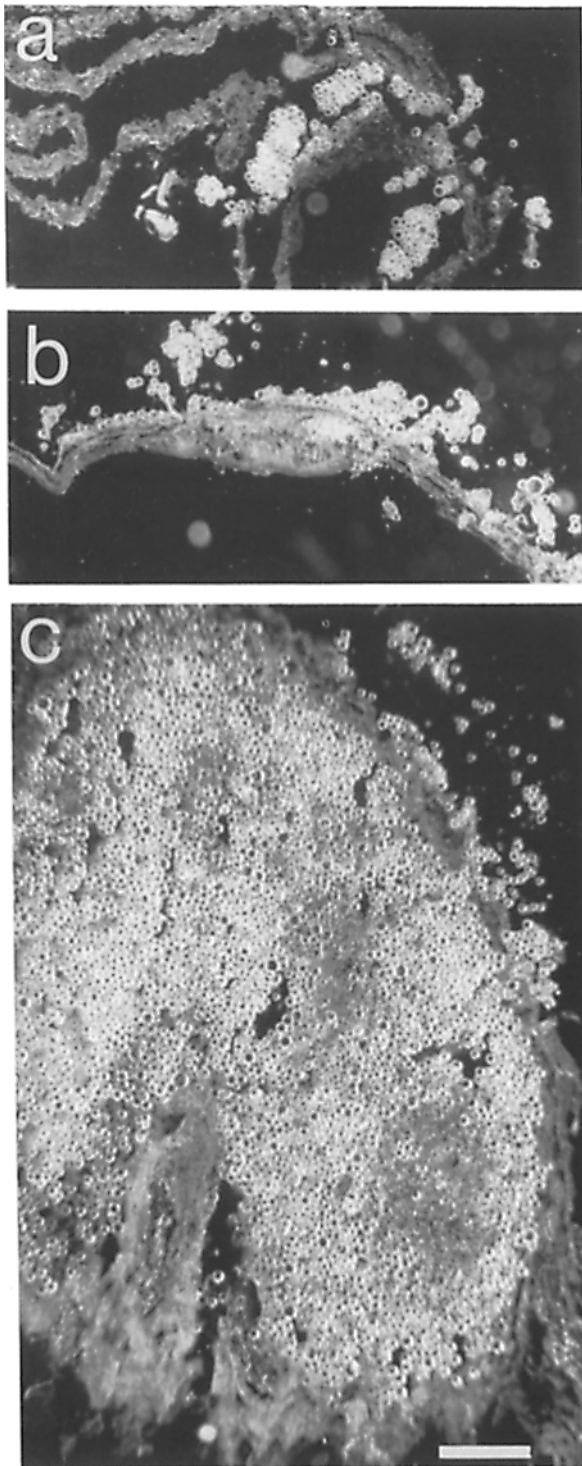


Figure 6. Nonspecific invasion of the CAM by polystyrene beads in the presence of neurofibroma Schwann cells. Polystyrene beads placed onto the CAM alone (a) or mixed with neurofibroma fibroblasts (b) stayed on top of CAM. In contrast, when beads were added to the CAM together with neurofibroma Schwann cells, beads entered the CAM mesoderm (c). Hematoxylin and eosin stained paraffin sections were photographed using dark field illumination. Bar, 176 μm .

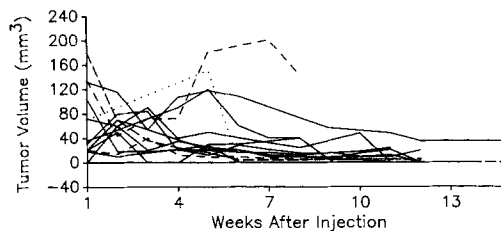


Figure 7. Neurofibroma cells do not form tumors in nude mice. A total of 20 neurofibroma samples were injected subcutaneously into nude mice; tumor volume was measured weekly. Each line represents measurements from a single sample. While many samples underwent transient increases in volume, all had completely regressed or stopped growing by 13 wk after injection.

Discussion

Our results show that neurofibroma Schwann cells produce angiogenic factor(s) and invade basement membranes. These properties are retained for at least 2 wk after separation of cells from their *in vivo* milieu. In contrast, neither fragments of normal human nerve nor fibroblasts purified from neurofibromas stimulate angiogenesis or invade basement membranes. These results indicate that angiogenic and invasive properties are restricted to Schwann cells within neurofibromas. Although unlikely, we cannot formally exclude the possibility that fibroblasts contribute to the expression of these characteristics by neurofibroma-derived Schwann cells, since all Schwann cell cultures retained some contaminating fibroblasts.

This report is the first to demonstrate that Schwann cells have angiogenic potential. Blood vessel formation can be stimulated by a number of polypeptides, including angiogenin, transforming growth factors α and β , epidermal growth factor, and the fibroblast growth factors, acidic and basic FGF; additional, less well characterized angiogenic factors have also been described (reviewed in reference 12). Which of these polypeptides contribute to stimulation of blood vessel formation by neurofibroma Schwann cells has not been addressed in this study. However, using biochemical and immunological criteria we have previously shown that the angiogenic factor bFGF is present in 60% of neurofibromas analyzed (29), suggesting that bFGF might contribute to neovascularization in most neurofibromas. Since all neurofibromas tested stimulated angiogenesis on the CAM, it is possible that additional angiogenic factors are produced in some, or all, neurofibromas. Alternatively, lesser amounts of bFGF, below the limit of our detection, may be produced in some neurofibromas; this explanation is consistent with the fact some neurofibromas placed onto the CAM required longer times than others to produce an angiogenic response.

The ability of neurofibroma cells to invade the CAM might be due to the production of a variety of enzymes, including collagenases and plasminogen activators, that have been implicated in the progression of normal to malignant cells (9, 21, 45, 46). Consistent with this proposition, proteolytic activity in neurofibroma homogenates is 10-fold higher than in normal human peripheral nerve (20). Many neurofibroma Schwann cells are separated from neurons *in vivo*. Since the amount of protease secreted by Schwann cells increases after denervation in a tissue culture model (3, 7), it was reasonable

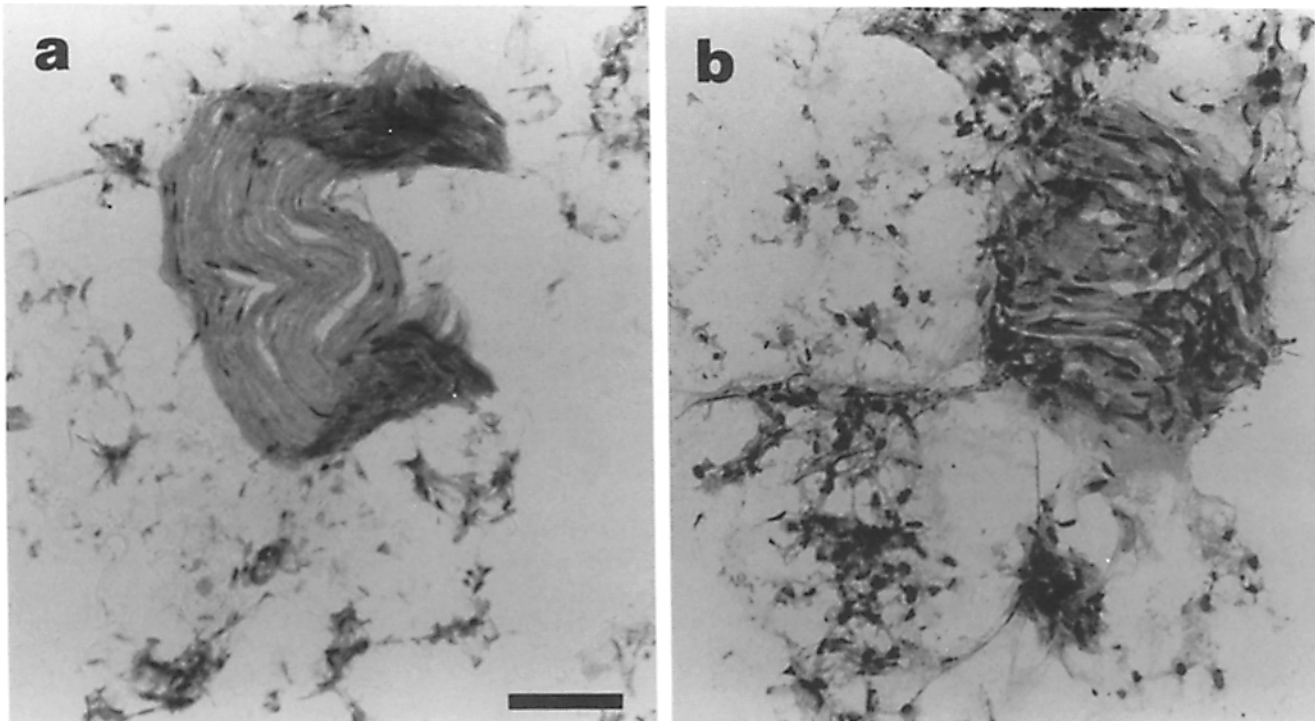


Figure 8. Neurofibroma cells in nude mice. Cryostat sections of stationary tumors were analyzed histologically after hematoxylin and eosin staining (*a*). A loose network of extracellular matrix in which spindle-shaped cells are embedded is shown, as is an example of a nerve bundle (*arrow*). Immunostaining for S100 (*b*) demonstrates that the spindle-shaped cells, both in the matrix and in the nerve bundle (*arrow*), are S100⁺ Schwann cells. Bar, 100 μ m.

to postulate that even normal Schwann cells, after separation from axons, would invade the CAM. However, neither fragments of adult human nerve containing Schwann cells nor embryonic rat Schwann cells separated from axons for three weeks are invasive in this system. These data suggest that axotomy alone is not sufficient to cause Schwann cells to become invasive.

After injection of neurofibromas into nude mice, tumor mass often underwent an initial increase. Transient increases in volume have been observed after injection of other benign tumors into nude mice (32, 34, 36) and may reflect vascularization of tumor samples or the differentiation of tumor cells in nude mice. Nerve bundles were present in sections of all the neurofibroma stationary tumors analyzed, suggesting that some Schwann cells injected into nude mice associate with host neurons. These data show that neurofibroma Schwann cells are competent to undergo the first step in Schwann cell differentiation characteristic of these sheath cells, adhesion to axons (38). Significantly, neurofibromas failed to form progressive tumors in nude mice, even after 5 mo. This observation is consistent with the clinical diagnosis of neurofibromas as benign tumors.

Our findings raise fundamental questions about the mechanisms of neurofibroma formation. Neurofibroma Schwann cells fail to proliferate *in vitro* unless stimulated by the addition of specific mitogens (20, 40), and fail to grow in nude mice. However, the transition from a normal to a neoplastic cell is thought to occur by a series of mutations which occur during cell proliferation (4, 13, 16, 43). Thus, hyperplasia serves as a contributing factor for the development of neoplasia. For example, it has recently been shown that angiogenic

activity in pancreatic islet cells appears after hyperplasia of cells but before the onset of tumor formation (13). In the absence of an intrinsic ability to proliferate, how does Schwann cell hyperplasia occur in patients with NF1? We have previously shown that Schwann cell mitogen is present in neurofibroma extracts (29). It is possible that mitogen production results in Schwann cell hyperplasia and leads to the formation of a population of Schwann cells that develops transformed properties. Other solid tumors are known to convert to more invasive variants by the outgrowth of small populations of cells that differ from the parental population (2, 18, 28). This model predicts that Schwann cell abnormality in neurofibromas is a consequence of genetic and/or epigenetic transformation event(s) secondary to the NF1 mutation, and therefore that Schwann cells not associated with neurofibromas in NF1 patients are untransformed. We favor this model since neurofibromas are present at some, and not all, locations in NF1 patients. However, it is also possible that the NF1 mutation itself causes Schwann cells to become invasive and to promote angiogenesis. If this is true, Schwann cells from peripheral nerves of patients with NF1 who do not have neurofibromas should display these phenotypes. Analysis of nonneurofibroma-related Schwann cells from NF1 patients in the transformation assays established in this paper will distinguish these hypotheses.

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