Neurofibromin-deficient fibroblasts fail to form perineurium in vitro

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SUMMARY

To identify cell type(s) that might contribute to nerve sheath tumors (neurofibromas) in patients with neurofibromatosis type 1, we generated cell cultures containing neurons, Schwann cells and fibroblasts from transgenic mouse embryos in which the type 1 neurofibromatosis gene was disrupted by homologous recombination (Brannan et al. (1994) Genes Development, 8,1019-1029). Normal fascicle formation by perineurial cells failed to occur in the absence of neurofibromin. Fascicles were reduced in number and showed abnormal morphology when normal neurons and Schwann cells were cultured up to 37 days with fibroblasts lacking neurofibromin. Proliferation was increased in a majority of fibroblast cell strains analyzed from embryos lacking neurofibromin. These observations suggest that mutations in the neurofibromatosis type 1 gene affect fibroblast behavior that might contribute to neurofibroma formation in patients with neurofibromatosis type 1.

Key words: neurofibromatosis, NF1, fibroblast, neurofibroma, perineurium, ras, transgenic mice, Schwann cell

INTRODUCTION

Patients with the inherited disease type 1 neurofibromatosis (NF1) develop benign peripheral nerve sheath tumors called neurofibromas, described as the hallmark feature of the disease by von Recklinghausen (Riccardi, 1981; von Recklinghausen, 1882). Neurofibromas occur as small, usually multiple, tumors associated with nerve branches or as tumor masses associated with major peripheral or visceral nerve trunks (plexiform neurofibromas) (Riccardi, 1981).

In normal peripheral nerves, contact between axons and Schwann cells is essential for Schwann cell proliferation and differentiation (Bunge, 1993; Bunge and Bunge, 1983; Eldridge et al., 1987; Webster and Favilla, 1993; Wood and Bunge, 1975). Specialized fibroblasts called perineurial cells surround axon-Schwann cell groups and provide a diffusion barrier (Thomas and Olsson, 1984). Inclusion of fibroblasts in neuron-Schwann cell co-cultures leads to differentiation of fibroblasts into perineurial cells and formation of fascicles that closely resemble those observed in vivo (Bunge et al., 1989; Williams et al., 1982; reviewed in Bunge, 1993).

Neurofibromas are caricatures of the normal organization of peripheral nerves. They contain mainly Schwann cells, fibroblasts, perineurial cells and axons (Fisher and Vuzevski, 1968; Hirose and Hizawa, 1986; Nakajima et al., 1982; Peltonen et al., 1988; Stefansson et al., 1982), embedded in abundant extracellular matrix (Penfield, 1932). Schwann cells make up 40-80% of the cells in neurofibromas as defined by positive staining for the S-100 protein (Peltonen et al., 1988); many Schwann cells within neurofibromas show no apparent contact to axons (Stefansson et al., 1982; Waggener, 1966). Cells that morphologically resemble fibroblasts, containing dilated cisternae of rough endoplasmic reticulum and no basal lamina, and perineurial cells, containing multiple smooth vesicles (called caveolae) and patchy basal lamina (Gamble and Eames, 1964; Peters et al., 1991; Thomas and Olsson, 1984; Ushigome et al., 1986), are both present in neurofibromas. It remains unclear how different cell types contribute to formation and growth of neurofibromas. Von Recklinghausen (1882) and Penfield (1932) suggested that the primary abnormal cell in neurofibromas is mesodermal cells. Fibroblasts produce extracellular matrix abundant in neurofibromas (Peltonen et al., 1984a,b, 1988). Neurofibroma-derived fibroblasts have been reported to show differences in expression of adrenergic receptors (Kaila et al., 1988), epidermal growth factor receptors (Zelkowitz, 1981), differential response to mitogens (Kadono et al., 1994) and altered organization of actin stress fibers (Hayashi et al., 1990; Peltonen et al., 1984c). However, other investigators have stressed the Schwann cell origin of neurofibroma cells (Fisher and Vuzevski, 1968; Murray et al., 1941; Waggener, 1966).

In 1990, the NF1 gene was identified and the NF1 cDNA cloned (Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990; Marchuk et al., 1991). The 220-280 kd protein
product of the Nf1 locus (neurofibromin) was subsequently identified (DeClue et al., 1991; Gutmann et al., 1991) and shown to be most abundantly expressed in the nervous system (Buchberg et al., 1990; Daston et al., 1992; Golubic et al., 1992), especially in neurons, Schwann cells and oligodendrocytes (Daston et al., 1992; Huynh et al., 1994). The NFI gene shares sequence homology with Ras GTPase activating proteins in Saccharomyces cerevisiae (IRA1 and IRA2) (Buchberg et al., 1990; Tanaka et al., 1990; Xu et al., 1990b) and mammals (GAP) (Trahey and McCormick, 1987; Xu et al., 1990a) suggesting that neurofibromin is a part of the Ras signal transduction pathway. As Ras has been shown to have multiple effects on cell proliferation and differentiation (Barbacid, 1987; Satoh and Kaziro, 1992), tumor formation in neurofibromatosis patients could be explained by deregulation of intracellular signaling events secondary to NFI mutations. In support of this hypothesis, neurofibromin levels are highly reduced while concentrations of GTP-bound p21Ras are elevated in cells derived from malignant neurofibrosarcomas (Basu et al., 1992; DeClue et al., 1992).

The role of mutations in the NFI gene remains to be determined for benign lesions of NF1, including neurofibroma formation. If mutations in the NFI gene are recessive at the cellular level, so that tumors arise only when both alleles acquire mutations, then the NFI gene could act as a tumor suppressor and fall into a group of genes including RB, WT1, P53 and NF2 (Marshall, 1991). However, markers on chromosome 17 on which the NFI allele is localized are not deleted in DNA from neurofibromas (Menon et al., 1990; Skuse et al., 1991), in contrast to results in malignant neurofibrosarcoma (Menon et al., 1990, Skuse et al., 1991).

In an attempt to define cells causally associated with neurofibroma formation and to test if alterations in both NFI alleles are required for abnormal cellular behavior in vitro, we have begun to study cell-cell interactions among cells from mice in which the NFI locus was disrupted by homologous recombination (Branman et al., 1994, Kim et al., 1995). Mice heterozygous for the NFI mutation do not develop neurofibromas. Mice in which both alleles of the NFI gene have been disrupted die in utero between embryonic days 11.5 and 14.5 (Branman et al., 1994, Jacks et al., 1994), so that tumor formation cannot be analyzed directly. To obtain cells deficient in neurofibromin, we generated cell cultures from embryonic day 12.5 NFI mouse embryos. Here, we report that, in our culture system, neurofibromin-deficient fibroblasts are sufficient to imitate in part the histologic phenotype of neurofibromas in human NF1 patients.

MATERIALS AND METHODS

Transgenic Nf1 mice

C57BL/6J mice in which one allele of the NFI gene has been targeted were generated as described elsewhere (Branman et al., 1994). In order to obtain NFI (−/−) embryos, male and female NFI (+/−) mice were mated for 12-16 hours, then separated and female mice checked for the presence of a copulatory plug. Pregnant mice were killed to obtain embryos after 12.5 days, with the day the plug was observed being day 0.5.

PCR conditions

For genotyping, embryo heads were removed, lysed in lysis buffer (Laird et al., 1991) overnight at 55°C and DNA isolated. One μl of head DNA was added to 25 μl of PCR reaction mixture containing 2.5 μl 10x reaction buffer (Perkin-Elmer Cetus), 200 ng of each oligonucleotide primer (NeoTkp, NF31a, NF31b) (Branman et al., 1994), 0.25 units of Taq polymerase (Perkin-Elmer Cetus) and a mixture of dATP, dCTP, dGTP and dTTP each at a final concentration of 0.2 mM. PCR was performed for 40 cycles using the following conditions: 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes. 8 μl of each amplification product was used for electrophoresis on a 1% agarose gel. A 194 bp band indicated a wild-type allele, a 340 bp band the mutant allele as described previously (Branman et al., 1994).

Tissue preparation from Nf1 mice and embryonic rats

Tissue cultures were obtained from E12.5 mouse embryos and from E15 rat embryos essentially as described (Bunge et al., 1983; Wood, 1976). Pregnant females were anaesthetized, using methoxyflurane (Metofane™, Pitman-More), a hysterectomy performed and embryos dissected out of amniotic sacs. Embryos were decapitated in Leibovitz’s L15 medium (Gibco). The spinal cord with dorsal root ganglia (DRG) attached was removed and DRG enzymatically dissociated in 0.25% trypsin in Hanks’ Balanced Salt Solution (Gibco) at 37°C on an orbital shaker at 40 revs/minute for 40 minutes. Ganglia were transferred to approximately 10 ml of Dulbecco’s Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco) and centrifuged at 800 revs/minute for 5 minutes. The pellet was resuspended in approximately 0.5 ml of ‘C’ medium (DMEM containing 10% human placental serum [provided by the delivery room of a local hospital] and 50 ng/ml nerve growth factor [2.5S NGF, Harlan Bioproducts]) and mechanically dissociated using a narrowed bore Pasteur pipette. This cell suspension (containing neurons, Schwann cell precursors and fibroblasts) was plated in single drops onto collagen-coated glass coverslips in 24-well plates, or on 35 mm dishes (Falcon), coated with ammoniated rat tail collagen. 5-6 individual drops of cell suspension were separately placed onto the hydrophobic collagen substratum so that drops did not flow together. Cells were allowed to attach overnight at 35°C and 7.5% CO2. The following day dishes/wells were flooded with ‘C’ medium. Medium was replaced at least twice per week.

Purified neuronal cultures

In order to obtain purified neuronal cell populations, rat cultures were treated with the antimitotics 5′-fluoro-2′-deoxyuridine and uridine (Sigma) added to ‘C’ medium at 10−5 M final concentration (‘CF’ medium) (Bunge et al., 1983). Cultures were cycled through two-three passages of C/CF; each medium was replaced after 3 days.

Preparation of rat primary Schwann cells

Primary Schwann cells were purified from sciatic nerve from postnatal day 1 rats as described previously (Brookes et al., 1979). Following isolation of Schwann cells by antimitotic treatment and complement-mediated lysis of residual fibroblasts, cells were maintained in DMEM plus 10% fetal bovine serum until plating onto neurons. In some experiments, Schwann cells were amplified for 1 week in recombinant human glial growth factor (rhGGF2, Cambridge Neuroscience) and 2 μM forskolin prior to addition to neurons.

Preparation of Nf1 mouse fibroblasts

Fibroblasts from E12.5 mouse embryos were obtained when embryos were dissected for DRG preparation. After removal of internal organs, head and spinal cord, remaining tissue of each embryo was transferred to a tube containing 2 ml of ice-cold 0.05% trypsin in 0.6 mM EDTA in Hanks’ Balanced Salt Solution and incubated overnight at 4°C. The following day, tubes were incubated for 20 minutes at 37°C, approximately 10 ml of DMEM containing 10% FBS added and the cell suspension centrifuged for 5 minutes at 800 revs/minute. Cells were resuspended in 1 ml DMEM with 10% FBS then mechanically dis-
sociated with a narrowed bore Pasteur pipette. The whole cell sus-
pension was plated onto one 100 mm tissue culture plastic dish con-
taining 10-15 ml of DMEM, 10% FBS, 1% penicillin-streptomycin and
maintained at 35°C and 7.5% CO₂ for several days until the cells
were nearly confluent. Fibroblasts were used immediately or at up to
3 passages for experiments.

Preparation of neuron, Schwann cell and fibroblast co-
cultures
2-week-old purified rat neuronal cultures on a collagen-coated 35 mm
dish were seeded with 0.3-0.5x10⁶ rat Schwann cells in ‘C medium’
and kept at 35°C and 7.5% CO₂ overnight. After 24 hours, cultures
were switched to a defined ‘N2’-medium (Bottenstein and Sato, 1979)
which stimulates Schwann cell proliferation but not myelination
(Moya et al., 1980) and maintained at 37°C and 10% CO₂. After 3-7
days, Schwann cells were distributed evenly over the whole neurite
outgrowth. At this time, medium was shifted to ‘C’ supplemented
with 50 μg/ml ascorbic acid (Sigma) to enhance Schwann cell differ-
entiation (Eldridge et al., 1987). At the same time 0.3-0.5x10⁶ Nf1
mouse fibroblasts per dish were added; cultures were maintained at
35°C and 7.5% CO₂.

Preparation of semithin sections
Cultures were fixed for 30 minutes at room temperature in 0.1 M
sodium cacodylate buffer pH 7.4 containing 2% paraformaldehyde,
1.25% glutaraldehyde and 100 mM sucrose. After 3 washes for 5
minutes each with 0.2 M cacodylate buffer, cultures were postfixed
for 60 minutes at room temperature with 0.1 M cacodylate buffer con-
taining 1% OsO₄ and 1.5% potassium ferrocyanide, washed again and
kept overnight at 4°C in 0.2 M cacodylate buffer. Cultures were dehy-
drated through 30%, 50% and 70% ethanol (5 minutes each), stained
en bloc for 30 minutes in 0.5% uranylacetate in 75% ethanol, further
dehydrated in 85%, 95% and 100% ethanol, and embedded in Epon.

Culture analysis
For EM analysis, ultrathin sections were stained with lead citrate and
uranyl acetate and photographed on a JOEL-10 CX electron micro-
scope at 10000-50000 magnification. Organotypic cultures derived from
4 wild-type, 14 Nf1 (+/−) and 7 Nf1 (−/−) embryos were evalu-
ed; 1-2 areas/culture were analyzed. For experiments in which
fibroblasts of different genotype were added to normal neurons and
Schwann cells for quantitative analysis, 3-8 different areas of each
culture were evaluated. Cultures from a total of 4 different embryos
were analyzed for each genotype. For all cultures, each block was
selected 2 mm outside areas containing neuronal cell bodies, areas
that are maximally differentiated (Obremski et al., 1993a). Semithin
plastic sections were analyzed at 400x magnification for fascicle
number. A fascicle was defined as a group of axons and Schwann cells
encircled for at least 75% of its circumference by one or more
elongated fibroblasts.

RESULTS

Cell cultures containing neurons, Schwann cell precursors and
fibroblasts were obtained by culturing dissociated cells from
sensory (dorsal root) ganglia from embryonic day 12.5 mice. These
organotypic cell cultures were prepared without prior
knowledge of the genotype from crosses of heterozygous males
and females. Within 16-24 hours after plating, viable cells had
attached to the collagen substratum. Neurons, identified as
round phase-bright cells, had extended processes. Phase-dark
cells (presumptive Schwann cell precursors and fibroblasts)
were located underneath the neuronal cell layer (not shown).
By 2-3 days in vitro, neurons became progressively more phase
dark and a population of cells attached to neuronal processes
could be defined, based on attachment to neurites, as Schwann
cells. Lack of neurofibromin did not by itself appear to cause
changes in neuron-Schwann cell adhesion or in neuronal differen-
tiation in response to nerve growth factor under these culture
conditions, although subtle abnormalities cannot be ruled out.
Fibroblasts continued to form a cellular background layer. No
obvious difference in viability or cell number was noted in
many cultures containing cells lacking neurofibromin as
compared to wild-type cultures until 9 days in vitro. However,
by 9 days in vitro, in some (60%) of null cultures axons
covered by Schwann cells appeared more obscured (Fig. 1C)
than in wild-type or heterozygous cultures (Fig. 1A,B) and
medium began to acidify more quickly than in cultures from
other genotypes. A possible explanation for these results was
increased cell number in some cultures lacking neurofibromin.
As we have shown that Schwann cell proliferation decreased
when null cells are in contact with axons (Kim et al., 1995), it
seemed unlikely that Schwann cell proliferation underlied
increased cell number. Therefore, we tested whether fibroblast
proliferation increased in the absence of neurofibromin.
Growth curves were constructed using fibroblasts of different
genotype in 10% fetal calf serum. In 4 out of 6 cell strains,
increased proliferation was detected in Nf1 (−/−) cells as
compared to wild-type or heterozygous cells (Fig. 2). In the
remaining Nf1 (−/−) cell strains, no difference in growth rate
was detected. This result has been replicated in 5-day growth
assays of fibroblasts from over 100 Nf1 mouse embryos; 80%
of Nf1 (−/−) cell strains showed increased growth over wild-
type cultures. Thus, fibroblast proliferation was increased in
null cell cultures from some but not all embryos. Western blot
analysis confirmed that fibroblasts from Nf1 (−/−) embryos
contained no detectable neurofibromin, while heterozygous
cells showed a reduction as compared to wild-type cells (not
shown).

After 18 days in vitro, cell cultures containing neurons,
Schwann cells and fibroblasts of each genotype were fixed and
analyzed in semi-thin plastic sections to determine if normal
organization of axons, Schwann cells and fibroblasts had
occurred. Cultures derived from 4 wild-type, 14 heterozygous
and 7 Nf1 (−/−) embryos were evaluated in a total of 4 experi-
ments.

As neuronal somata had been plated in drops on collagen
substrata, each sample contained discrete spots containing
neuronal cell bodies; neuritic processes extended from each
group of neuronal cell bodies radially. Cultures were fixed and
embedded in plastic; samples for analysis were selected 2 mm
outside the central area containing neuronal cell bodies in
regions that are maximally differentiated (Obremski and
Bunge, 1993a). Examination of semi-thin plastic sections by
light microscopy revealed a striking difference among cultures
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Fibroblasts in NF1
cell units and more space was evident between adjacent fibroblasts (Fig. 3C). In some NF1 (--/--) sections even this pattern of organization was missing (Fig. 3D) and fibroblasts were intermingled with axon-Schwann cell units and not confined to two separate layers surrounding axons and Schwann cells. Sections from NF1 (+/-) cultures had an intermediate phenotype (Fig. 3B).

As the fibroblasts in neurofibromin-deficient cultures appeared to behave abnormally, it seemed plausible that loss of neurofibromin in fibroblasts themselves might underlie this organizational deficit. To test this hypothesis, fibroblasts were expanded from E12.5 mouse embryos and 0.3×10^6 added to cultures of normal rat dorsal root ganglion neurons that were fully populated with rat Schwann cells. Rat-derived cells were used in this experiment because defined methods exist for purification of rat neurons and of highly purified rat Schwann cell cultures. It is not yet possible to generate fibroblast-free mouse Schwann cells. 17, 30, or 37 days after addition of fibroblasts, culture organization was analyzed by assessment of semi-thin plastic sections. At 17 days in vitro sandwiching of groups of axons and Schwann cells between multiple layers of fibroblasts was observed in all sections from cultures containing wild-type fibroblasts. Sections from cultures with Nf1 (--/-) fibroblasts did not show this organizational pattern and resembled those sections in which all three cell types lacked neurofibromin. Cultures containing Nf1 (+/-) fibroblasts did not show this organizational pattern and resembled those sections in which all three cell types lacked neurofibromin. Cultures containing Nf1 (+/-) fibroblasts exhibited an intermediate phenotype (data not shown). By 30 and 37 days in vitro, fibroblasts in wild-type cultures appeared increasingly flattened and had formed perineurium, defined as concentric layers of elongated, spindle-shaped fibroblasts closely surrounding groups of axons and Schwann cells (Fig. 4A). In wild-type cultures and those containing Nf1 (+/-) fibroblasts (Fig. 4B), most groups of axons and Schwann cells were organized into these fascicle-like groups that were further enclosed by multiple layers of tightly packed fibroblasts (Fig. 4A,B). Cultures with Nf1 (+/-) fibroblasts showed subtle differences from wild-type cultures including less compact bundling of axon-Schwann cell units and incomplete enclosure by fibroblasts (Fig. 4B). When cultures consisted of Nf1 (--/-) fibroblasts together with normal neurons and Schwann cells, fascicles and perineurium formation could be found only rarely; most semithin cross sections did not show any fascicle formation at all (Fig. 4C).
To quantitate the relative number of fascicles in cultures containing fibroblasts of different genotype, the number of perineurium-surrounded fascicles per culture was counted in 1 μm cross sections (Table 1). At 30 days in vitro, Nf1 (+/−) cultures showed 1% as many as those from wild-type sections. Cultures with Nf1 (+/−) fibroblasts contained 60% as many fascicles as wild-type cultures. In two experiments, the number of fascicles was counted after 37 days in vitro. Cultures containing fibroblasts without neurofibromin showed 17.7% and 23.8% as many fascicles as those with wild-type fascicles. As the number of fascicles in cultures containing Nf1 (−/−) fibroblasts was significantly higher after 37 days compared to only 30 days in vitro, it seems possible that the organizational deficit secondary to neurofibromin deficiency in fibroblasts might further improve over a longer incubation period. By 37 days, fascicle number in cultures containing Nf1 (+/−) fibroblasts was similar to that in control cultures.

When sections containing Nf1 (−/−) fibroblasts were evaluated using the electron microscope, it became apparent that even those regions of sections scored as containing fascicles at the light microscope level were in fact strikingly different from wild-type controls. Fascicles that appeared normal at low magnification (Fig. 5C) differed morphologically from those found in wild-type cultures when examined at higher magnification: increased endoneurial space was evident and fibroblasts did not completely enclose groups of axons and Schwann cells (Fig. 5D). More typically, axon-Schwann cell units were distant from the multiple fibroblast layers and were not collected into bundles (Fig. 5A,B). Myelinated and non-myelinated axons were present in all cultures, independent of fibroblast genotype (not shown). Schwann cells were identified by association with axons. No evidence for Schwann cells that lost axonal contact could be found, either in wild-type cultures or in cultures containing Nf1 (+/−) or Nf1 (−/−) fibroblasts.

Perineurial cells are defined as elongated cells containing clear vesicles called caveolae and covered by basal lamina on both surfaces; this basal lamina may be patchy (Thomas and Olsson, 1984). Cells with these characteristics were identified in cultures containing both Nf1 (−/−) and wild-type fibroblasts (Fig. 6A,B), suggesting that even fibroblasts without neurofibromin are capable of responding to cues from neurons and Schwann cells that result in perineurial cell differentiation. In addition, collagen fibers with similar diameter were present in different from wild-type controls. Fascicles that appeared normal at low magnification (Fig. 5C) differed morphologically from those found in wild-type cultures when examined at higher magnification: increased endoneurial space was evident and fibroblasts did not completely enclose groups of axons and Schwann cells (Fig. 5D). More typically, axon-Schwann cell units were distant from the multiple fibroblast layers and were not collected into bundles (Fig. 5A,B). Myelinated and non-myelinated axons were present in all cultures, independent of fibroblast genotype (not shown). Schwann cells

![Image A](image1.png)

![Image B](image2.png)

![Image C](image3.png)

![Image D](image4.png)

**Fig. 3.** Failure of organization in cultures lacking neurofibromin. Cell cultures containing neurons, Schwann cells and fibroblasts were obtained from wild-type, Nf1 (+/−) or Nf1 (−/−) mouse embryos, fixed after 18 days in vitro and analyzed in semi-thin plastic sections. Wild-type cultures showed a high level of organization with axon-Schwann cell units sandwiched between multiple layers of elongated fibroblasts (A). In most cultures lacking neurofibromin, fibroblasts were loosely arranged in two multilayers sandwiching axon-Schwann cell units (C). In some Nf1 (−/−) cultures, this organizational pattern was completely disrupted (D) and fibroblasts were intermingled with axon-Schwann cell units. Cultures from Nf1 (+/−) animals exhibited an intermediate phenotype (B). Arrows point to layers of fibroblasts above and below axon-Schwann cell units, arrowheads to axons associated with Schwann cells. In C, * denotes spaces between adjacent fibroblasts layers. Bar, 30 μm.

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<th>No. of Fascicles ± SEM</th>
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**Table 1. Reduced fascicle formation in cultures of wild-type neurons and Schwann cells with mutant fibroblasts**

Rat sensory neurons were fully populated with rat Schwann cells. Mouse fibroblasts of different genotype were added and cultures maintained for the designated number of days in differentiation-promoting medium. After fixation and embedding, at least 3 and up to a maximum of 9 blocks from each different embryo were evaluated in semithin plastic sections for fascicle formation. Fibroblasts from 4-5 different embryos were evaluated for each genotype. Within each experiment, fascicle number was averaged over all the blocks analyzed. Note that large variability in number of fascicles per section generated large standard errors.
wild-type and \( Nf1 (-/-) \) cultures (average fiber diameter in cultures of both genotypes was 18 nm, based upon measurement of several hundred collagen fibers); this fiber diameter is a characteristic of normal cultures containing fibroblasts together with axons and Schwann cells (Bunge et al., 1980). However, in cultures with wild-type fibroblasts, cells with characteristics of perineurial cells were the same cells that ringed bundles of axons and Schwann cells; fibroblasts distant from axons and Schwann cells were progressively less differentiated. In contrast, in cultures containing fibroblasts lacking neurofibromin, perineurial cell differentiation was observed in cells throughout the sections, with no preferential differentiation in cells close to axons and Schwann cells.

**DISCUSSION**

We used cell culture as a model system to study interactions among cells of the peripheral nerve that occur in the absence of the \( NF1 \) gene product, neurofibromin. Heterozygous mice in which the \( Nf1 \) locus was targeted by homologous recombination do not develop neurofibromas (Brannan et al., 1994; Jacks et al., 1994). However, we have now shown that, in cultures derived from these mice, several features of human neurofibromas are recapitulated.

Cultures containing normal fibroblasts and Schwann cells form cordons with Schwann cells inside a sleeve of fibroblasts (Obremski et al., 1993b) and fibroblasts form fascicles around axon-Schwann cell bundles when neurons are also present (Bunge et al., 1989; Williams et al., 1982, 1983). In contrast, as in human neurofibromas (Hirose and Hizawa, 1986; Ushigome et al., 1986), cultures consisting of normal neurons and Schwann cells together with neurofibromin-deficient fibroblasts contain cells with the typical morphology of fibroblasts and cells with characteristics of perineurial cells that are close to but do not contact or segregate Schwann cells. In this sense, the culture system that we have developed models cellular behavior in neurofibromas.

The mechanism(s) underlying the abnormal fibroblast behavior that we have observed remain unknown. Reciprocal signals between Schwann cells and fibroblasts that underlie peripheral nerve organization are also unknown (Bunge, 1993). Proximity to Schwann cells (and neurons) causes fibroblasts to differentiate into perineurial cells (Bunge et al., 1989) and fibroblasts are known to stimulate Schwann cell basal lamina deposition (Obremski et al., 1993b) suggesting that reciprocal fibroblast-Schwann cell signals underlie nerve fascicle formation. We conclude that neurofibromin is required for fibroblasts to form perineurium-like sheaths around axons and Schwann cells. Fibroblasts deficient in neurofibromin may fail to recognize and/or respond to signals from Schwann cells. However, fibroblasts lacking neurofibromin do respond to signals from Schwann cells sufficiently to differentiate into perineurial cells as assessed by expression of caveolae and basal lamina. It seems more likely that fibroblasts fail to send information to Schwann cells that results in fascicle formation. Our data suggest that molecules in the Ras signalling pathway might be crucial for neuron-Schwann cell-fibroblast interactions, as the only known function of neurofibromin is as a modulator (or possibly effector) of Ras signals (McCormick, 1994). Recent evidence suggests that early embryonic neurofibromin-deficient fibroblasts show elevated basal levels of Ras-GTP but no significantly enhanced response to the serum mitogen PDGF (van der Geer, P., Henkemeyer, M., Jacks, T. and Pawson, T., unpublished data). While in some cells loss of neurofibromin is correlated with increased GTP-bound Ras (Basu et al., 1992; DeClue et al., 1992; Kim et al., 1995), in other cell types such as melanoma and neuroblastoma cells (Johnson et al., 1994)) it is not.
Fig. 5. Abnormal fascicles in representative cultures containing Nf1 (−/−) fibroblasts. Most cultures containing fibroblasts without neurofibromin (prepared as described in Fig. 4) did not show any fascicle-formation at all (A,B). Axon-Schwann cell units (arrows) remained distant from each other and were intermingled with fibroblasts (short filled arrows). Some regions, however, showed a high level of organization with apparently normal fascicle-formation (C). Examination at higher magnification (D) revealed that ensheathment of axon-Schwann cell bundles by perineurial fibroblasts (open arrow) was in fact incomplete (double arrow) and endoneurial space was increased (asterisk). Bars: A,C, 10 µm; B,D, 2 µm.

Fig. 6. Perineurial cells differentiate in the absence of neurofibromin. Cultures were prepared as described in Fig. 4. Both wild-type (A) and Nf1 (−/−) (B) mouse fibroblasts exhibit characteristics of perineurial cell differentiation. Cells become elongated, are covered with patchy basal lamina (open arrows) on both sides and contain caveolae (arrows). Collagen fibers of similar diameter are present in both cultures (short filled arrows). Basal lamina also covered Schwann cells in cultures (curved arrows). In neurofibromin-deficient cultures, however, relatively undifferentiated fibroblasts (with distended rER) occurred adjacent to axon-Schwann cell units (double arrow). Bar, 0.25 µm.
Our data suggest that growth of mouse fibroblasts lacking neurofibromin is frequently, but not always, increased as compared to wild-type cells. Fibroblasts may be especially sensitive to fluctuations in levels of other gene products; this would result in increased growth only in certain cell populations. Differences between fibroblasts from different transgenic embryos might also reflect the influence of modifier genes which have been shown to affect the expression of the NF1 phenotype in humans (Easton et al., 1993), even on the relatively homogenous C57 background. Several studies using human fibroblasts from neurofibromas reported altered growth compared to normal fibroblasts. Krone et al. (1981) reported enhanced growth potential more often for NF1 fibroblast strains (in response to serum); Kadono et al. (1994) reported enhanced proliferation in response to TGFβ1 and PDGF BB. Others reported no change in growth rate (Riccandi and Margos, 1981) or even reduced growth (Zelkowitz, 1981), suggesting that heterogeneity may be characteristic of human as well as mouse fibroblasts with lowered neurofibromin.

It has been suggested (Brannan et al., 1994) that a feature of mice lacking neurofibromin is developmental delay. It remains possible that abnormalities that we have observed in neurofibromin-deficient fibroblasts would be ameliorated with sufficient time in vitro. While mouse fibroblasts heterozygous at the NF1 locus showed a qualitative phenotype intermediate between normal and null cells (Figs 3, 4), statistically significant differences in fascicle number between wild-type and heterozygous cells were not observed (Table 1). It is not known if abnormal cellular behavior in neurofibromas occurs in heterozygous cells or if loss of the previously normal somatic allele of the NF1 locus is required. Loss of heterozygosity on human chromosome 17 was not detected in neurofibromas (Menon et al., 1990; Skuse et al., 1991); however, these data are inconclusive because, if mutations at the NF1 locus were solely in fibroblasts, they might be obscured by normal NF1 alleles present in the more abundant Schwann cells. While data presented here suggest that loss of one wild-type allele at the NF1 locus could contribute to functional deficits in fibroblasts resulting in disturbed perineurium function, genetic tests are required to ascertain if mutations are present in both NF1 alleles in neurofibroma fibroblasts (or Schwann cells).

The perineurial sheath acts as a barrier regulating access of serum proteins to endoneurial cells (Thomas and Olsson, 1984). A failure of NF1 fibroblasts to form perineurium in vivo is predicted to result in a breakdown of this barrier function. Consistent with this view, plasma-derived fibronectin is present in neurofibromas (Peltonen et al., 1988). Breakdown of the perineurium could stimulate proliferation of endoneurial cells (fibroblasts and Schwann cells) by providing access to serum growth factors. Increased cell proliferation in response to serum growth factors might facilitate accumulation of mutations (at the NF1 locus or at other loci), contributing to neurofibroma formation. Thus, abnormalities in NF1 fibroblasts could provide the ‘ground’ on which additional mutations lead to tumor formation.

While the behavior of fibroblasts in culture is strikingly similar to that of fibroblasts in neurofibromas, behavior of Schwann cells in the cultures does not mimic behavior in tumors. In neurofibromas Schwann cells occur without apparent contact with axons (Stefansson et al., 1982; Waggener, 1966); however, we have not observed any Schwann cells lacking axonal contact in electron micrographs from many sets of cultures from cells without neurofibromin (Rosenbaum et al., unpublished data). Neurofibroma-derived human Schwann cells, like those from mice, associate with neurons and assemble basal lamina (Baron and Kreider, 1991). It is possible that epigenetic events modulate Schwann cell behavior in neurofibromas. As neurofibroma-derived Schwann cells exhibit properties different from normal Schwann cells (Kim et al., 1995; Sheela et al., 1990), it is likely that Schwann cell abnormalities, in addition to fibroblast abnormalities, contribute to neurofibroma formation.

We conclude that fibroblasts might play an important role in the formation of human neurofibromas. Our findings corroborate that neurofibromatosis should not be defined as a neurocristopathy (originally proposed by Bolande, 1974) as we have clearly demonstrated that loss of neurofibromin affects fibroblasts, cells of mesenchymal origin.

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