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epidermal growth factor
fibroblast

neurofibromatosis
ontogeny

Short Communication: Neurofibromatosis Fibroblasts: Slow Growth and Abnormal Morphology

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Summary

We hypothesized that skin fibroblasts from patients with neurofibromatosis (NF) may have abnormalities of growth in tissue culture to correlate with the clinical abnormalities of overgrowth and malignancy seen in this disease. Using five lines of NF cells, age- and passage-matched to normal controls, we found that NF fibroblasts grew more slowly and stopped growing at a lower population density than normal cells ($P < 0.0005$). The same cells also incorporated [^{3}H]thymidine at a lower rate than normal skin fibroblasts (9,330 ± 3,240 *versus* 42,100 ± 6,840; $P < 0.01$). The addition of epidermal growth factor to the medium stimulated the growth of both the normal and the NF fibroblasts; however, the stimulation of the NF fibroblasts was inadequate to fully correct the slow growth rate ($P < 0.025$). NF cells ($N = 5$) were found to be morphologically different from normal skin fibroblasts ($N = 5$) in culture by light microscopy. NF cells were larger (approximately $9 \times 10^4 \times 2 \times 10^4$ *versus* $2 \times 10^4 \times 2 \times 10^4$A), pleomorphic, and failed to form confluent monolayers when growth ceased.

Speculation

These data indicate that there may be an underlying abnormality of growth regulation in neurofibromatosis. The slow growth of neurofibromatosis fibroblasts, and their diminished response to epidermal growth factor, provides a means for studying the growth abnormality of neurofibromatosis in tissue culture. In addition, the expression of this abnormality may serve as a marker for the disease.

Neurofibromatosis (NF), also known as von Recklinghausen's disease, is a common autosomal dominant disease with a high incidence of disorders of growth, including malignant degeneration in excess of 15% (13, 20). At least nine different types of tumors, both benign and malignant, have been associated with NF (1, 5, 7, 8, 11, 13, 17, 19, 22, 24, 27, 30). Any cellular element may be involved, and disorders involving ectodermal, mesodermal, and endodermal overgrowth are all well described (12, 24, 29). These may include gross skin changes (8, 28), aberrations of bony growth (2, 12–16, 26), and hyperplastic changes of blood vessels (12, 23). The spectrum of these aberrations suggests a cellular defect in growth regulation as a primary abnormality. This study was undertaken to define whether growth abnormalities of NF fibroblasts from normal skin of affected patients could be seen in tissue culture.

MATERIALS AND METHODS

SOURCE OF FIBROBLASTS

Skin fibroblasts from normal skin of the forearm were either obtained commercially from the Human Mutant Genetic Cell Repository, Camden, NJ, or by skin biopsy, with informed consent.

TISSUE CULTURE

Approximately 3×10^4 cells were plated on 60 mm plastic Petri dishes (Falcon; Becton Dickinson, Cockeysville, MD) in 3.0 ml of Dulbecco's modified Eagle's medium, containing 10 units of penicillin and 10 mg of streptomycin per ml, as well as 10% fetal bovine serum and 10% calf serum (Grand Island Biological Co., Grand Island, NY). The cells were grown in an incubator (Forma Scientific model 3157) at 37°C with controlled humidity and 5% CO_2 and air. The medium was changed twice per week. All NF cell lines ($N = 5$) were matched as closely as possible for age of donor and passage number with normal controls ($N = 5$) (Table 1), and all were used for all studies.

GROWTH CURVES

Fibroblasts were dispersed in 0.25% trypsin, centrifuged to a small pellet, and resuspended in 1.0 ml of Hanks' balanced salt solution (HBSS) before cell counts by hemocytometer and/or protein determination by the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Richmond, CA). Cells were routinely screened for mycoplasma by Chen's method (6). For epidermal growth factor (EGF) (gift of B. G. W. Arnason, University of Chicago) stimulation experiments, the hormone was added in serum-free medium at a concentration of 40 ng/ml.

[^{3}H]THYMIDINE INCORPORATION

The medium was removed from the plate, and the cells were washed twice with HBSS, trypsinized at 37°C for 5 min, and centrifuged at 1500 rpm (International Equipment Co.; model K centrifuge). An aliquot was then counted, and the remainder was washed twice with HBSS and resuspended in medium with serum. The cells were then corrected to a concentration of 10^5 cells/ml, and 200 μl aliquots were placed in each of four wells in a plastic culture dish. For EGF influence, the experiment was done with 8 ng of EGF added to each well.

After a 48-hr incubation, the [^{3}H]thymidine (40 μCi/ml; 25 μl/well) was added, and the incubation continued for another 5 hr. The cells were then harvested onto filter paper using a multiple automatic cell harvester, dried overnight in a 60°C oven, and counted in an omnifluor-toluene counting fluid in a Beckman LS 7000 liquid scintillation counter.

LIGHT MICROSCOPY

Monolayers of cells were photographed *in vivo* using an inverted phase contrast microscope and a Nikon 35S camera at ×100 magnification.

RESULTS

GROWTH (FIG. 1)

Normal fibroblasts grew rapidly, achieved a high cell population density during their rapid growth phase, and stopped growing as they approached confluency. The NF cells grew slowly and went through a log phase, but stopped growing at a significantly lower cell density than NF fibroblasts ($P < 0.0005$). Control fibroflasts continued to grow at a normal rate for at least 20 passages, whereas the NF fibroblasts grew at the same slow rate until the tenth or eleventh passage, when they stopped dividing. Neither transporting the cells from an outside source nor freezing them in liquid nitrogen and thawing affected their growth or morphology. There was on average 0.1 mg protein per plate for both NF and normal cells (approximately 10^6 cells). Attempts to increase the viability of the NF cells by stimulation with EGF changing the medium daily or by increasing the concentration of fetal bovine serum up to 30% did not change the growth characteristics of the cells.

Transfer of the medium from NF cells to normal cells in culture did not change the growth characteristics of the normal cells. Neither did medium from normal cells correct the growth defect in the NF cells.

Both the normal and NF fibroblasts incorporated [^{3}H]thymidine, a specific DNA precursor. However, the NF cells incorporated it at a significantly lower rate ($P < 0.01$) (Table 2), suggesting a decreased synthesis of DNA by NF fibroblasts in this system.

EGF was placed in media of both cell types at a supraphysiologic concentration (40 ng/ml) to assure a maximal response. EGF increased the normal cell count from $45.5 \pm 3.3 \times 10^4$ to $197.8 \pm 70.9 \times 10^4$ cells/dish and the NF cell count from $8.8 \pm 2.9 \times 10^4$ to $10.6 \pm 3.3 \times 10^4$ ($\pm$ S.E.). The response of the normal cells was significantly greater than that of the NF cells ($P < 0.025$), with no overlap. There was an increase in [^{3}H]thymidine incorporation in both groups. However, the NF fibroblasts did not increase their rate of [^{3}H]thymidine incorporation to normal levels (Table 2).

Table 1. *Fibroblast culture*[1]

Cell line	Source	Diagnosis	Age	Sex	Passage
KY	UCHC	nl	3	F	7
RT	UCHC	nl	3	M	5
GM 2185	Camden	nl	36	M	7
GM 2987	Camden	nl	19	M	7
JK	UCHC	nl	2	F	6
GM 622	Camden	NF	8	M	6
SP	UCHC	NF	12	F	3
GM 1860	Camden	NF	41	M	6
GM 1633	Camden	NF	61	M	8
GM 1639	Camden	NF	19	F	8

[1] Source: Human Mutant Genetic Cell Repository, Camden, NJ.; University of Chicago Hospitals and Clinics.

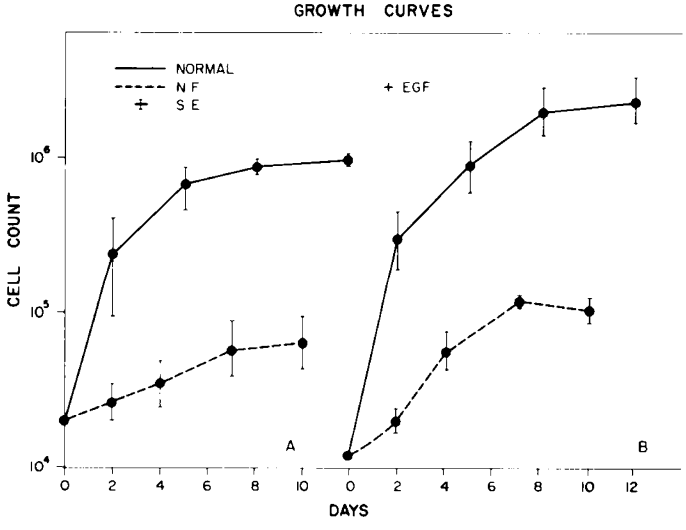

Fig. 1. *A*, normal and NF fibroblast growth curves over time in days (*abscissa*). *B*, growth response of normal and NF fibroblasts to EGF. *Vertical bars*, S.E.

MORPHOLOGIC STUDIES

The NF fibroblasts, all obtained from sections of normal skin, were morphologically abnormal by the fourth passage. Light microscopic examination of the cells (Fig. 2) showed them to be larger than normal fibroblasts and to lack the usual bipolar configuration (approximate dimensions: NF = $9 \times 10^4 \times 2 \times 10^4$Å; normal = $2 \times 10^4 \times 2 \times 10^4$Å). Whereas at confluency normal skin fibroblasts maintained their elongated bipolar shape and were aligned side by side, the NF cells maintained a stellate shape with multiple processes and showed a disoriented pattern. We did not observe similar changes in normal fibroblasts at the fourth passage. In addition, when normal cells became stressed (fungal or bacterial infection), they never assumed the configuration of the NF fibroblasts.

We have noted that these changes in growth and morphology of the NF fibroblasts are reversible by transferring the cells to Ham's F-12 medium. That these are reversible changes strongly indicates a critical need of the NF cells, not contained in Dulbecco's minimal essential medium. Experiments to determine these requirements are under way.

DISCUSSION

Neurofibromatosis is a common (incidence, 1:2000), but poorly understood, autosomal dominant disease. The disease is highly variable in its expression, and diagnosis can only be made by classical physical signs. It is, therefore, impossible to know if a seemingly unaffected family member carries the genetic defect.

NF is a disorder manifested by multitudinous growth abnormalities, and need not, therefore, be a "neurocristopathy" as Bolande suggests (3). Tumors are among the growth aberrations most frequently described. Among these tumors, acoustic neuroma, optic glioma, and meningioma are the most common. The more malignant meningiosarcoma is also more common in children with NF (7). Delleman *et al.* (10) describes a family with neuromas and multiple meningiomas through two generations involving five members. The increased incidence of rhabdomyosarcoma (18) and second malignancy (20) in patients with NF also supports the concept of a growth abnormality as the primary defect, but not necessarily an abnormality of neural crest origin.

Nonlymphocytic leukemias, which are rare in children, are

Table 2. *Effect of EGF on [^{3}H]thymidine incorporation in normal and NF fibroblasts*[1]

Cells	[^{3}H]Thymidine incorporation (cpm per 10^6 cells) −EGF	+EGF
Normals ($N = 5$)	42,100 ± 6,840[2]	62,950 ± 12,270
NF ($N = 5$)	9,330 ± 3,240	12,690 ± 3,770
P	<0.01	<0.01

[1] Experiments were performed as described in the text. All cells were age- and passage-matched, and all studies were done after 48 hr of subculture.

[2] Mean ± S.E.

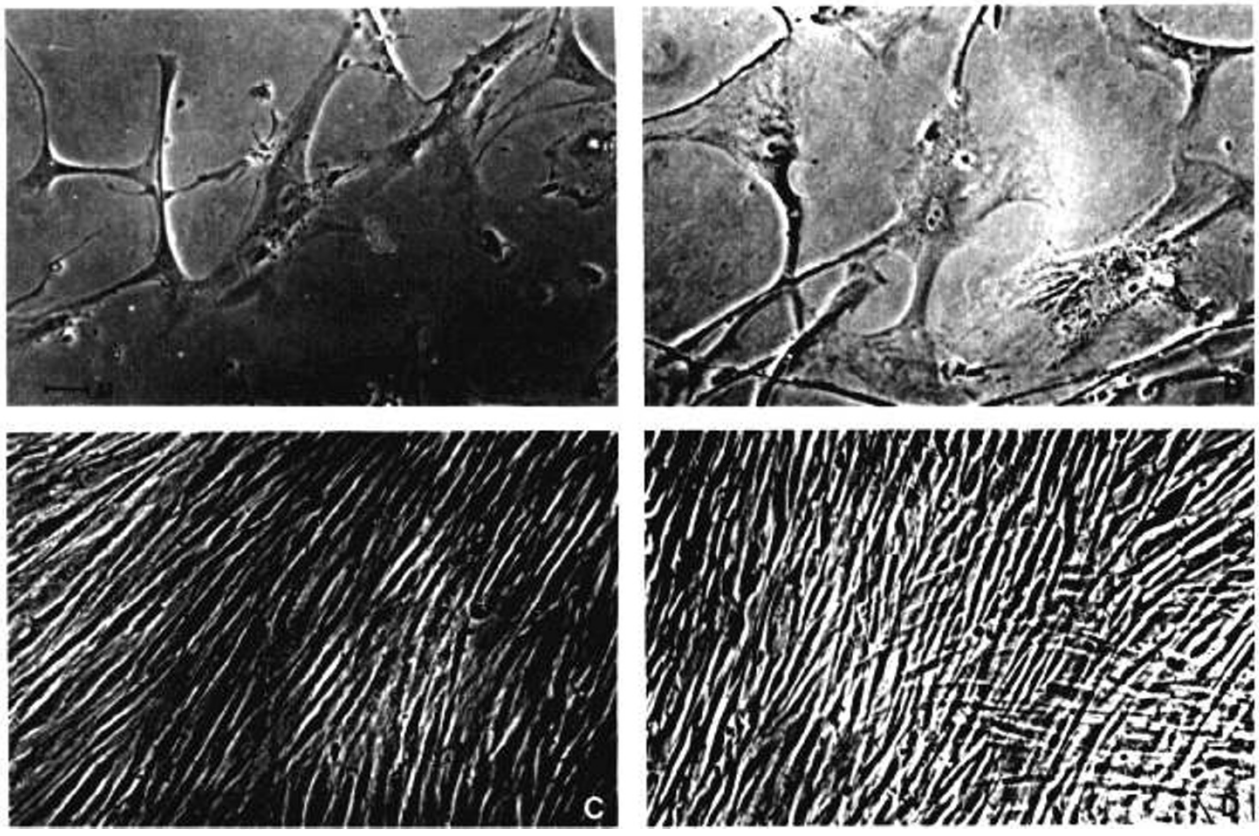

Fig. 2. *Top panels*, morphology and cellular density of NF cells at day 7 without (*left*) and with EGF (*right*). When stimulated with EGF, the NF cells became multinucleated (*center*), and had more distinct cellular outlines. *Bottom panels*, normal fibroblasts at day 7 without (*left*) and with EGF (*right*). All photographs are at the same magnification (× 100).

more common in children with NF. The unusual distribution and increased incidence of nonspecific nonneural crest leukemias [erythroleukemia (25), xantholeukemia (27)] in NF suggests a genetic predisposition to this type of disordered growth.

Our data indicate that NF fibroblasts grow slower than normal fibroblasts and incorporate significantly less [^{3}H]thymidine. NF cells also have a diminished ability to respond to EGF and are morphologically abnormal when compared to normal cells.

NF fibroblasts stopped growing at a significantly lower population density than did normal fibroblasts. NF cells stopped growing by the tenth or eleventh passage, whereas normal cells have continued to thrive for at least 20 passages.

When NF cells were stimulated with epidermal growth factor, they did not increase their rate of thymidine incorporation to the rate of the normal cells. The growth rates and thymidine data for our normal cells agree with the results of Carpenter and Cohen (4).

The experiments transferring media from NF cells onto normal cells indicates that NF cells do not secrete a growth inhibitory substance such as a "false" epidermal growth factor than inhibits growth.

The cellular morphology shown by light microscopy shows that the normal cells are morphologically similar to those in published studies (4, 18). Also, the media transfer experiment did not induce changes in the normal cells, further supporting the hypothesis that the changes in the NF cells are not the result of a secreted substance from the cells that inhibits growth. Whereas at confluency normal skin fibroblasts are elongated and aligned side-by-side, the NF cells maintained a disoriented pattern and continued to have multiple processes. NF cells were larger, pleomorphic, and failed to form confluent monolayers when growth ceased.

These observations may provide a means for determining whether NF is a heterogeneous disorder. Although some authors suggest that the central and peripheral forms of the disease are genetically distinct, other case reports argue for a more heterogeneous expression (1, 10). Examining fibroblast growth rates, thymidine incorporations, and their response to epidermal growth factor could provide a biological measure that could determine whether or not NF is truly a homogeneous disease.

We suggest that the manifestations of abnormal growth in NF are the result of intrinsic underlying abnormalities of growth regulation. These abnormalities are reflected in the skin fibroblast, which provides a tool to study and/or diagnose neurofibromatosis. Some of these critical "factors" or "nutrients" are apparently lacking in the Dulbecco's minimal essential medium, but not in the Ham's F-12 medium. Experiments to determine a critical factor necessary for the growth of the NF cells have been fruitless thus far. The diminished response of the NF cells to EGF provides a further clue to the growth abnormality of the NF fibroblast.

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LABORATORY DESIGN SEMINAR

The First Southeastern Regional Laboratory Design Seminar will be held in Atlanta, Georgia, Marrch 25-27, 1981.

This Seminar will cover the planning process, laboratory ventilation, energy conservation, utility requirements, laboratory furniture selection, biohazard and carcinogen control, building technology, and design innovations that can provide maximum, adaptability and optimum performance. Laboratory tours will be available.

Further information and registration forms can be obtained from Norman V. Steere & Associates, Inc., 140 Melbourne Ave., S.E., Minneapolis, Minnesota 55414. Phone: 612-378-2711.

Registration fee is $400 which includes course material and luncheons.

ANNOUNCEMENT

The annual meeting of the American Society of Pediatric Nephrology will be held at the Hilton Hotel in San Francisco, California, on Tuesday evening, April 28, 1981. This year the symposium will be entitled, "State of the Nephron: The Pediatric Nephrologist View." For further information, please contact Dr. Russell Chesney, Secretary-Treasurer, American Society of Pediatric Nephrology, University of Wisconsin Clinical Sciences Center, 600 Highland Avenue, H4/452, Madison, Wisconsin 53792.