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Nerve growth factor receptors on human melanoma cells in culture

(membrane receptors/cell culture/neural crest/binding assay)

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ABSTRACT **Purified mouse nerve growth factor (NGF) radiolabeled with ^{125}I was tested for its ability to bind to a variety of different cultured cells. NGF binds readily to human and hamster melanoma cells but does not bind to many other cell lines. The three cell lines with the highest number of NGF receptors were derived from metastatic melanomas. One of these lines, A875, was studied in detail and was shown to have approximately 7×10^5 NGF receptors per cell with an association constant of 1.0×10^9 liters/mole. The use of these cells in competition binding assays permits the detection of 0.25 ng of NGF in various biologic fluids. NGF can be shown to increase the survival, but not the division, of melanoma cells maintained in medium depleted of serum growth factors. This effect of NGF as a specific "survival factor" appears analogous to its effect on cultured sympathetic ganglion cells and on other cells derived from the neural crest.**

Nerve growth factor (NGF) (1) binds to specific membrane receptors on the surface of relatively few cell types in culture. It will stimulate the outgrowth of axons from sympathetic ganglia, providing the basis for an assay of its biologic activity (2). Mouse neuroblastoma cells in culture have also been reported to have specific NGF binding sites (3). In this study we show that the great majority of cultured cells (fibroblasts and epithelial cells from various species, and normal as well as tumor cells) have no detectable NGF receptors. In striking contrast, human and hamster melanoma cells have abundant NGF receptors. The availability of continuous cell lines with large numbers of high affinity NGF receptors has made it possible to develop a competition binding assay that readily detects 0.1 biologic unit (1 ng) of NGF.

MATERIALS AND METHODS

Cells. The majority of the human tumor lines tested, including five of the six melanomas, were developed in this laboratory; two of them (A375 and A875) have been described previously (4). Melanoma line Hs294 was obtained from the Naval Biological Research Laboratory, Oakland, Calif., as was the embryonic fibroblast line Hs0161 (AA422). Several of the nonhuman cell lines were obtained from the American Type Culture Collection. The pertinent clinical and cell culture history on the line chosen for detailed study in this report, A875, is as follows:

A 36-year-old woman underwent wide excision of an irregular, black, elevated mole at the left scapular area. Pathological examination revealed invasive, malignant melanoma. The patient was re-admitted several months later for excisional biopsy of recurrent melanoma at the left side of the jaw. At that time, left axillary adenopathy was noted; of 20 nodes excised, one was positive for metastatic melanoma. During the following 2 years the patient received radiation therapy to the cranial vault as well as chemotherapy for systemic melanoma. However, a space-occupying lesion developed deep in the right frontal lobe and metastatic melanoma was excised. Part of this tumor was sent to our laboratory for initiation of the A875 line. The pathological report on this specimen indicated a $5 \times 3.3 \times 0.3$ cm mass, containing numerous mitotic figures, giant cells, and pleomorphic cells containing dark brown melanin granules. After a progressively deteriorating course, the patient expired 6 months after surgery.

The A875 cells grew rapidly in primary culture with a mass doubling time of approximately 4 days on early passages. The cells grow in soft agar, on monolayers of normal cells, and are tumorigenic in *nude* mice. In culture they contain abundant intracytoplasmic pigment granules. Frozen samples are available from the fourth through sixty-fifth passages. The experiments presented in this paper were done with cultures at passage 20 to 25. Stock cultures were maintained at 37° in plastic T-75 flasks with Dulbecco's modification of Eagle's medium supplemented with 10% fetal calf serum (Colorado Serum Co.).

Iodination of NGF. The 2.5S preparation (β subunit) of nerve growth factor (NGF) used in these experiments was a generous gift of Dr. Rita Levi-Montalcini. One biological unit (BU) corresponds to 10 ng of protein, using the conventional bioassay described by Levi-Montalcini *et al.* (2). Gel electrophoresis performed in the presence of sodium dodecyl sulfate (5) showed a single band with an apparent molecular weight of 13,000. Twenty micrograms of NGF were mixed with 1 mCi of carrier-free $Na^{125}I$ (Amersham Searle) in 50 μl of 0.4 M phosphate buffer, pH 7.5. Ten microliters of chloramine T (6) were added (1 mg/ml), and after 30 sec, the iodinated protein was separated from the other reaction products by passing the reaction mixture through a 14×1 cm column of Sephadex G-15; the void fractions were pooled. The specific activities of labeled preparations ranged from 5 to 23 μCi/μg. ^{125}I-labeled NGF was active in binding assays after storage at −60° for at least 2 months.

The 6.5S form of NGF was prepared from male mouse submaxillary glands using the method of Varon *et al.* (7), and was iodinated as above. This preparation was used in some experiments and showed the same specificity for binding, as did the iodinated 2.5S preparations.

Binding Assay. Human melanoma cells (A875) (1 to 5×10^5) grown in 60 mm petri dishes (Falcon Plastics, no. 3002) were washed twice with modified Eagle's medium containing 0.1% bovine serum albumin and buffered to pH 6.8 using 0.05 M *N,N'*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (Bes) (binding buffer) (8). Twenty nanograms of ^{125}I-NGF were added in 1.5 ml of binding buffer and incubated for 60 min at room temperature. Unbound ^{125}I-NGF was then removed and the cells were washed four times with binding buffer. The bound ^{125}I-NGF was determined by disrupting the cells with 0.75 ml of lysing buffer (0.5% sodium dodecyl sulfate, 10 mM Tris at pH 7.4, and 1 mM EDTA) and transferring the fluid to scintillation vials containing 15 ml of Aquasol (New England

Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor.

Table 1. Binding of nerve growth factor (NGF) and epidermal growth factor (EGF) to a variety of normal and tumor cells in culture

Cells	NGF (femtomoles bound per 10^6 cells)	EGF (femtomoles bound per 10^6 cells)
Human		
Normal cells		
Hs0161, embryonic fibroblast	0.7	11
M413, embryonic fibroblast	0.7	17
A1707, normal skin fibroblast	0	24
Melanomas		
A375	1.2	1.5
A875	127	2
A1502	6	0.5
A2018	98	4
A2058	15	0.5
Hs294	118	2
Other tumor cells		
IMR-32, neuroblastoma	1	2
Gol B, glioma	0.1	29
A172, glioblastoma	0.4	22
A382, astrocytoma	0	52
HT1080, fibrosarcoma	0	86
8387, fibrosarcoma	0.5	16
A431, epidermoid carcinoma of vulva	0.8	203
A1663, carcinoma of bladder	0	38
WI38, VA13, SV40-transformed lung	0.1	15
Nonhuman		
Normal cells		
FCf2Th, dog thymus	0	10
MCDK, dog kidney	0.3	10
SIRC, rabbit cornea	0.1	6
Mv1Lu, mink lung	0.7	70
FEC, cat embryo	0.6	44
NRK, rat kidney	0.3	22
BALB/3T3, mouse embryo	0.4	57
Tumor cells		
RPMI-1846, hamster melanoma	22	0.3
Y22, hamster melanoma	12	0
NCTC 3959, mouse melanoma	0.9	0.9
NB41A3, mouse neuroblastoma	13	0.5

Nuclear). The dishes were washed twice more with 0.5-ml portions of lysing buffer, the contents were transferred to the counting vial, and the radioactivity was determined using a Beckman LS-250 liquid scintillation counter. Nonspecific binding was estimated from the cell-bound radioactivity in the presence of 3 μg of unlabeled 2.5S NGF or 6.5S NGF (7). NGF present in either form competed for the binding of the radiolabeled 2.5S NGF. Assays were performed 24–48 hr after subculture under conditions where binding was linearly related to cell number (see Fig. 2).

Competition Assay. Competition studies were performed using A875 cells seeded in FB-16-24 TC Linbro plates (24 wells per plate; 4×10^4 cells per well). After the cells were washed twice with binding buffer, unlabeled competing samples were incubated with the cells in a 250-μl volume for 60 min at room temperature. Three nanograms of ^{125}I-NGF in 25 μl were then added to wells and allowed to incubate another 60 min. Cells were washed four times with binding buffer and lysed; the radioactivity was determined as described.

Table 2. Human melanoma cell lines tested for NGF specific membrane receptors

Line	Age & sex	Site	Primary (P) or metastasis (M)	NGF receptors
A375	54 F	Leg	P	–
A875	78 M	Brain	M	++++
A1502	84 F	Lymph node	M	++
A2018	74 M	Brain	M	++++
A2058	43 M	Lymph node	M	++
Hs294	56 M	Lymph node	M	++++

RESULTS

Table 1 shows the cell lines that were tested for their ability to bind ^{125}I-labeled NGF. Three human melanomas [A875 (4), A2018, and Hs294] showed the highest levels of NGF binding. Two other human melanoma lines, A1502 and A2058, also showed considerable NGF binding, while a sixth melanoma line, A375, showed little or no binding. The transplantable malignant melanoma cell line, RPMI-1846 (9), derived from a Syrian hamster and the Chinese hamster melanoma line, Y22 (10), also bound NGF. Like A375, no significant binding was observed with the mouse melanoma line, NCTC 3959. Low levels of binding were seen with a mouse neuroblastoma line, C1300 clone NB41A3; the observed levels of binding, however, were low when compared to the majority of melanoma cell cultures. By contrast, a variety of cell lines established from normal cells or tumor cells of other histologic types failed to show receptors for ^{125}I-NGF.

To show whether the results obtained with ^{125}I-NGF reflected the presence of specific NGF receptors on melanoma cells, binding experiments were also performed using a second growth hormone with specific membrane receptors, epidermal growth factor (EGF) (11). EGF has a very different pattern of binding, which corresponds to its ability to stimulate cell division in epithelial cells and in various other cells of mesodermal origin (12–14). As described by others (14), EGF and NGF do not compete with one another for receptor sites. Table 1 shows that EGF did not bind to any of the melanoma cell lines tested or to the human neuroblastoma cell line, IMR-32; by contrast, EGF receptors were readily detected on both normal and tumor cells derived from several mammalian species.

The origin of the human melanoma lines used in this study is summarized in Table 2. Two of the high NGF binders were derived from malignant melanomas that had metastasized to the central nervous system, while a third line, HS294, came from a peripheral lymph node metastasis. The one melanoma (A375) that showed little or no NGF specific binding was derived from a primary tumor.

Fig. 1 shows the time course of NGF binding to the human melanoma line, A875, at 4°, 22°, and 37°. The binding assays performed at 22° and 37° gave comparable results; at 4°, however, not only was the rate of binding somewhat slower, but the final extent of binding was also diminished. The nonspecific binding was determined by adding a large excess of unlabeled NGF (3 μg) to the binding mixture containing the ^{125}I-NGF (20 ng). This reduced the binding of labeled NGF to the levels seen with empty petri dishes or with cultures of cell lines failing to show specific NGF binding. The amount of specific binding was determined by subtracting the nonspecific bound from the total amount bound. The ratio of specific to nonspecific binding for A875 was 10–15/1. Fig. 2 shows the effect of cell number

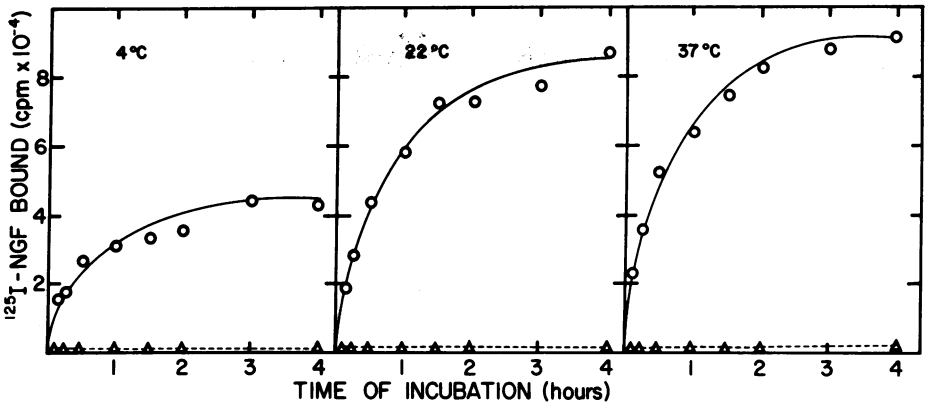

FIG. 1. Cultures of A875 (○) and HT1080 (△) were grown to a density of 4×10^5 per 60-mm petri dish at 37°. Assays were performed using 20 ng of ^{125}I-NGF (140,000 cpm) in 1.5 ml of binding buffer, at three temperatures, 4°, 22°, and 37°. At the indicated times, the amount of ^{125}I-NGF bound to the cells was determined.

on the specific binding of ^{125}I-labeled NGF to A875 cells. At lower cell concentrations, binding was a linear function of cell number, indicating first-order kinetics with respect to receptors. At higher cell concentrations, however, the amount of NGF bound began to plateau, implying an excess of receptors. By studying the binding of increasing concentrations of NGF to a fixed number of cells it was possible to estimate the number of NGF specific receptors per cell. A Scatchard plot (15) (Fig. 3) shows the data obtained with the human melanoma line, A875. We calculate that there are 6.9×10^5 NGF receptors per cell with an association constant ($K_{apparent}$) of 1.0×10^9 liters/mole. By comparison, most epithelial cells have only 5×10^4 to 1×10^5 EGF receptors per cell (14, 16). The association constant for NGF binding to the melanoma cells is comparable to that previously reported for NGF binding to embryonic sensory ganglion cells (17) and sympathetic ganglion cells (18).

The dissociation of bound NGF from human melanoma cells was studied by first incubating ^{125}I-labeled NGF with A875 cells, washing the cells several times in the absence of NGF, reincubating the cells in binding buffer, and then measuring the radioactivity remaining bound to the cells as a function of time (Fig. 4). The $T_{1/2}$ for dissociation at 4° was determined to be 110 min, a value comparable to that obtained using homogenates of a sympathetic ganglion microsomal fraction (17). No dilution dependency could be found over a 10-fold range for the dissociation of bound ^{125}I-NGF from the A875 cells.

The relatively large number (6.9×10^5 per cell) of high affinity NGF receptors on A875 cells has made it possible to develop a sensitive, specific, and rapid competitive binding assay capable of measuring NGF. Fig. 5 shows the results of a competitive binding assay for NGF. In the assay shown, 50% of the maximal competition was seen with 1.5 ng of NGF, while 15% inhibition required 0.25 ng of NGF. The latter figure corresponds to 0.025 biologic unit (BU) of activity. In contrast, 5 μg of EGF in the same experiment showed no detectable competition for NGF binding sites. The sensitivity of the assay could be further increased by using still fewer target cells and/or by increasing the specific activity of the radiolabeled NGF.

Human melanoma A875 cells were seeded into microtiter wells at 2×10^4 cells per well in serum-depleted medium (19) supplemented with various concentrations of NGF ranging from 24 pg to 15 ng. Two other human cell cultures lacking detectable NGF receptors, a normal second trimester embryo cell strain (Hs0161) and a human fibrosarcoma (HT1080), were similarly tested. Only in the case of the A875 cells could a survival enhancing effect be seen. The 2×10^4 cells per well seeded

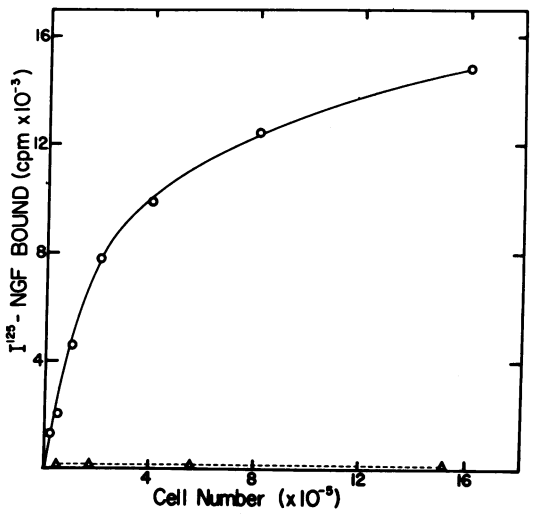

FIG. 2. Cultures of A875 (○) and HT1080 (△) were seeded in serial dilutions from 1.6×10^6 to 2.5×10^4 cells in 60-mm petri dishes at 37°. After cells were washed twice with the binding buffer, 20 ng of ^{125}I-NGF (45,000 cpm) was added to the cells for a 60-min incubation at 22°. The radiolabeled NGF was removed and cells were washed and lysed as described. The bound ^{125}I-NGF is plotted as a function of the cell number.

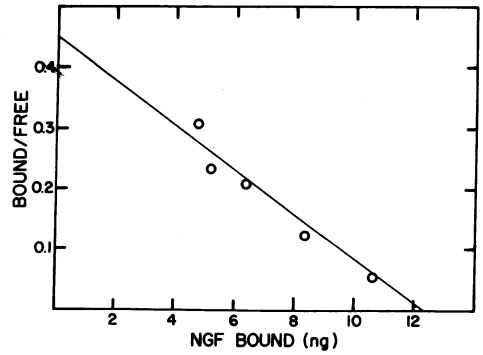

FIG. 3. Cultures of A875 were grown to a density of 4×10^5 cells per 60-mm petri dish. After cells were washed twice with binding buffer, 20 ng of ^{125}I-NGF (170,000 cpm) mixed with various known amounts of cold NGF were added to the dishes in 1.5 ml of binding buffer. These known amounts included a range from 1.5 ng to 4.5 μg. After a 60-min incubation at room temperature, the dishes were washed and cells were lysed as described. The ratio of bound/free ^{125}I-NGF was determined and plotted as a function of the ng of NGF bound (15).

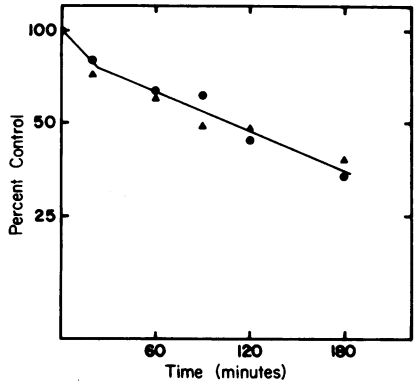

FIG. 4. A875 cells were grown to a density of 4×10^4 per well in Linbro plates. After cells were washed twice with binding buffer, 3 ng of ^{125}I-NGF (23,000 cpm) was added in 250 μl to the wells for a 60-min incubation at room temperature. ^{125}I-NGF was removed and wells were washed 4 times with cold binding buffer. To half the wells, 200 μl of binding buffer alone was added (●); to the remaining half 2 ml of binding buffer was dispensed (▲). After all samples were incubated at 4 ° for various times, duplicate wells were lysed as described and the amount of ^{125}I-NGF remaining was determined.

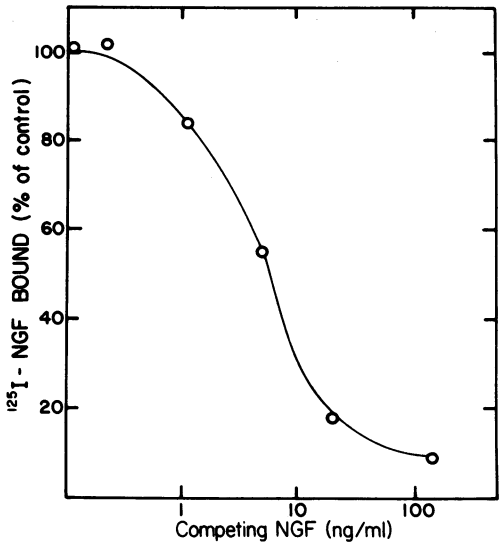

FIG. 5. Cultures of A875 were grown to a density of 4×10^4 per well in Linbro plates. After the cells were washed twice with binding buffer, various amounts of unlabeled NGF were added in 250 μl. After a 60-min incubation at room temperature, 2 ng of ^{125}I-NGF (13,000 cpm) was added in 25 μl to the unlabeled NGF. The mixture was removed after 60 min, the cells were lysed, and the bound radioactivity was determined as described. The percentage of control binding is indicated on the ordinate.

in depleted medium with 600 pg/ml or more of added NGF remained essentially constant (1.0 to 2.0×10^4 cells per well) over the next 5-day period, while those not supplemented with NGF or with 120 pg/ml or less of NGF failed to survive ($<5 \times 10^3$ cells per well). In contrast, neither the embryo fibroblasts nor the fibrosarcoma line could be protected by NGF addition. The results demonstrate that the NGF receptors found on melanoma cells are biologically active and that, at least under certain cell culture conditions, NGF can act as a potent and relatively specific survival factor for melanoma cells. Unlike the actions of EGF and fibroblast growth factor (20) on fibroblasts in cell culture, we have been unable to demonstrate a direct mitogenic effect of NGF on the melanoma cells.

DISCUSSION

The studies presented here lead to the conclusion that metastatic melanoma cells derived from both hamsters and man possess specific receptors for NGF. Previous studies have defined and characterized specific receptors in sympathetic ganglia and in one neuroblastoma cell line; thus the detection of NGF receptors on melanoma cells may not be surprising in view of the embryologic relationships among the cell types. The pigment cells, like the sympathetic ganglia and the nerve sheaths, are all derived from the neural crest (21). The melanoma cells, then, may have partially regained the pluripotency of a more primitive neural crest cell. Whether this represents the dedifferentiation and expression of a specific class of embryonic antigens or whether normal pigmented melanocytes also have specific NGF receptors remains to be determined.

The finding of NGF receptors on human melanoma cell lines has enabled us to develop a simple and sensitive receptor binding assay for this protein. In normal human sera the values obtained by this assay have ranged between 200 and 500 ng/ml (unpublished experiments). This compares with values of 20–40 ng/ml determined by radioimmunoassay for the biologically active 2.5S subunit (22). A 15% displacement of receptor binding is obtained in the present assay with 250 pg of NGF, corresponding to 0.025 biologic units. The melanoma cell binding assay, then, may prove valuable, along with the biologic and the immunologic assays, in detecting and characterizing nerve growth factor.

The identification of specific membrane receptors for NGF in melanoma cells may provide new approaches for the detection and perhaps for the prevention of spread of melanoma cells in the body. Antiserum to NGF has been shown to produce a functional sympathectomy (23). What effect such treatment might have on the course and the spread of melanotic disease would appear to merit consideration. Similarly, antiserum to a purified preparation of NGF receptor (3) might specifically inhibit the spread of malignant melanomas in the intact animal. The three cell lines with the highest NGF receptor levels were all derived from metastatic human melanomas. A larger sample of melanomas will have to be studied to see whether our results reflect the possibility that variant melanoma cells with high NGF receptor levels are more likely to survive and metastasize.

Although most melanoma lines tested have high levels of NGF receptors, two of the lines studied, one of human and one of mouse origin, have no detectable NGF receptors. The significance of this finding is not yet clear but may reflect the possibility that, like certain cell cultures, such as mouse sarcoma 180, 3T3, and L cells (24) and chick embryo cultures (25), these cells may endogenously produce NGF and by so doing be unable to bind exogenously applied NGF.

The availability of pure populations of cells with high levels of specific NGF receptors that both bind NGF and are biologically responsive to its action should facilitate our understanding of NGF and the mechanisms by which it interacts with cellular receptors.

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