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Human melanoma cells have both nerve growth factor and nerve growth factor-specific receptors on their cell surfaces

(membrane immunofluorescence/neural crest cell property/tumor-associated antigen)

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ABSTRACT **Human melanoma cells were examined in an indirect membrane immunofluorescence assay for surface nerve growth factor (NGF) and NGF receptors. This assay revealed that human melanoma cells have various levels of NGF and NGF receptors on the plasma membrane, whereas a variety of human sarcoma and carcinoma tumor cells and normal human fibroblasts are negative. Surface NGF could be detected on melanoma cells with a rabbit antiserum directed to NGF at titers as high as 1:64; prior adsorption of this antibody with mouse 2.5S NGF resulted in a loss of fluorescence. The melanoma cells were positive whether or not they were grown in the presence of fetal calf serum. NGF production by human melanomas is a previously unrecognized property of this differentiated cell type. Although other cells in culture have been shown to produce NGF, the association of NGF production with the presence of NGF receptors on the cell surface is rare among tumor cells, and may represent an opportunity for "autostimulation" of melanoma cells by this growth factor.**

The presence of membrane receptors for specific polypeptide growth factors appears to be a characteristic property of differentiated cells growing *in vitro* (1). We have previously reported that human melanoma cells in culture have specific cell surface receptors for nerve growth factor (NGF) (2). NGF is a small polypeptide hormone capable of stimulating axonal outgrowth from sympathetic ganglia and interacting with a variety of other neural tissues (3, 4). The presence of NGF receptors on melanoma cells may reflect their embryologic origin from the neural crest. In contrast to melanoma cells, NGF receptors are not found on normal human fibroblasts or on human tumor cells of mesodermal and endodermal origin, including a variety of sarcomas and carcinomas.

NGF receptors have been previously identified by binding ^{125}I-labeled NGF (^{125}I-NGF) to the cell surface (2, 5). In this report we describe a specific membrane immunofluorescence assay that can detect NGF receptors on plasma membranes of human melanoma cells. In addition, we demonstrate by this technique that melanoma cells have NGF or a closely related substance on their cell surface, whereas other human tumor cells lack surface NGF. NGF production by melanoma cells and its accumulation on the cell surface may represent a previously unrecognized property of this differentiated cell type.

MATERIALS AND METHODS

Cells. The majority of human tumor cell lines used in these experiments, including five of the six melanoma cell lines, were developed in this laboratory. Several of these lines have been described previously (6, 7). The following cell lines were obtained from other sources: HS294 and HT1080 (Naval Biological Research Laboratory, Oakland, CA), and HEL299 (American Type Culture Collection). Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% fetal calf serum (FCS) (GIBCO). Cells conditioned to grow in FCS-free medium were treated as follows: A 50–75% confluent monolayer of cells was washed four times at 30-min intervals with FCS-free medium. The cells were then grown in FCS-free medium for at least 7 days prior to testing.

NGF and NGF Antibody. The mouse 2.5S NGF used in immunofluorescence and binding assays, prepared as described by Bocchini and Angeletti (8), was the generous gift of Rita Levi-Montalcini. Mouse 7S NGF was prepared according to published methods (9) and inoculated into rabbits to prepare antibody to NGF. This antibody was adsorbed with human A549 lung tumor cells to remove nonspecific anti-human cellular determinants. A rabbit antibody prepared to the β subunit of mouse NGF, kindly provided by Eric Shooter, was used interchangeably (10).

Immunofluorescence Assay. All cells tested were detached from monolayers with 0.1% trypsin in phosphate-buffered saline, resuspended in their growth medium, and gently agitated for three hours at 37°C prior to use. An indirect membrane immunofluorescence test was carried out as follows. Each reaction mixture contained approximately 10^6 cells to which 20 ng of 2.5S NGF suspended in 50 μl of binding buffer (11) was added. This mixture was shaken, incubated at 22°C for 30 min, and subsequently washed two times with binding buffer. To the washed pellet of cells, 50 μl of an appropriate dilution of antiserum to NGF (or normal rabbit serum or buffer controls) was added. This mixture was shaken, incubated at 37°C for 30 min, and subsequently washed two times with binding buffer. Finally, to the washed pellet of cells, 50 μl of a 1:20 dilution of fluorescein-conjugated goat antiserum to rabbit IgG (National Institutes of Health, Bethesda, MD) was added. This mixture was shaken, incubated at 37°C for 30 min, and washed two times with binding buffer. The cell pellet was suspended in 50 μl of binding buffer and stored at 4°C until read.

^{125}I-NGF Binding Assay. The NGF receptor assay, as measured by the specific binding of ^{125}I-labeled 2.5S NGF to the cell surface, was performed as described (2).

RESULTS

Human cells, including the melanoma lines A375 and A875, were examined for the presence of NGF and NGF receptors in an indirect membrane immunofluorescence test. Cells were incubated with rabbit antiserum to mouse NGF and fluorescein-conjugated goat antiserum to rabbit IgG in both the presence and the absence of mouse 2.5S NGF. As shown in Table 1, the majority of human tumor cells show no detectable immunofluorescence (<10% positive cells) when tested with NGF antibody either with or without added NGF. Even at an added NGF concentration of 400 ng/ml no specific fluores-

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Abbreviations: NGF, nerve growth factor; FCS, fetal calf serum.

Table 1. Immunofluorescence and 2.5S ^{125}I-NGF binding assays for NGF and NGF receptors

Cell lines	% of cells demonstrating immunofluorescence*		Binding of 2.5S ^{125}I-NGF, fmol/10^6 cells
	+ 2.5S NGF	− 2.5S NGF	
Human melanoma cells			
A375	68–90	68–90	1.2
A875	88–100	<10	127.0
Other human cells			
A388 (epidermoid carcinoma)	<10	<10	<0.5
A498 (renal carcinoma)	<10	<10	<0.5
A549 (lung carcinoma)	<10	<10	<0.5
A673 (rhabdomyosarcoma)	<10	<10	<0.5
A1165 (pancreatic carcinoma)	<10	<10	<0.5
A1589 (epidermoid carcinoma)	<10	<10	0.8
A1663 (bladder carcinoma)	<10	<10	<0.5
8387 (fibrosarcoma)	<10	<10	0.5
HT1080 (fibrosarcoma)	<10	<10	<0.5
NC-37 (lymphoid cells)	<10	<10	<0.5
HEL299 (normal fibroblast)	<10	<10	<0.5

* The NGF antibody (previously adsorbed with human A549 cells) was used at 1:8 dilution. The concentration of added 2.5S NGF was 400 ng/ml.

cence could be detected on these cells. Therefore, these cells lack NGF receptors whether tested by immunofluorescence or direct ^{125}I-NGF binding. The two melanoma cell lines show a distinctly different pattern from the other tumor cells, as well as from each other. One cell line, A875, shows specific immunofluorescence only when NGF is added. The other cell line, A375, shows this membrane fluorescence even without added NGF. The percent of A375 cells that were positive varied between 60% and 90% depending on the assay, but was reproducibly equally positive with and without added NGF. A875 cells consistently were between 88% and 100% positive, but only in the presence of 2.5S NGF. Without added NGF, these cells were negative (<10% positive cells).

Fig. 1 shows fluorescence photomicrographs of A875 cells in the presence of 20 ng of 2.5S NGF (*A*) and A375 cells in the absence of added NGF (*B*). Although both cells are clearly fluorescence-positive, there appear to be differences in the pattern and intensity of staining. A875 shows a characteristic patchy fluorescence at this concentration of added NGF. However, with lower concentrations of NGF, the pattern of fluorescence with A875 resembles the punctate staining seen here with A375. The intensity of staining with A875 in the presence of 20 ng of mouse 2.5S NGF is stronger than that seen with A375. This difference in intensity of staining may reflect an incomplete crossreaction between the NGF produced by human melanoma cells and an antibody prepared to mouse NGF. The degree of immunologic crossreaction between mouse and human NGF is not known, but the reaction may be considerably less intense than the homologous reaction (12). Alternatively, the difference in intensity may simply indicate a lower concentration of endogenously produced NGF on A375 as compared to the amount of mouse NGF that can be added to the A875 cells. In fact, the intensity of fluorescence on A375 cells depended in part on the length of time the cells were incubated after trypsinization. When the cells were incubated for 16–24 hr, the intensity of fluorescence in the absence of added NGF increased, suggesting an increased level of endogenous NGF production. However, such long incubation periods were not used routinely because of diminished cell viability.

In order to demonstrate that the positive fluorescence on A375 cells detected by NGF antibody represents NGF and not some other membrane component, the antibody was adsorbed with increasing amounts of purified mouse 2.5S NGF prior to testing. As shown in Fig. 2, with increasing levels of antibody adsorption by mouse NGF there is a marked decrease in the fluorescence on A375 cells. A 50% reduction in the number of positive cells required approximately 90 ng of NGF to adsorb the antibody. To demonstrate that A375 cells had not picked up NGF or a related substance from the FCS in the medium, the cells were conditioned to grow in serum-free medium (see *Materials and Methods*) and then tested for the presence of surface NGF. The result was identical to that for A375 grown in 10% FCS. The experiment showed that NGF is indeed easily detectable on the surface of A375 cells grown and tested in serum-free medium. Taken together, these results demonstrate the specificity of the fluorescence assay for NGF and show that the fluorescence seen on A375 cells with this antibody represents endogenously produced NGF.

Experiments were performed to determine the sensitivity of the immunofluorescence assay for NGF and its receptors. A375 cells were incubated with serial dilutions of the rabbit antiserum to NGF in the absence of added NGF. As shown in Fig. 3*A*, significant immunofluorescence can be detected on A375 melanoma cells at an antibody titer of 1:32. At the same antibody dilution no immunofluorescence is seen with A875 melanoma cells or the human sarcomas and carcinomas tested (see legend to Fig. 3). At an antibody dilution of 1:2, a low level (20%) of A875 cells are positive in comparison to the nonmelanoma tumor cells, suggesting that a small amount of NGF may also be present on their cell membranes. Fig. 3*B* shows the results of experiments in which A875 and A375 melanoma cells and other human tumor cells were incubated with various concentrations of 2.5S NGF prior to the addition of antibody and conjugate. The fluorescence of A375 cells is entirely independent of the addition of NGF, which is the expected result if the fluorescence of these cells represents endogenous NGF secreted onto the cell surface. In contrast, A875 cells show greater fluorescence as the concentration of added NGF is increased. This result indicates that A875 has free surface receptors for NGF and that these receptors can be detected in an immunofluorescence assay by the addition of as little as 0.1–1.0 ng of NGF. At NGF concentrations as high as 400 ng/ml, no NGF receptors can be detected on any of the other nonmelanoma human cells tested.

In order to test whether the NGF-like substance produced

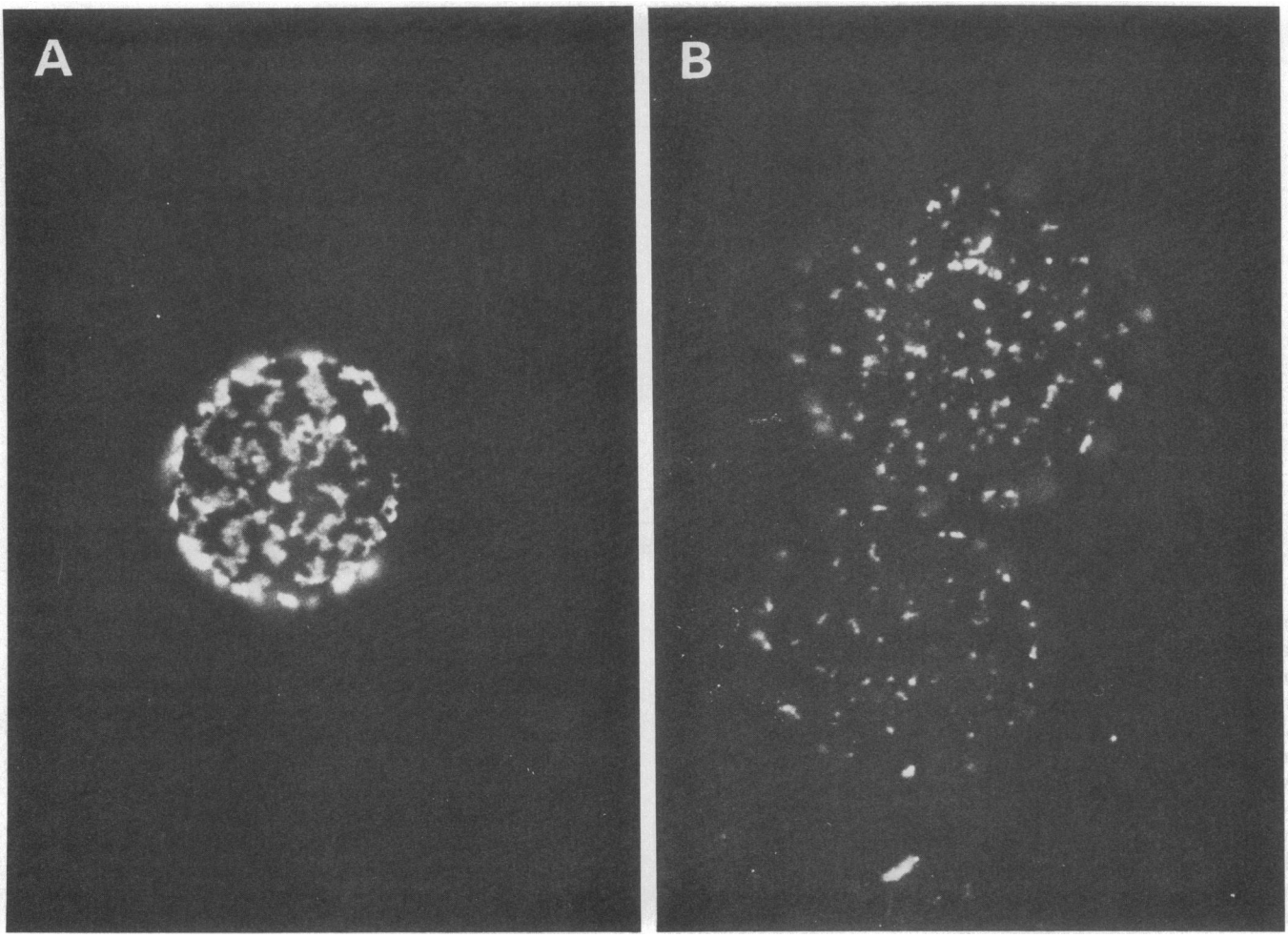

FIG. 1. Fluorescence photomicrographs of A875 melanoma cells in the presence of mouse 2.5S NGF and rabbit NGF antibody (*A*) and A375 melanoma cells in the presence of rabbit NGF antibody without added 2.5S NGF (*B*). The NGF antibody was used at a dilution of 1:8 and had been previously adsorbed with human lung tumor A549 cells. Fluorescein-conjugated goat anti-rabbit antibody was used to stain both cell types. (×1400.)

by A375 cells would bind to the NGF receptors on A875 cells, we designed an experiment in which the two cells were mixed in suspension, incubated for 8 hr at 37°C, and tested for surface NGF in the absence of added NGF. When the two cells were combined in equal proportions, 90% of the cells were fluorescence-positive (instead of the expected 50%), indicating that as many as 80% of the A875 cells in the mixture had become positive. The most likely explanation for the fluorescence of A875 under these circumstances is that an NGF-like substance produced by A375 had bound to available NGF receptors on A875. This result suggests that the NGF produced by A375 is both secreted into the culture medium and capable of binding to the NGF receptors on melanoma cells having a high level of available NGF receptors.

To determine whether NGF production is a general property of human melanomas, other human melanoma cell lines were examined by immunofluorescence for the presence of surface NGF. The melanoma cells tested were incubated with serial dilutions of antibody to NGF in the absence of added NGF. As shown in Fig. 4, all of the melanoma cells tested have various levels of NGF or a closely related substance on the cell surface. In this experiment, several other human tumor cells and normal human fibroblasts were negative for surface NGF. Certain melanomas appear to be producing more of this substance than A375 cells (for example, HS294 and A1502) and others less (A2018, A2058, and possibly A875). All of these melanoma cells (except A375) have been previously shown to have NGF receptors (2). One line, HS294, which was shown to have high levels of receptors by ^{125}I-NGF binding also appears to be producing high levels of NGF on its cell surface. Moreover, we have been able to detect available NGF receptors on HS294 cells by adding mouse NGF and noting a marked increase in the intensity of immunofluorescence. The presence of different levels of surface NGF in association with different numbers of NGF membrane receptors therefore appears to be a general property of human melanoma cells in culture.

DISCUSSION

In this study, we describe an indirect membrane immunofluorescence assay for the surface-bound NGF and the NGF receptor. With this assay we show that most human melanoma cells in culture have NGF on their cell surfaces. The data presented indicate the following. First, the method of detection of endogenously produced NGF on melanoma cells is specific, because detection can be prevented by the prior adsorption of the NGF antibody with purified mouse 2.5S NGF. Second, melanoma cells grown in serum-free media remain positive for secreted NGF, indicating that the surface NGF is endogenously produced by the melanoma cells. Third, the NGF produced by one melanoma cell line, A375, is capable of binding to the NGF receptors of another line, A875. And fourth, NGF antibody previously adsorbed with human cells detects surface NGF at

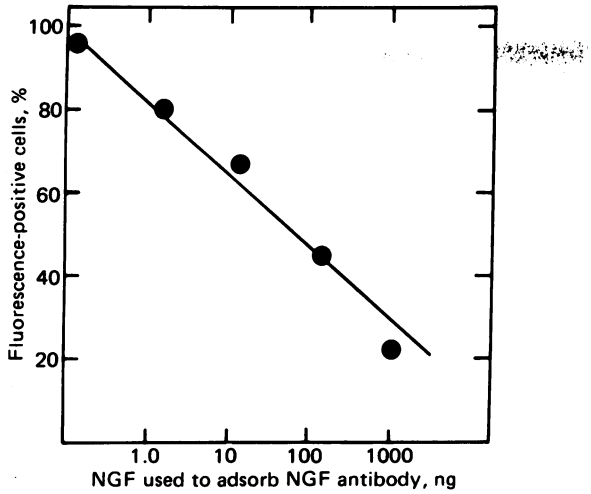

FIG. 2. Immunofluorescence of A375 melanoma cells after adsorption of the NGF antiserum with increasing amounts of mouse 2.5S NGF. Adsorption was carried out as follows: Fifty microliters of a 1:8 dilution of antibody to NGF was mixed with 50 μl of serial concentrations of 2.5S NGF in binding buffer and incubated at 37°C for 30 min. This mixture was then used as the adsorbed serum at an effective dilution of 1:16 and incubated with the cells for 30 min at 37°C.

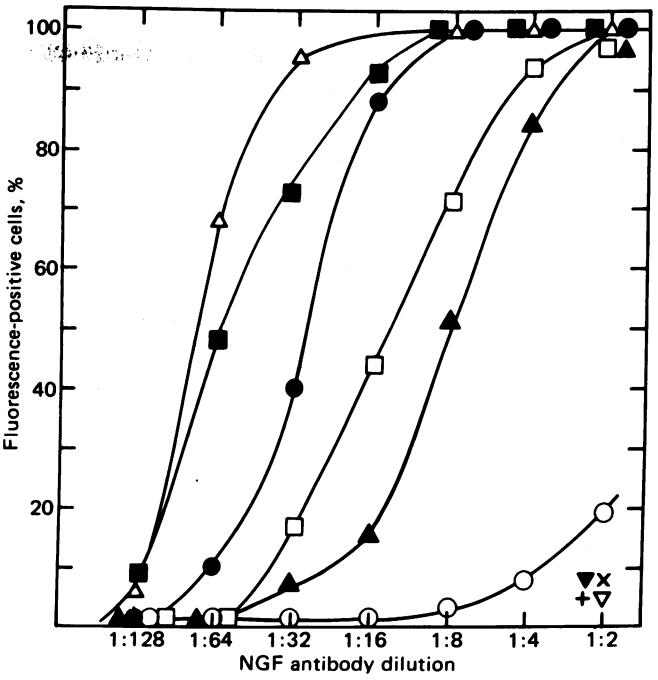

FIG. 4. Immunofluorescence of various human melanoma cells and other human cells with NGF antibody. Test cells were incubated with serial dilutions of the rabbit NGF antibody (previously adsorbed with human A549 cells) without added 2.5S NGF and then stained with fluorescein-conjugated goat anti-rabbit antibody. Cells tested included the following human melanoma cell lines: A375 (●), A875 (○), A1502 (△), A2058 (▲), A2018 (□), and HS294 (■). Other human cells included HT1080 fibrosarcoma (▼), A549 lung carcinoma (×), A1663 bladder carcinoma (+), and HEL299 normal fibroblasts (▽).

titers as high as 1:64, and detects NGF receptors with only 0.1–1.0 ng of added mouse NGF.

The presence of surface NGF molecules appears to be a general characteristic of human melanoma cells in culture and is a previously unrecognized property of this differentiated cell type. However, human melanoma cells are not unique in their ability to secrete NGF. NGF-producing cells include primary chick fibroblasts (13), rat C6 glioma cells (14), rat adrenal medullary cells in organ culture (15), and various transformed mouse cell lines such as L929 (16) and neuroblastoma C1300 (17). In addition, there are reports of NGF production by human cells in primary cultures of skin fibroblasts (18) and glioblastoma (18, 19) cells. While NGF production may not be unique to melanoma cells, the presence of specific NGF receptors as detected by either immunofluorescence or ^{125}I-NGF binding is rare among cell lines in culture. Thus far, only cultured neuroblastoma cells (5) have been shown to share this property with human melanoma cells. The presence of NGF receptors on these particular cell types most likely reflects their common embryologic origin from the neural crest and may suggest a role for NGF and related substances in embryogenesis (4). For example, the peripheral migration of neural crest derivatives, such as nerve sheath cells, adrenal medullary cells, and melanocytes might occur along NGF gradients and thus require that specific NGF receptors be present on these cells

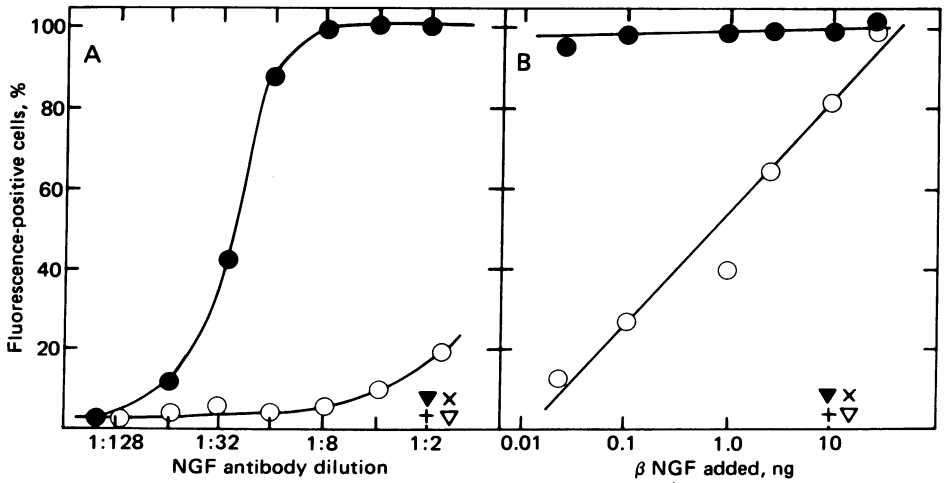

FIG. 3. Effect of NGF antibody dilution and added 2.5S NGF concentration on the immunofluorescence of human melanoma and other human tumor cells. (*A*) Test cells were incubated with serial dilutions of rabbit NGF antibody (previously adsorbed with human lung A549 cells) without added 2.5S NGF and were then stained with fluorescein-conjugated goat anti-rabbit antibody. (*B*) Test cells were first incubated with increasing amounts of mouse 2.5S NGF and then incubated with the NGF antibody at a dilution of 1:8 and conjugate. The cells tested include: A375 melanoma (●), A875 melanoma (○), HT1080 fibrosarcoma (▼), A549 lung carcinoma (×), A1663 bladder carcinoma (+), and HEL299 normal fibroblasts (▽).

(20–22). Therefore, under normal conditions of embryogenesis, and perhaps in the adult as well, it may be required that no one cell type both produces NGF and has NGF receptors. This particular combination of properties—the production of NGF as well as the presence of receptors to which it can bind—may be limited to malignant derivatives of neural crest cells.

The presence of both NGF and NGF receptors on the surface of human melanoma cells is of interest because the endogenously produced NGF is able to bind to available membrane receptors and may therefore be able to act on the very cell that produces it. To the extent that NGF is necessary for the growth or survival of melanoma cells, NGF production by melanoma cells might foster the growth of these cells and therefore be an example of "autostimulation" by a hormone-secreting tumor cell (17, 23). In this respect it is of interest that the addition of NGF to the culture medium has been shown to favor the survival of neuronal cells (24) and pheochromocytoma cells (25), as well as melanoma cells (2). Similar models with growth advantage to the producer cell have been suggested to explain the production of sarcoma growth factor (SGF) by mouse sarcoma virus-transformed cells (26) and a somatomedin-like growth factor by human fibrosarcoma cells (27). Hormonally responsive differentiated cells, when they acquire the ability to produce growth factors to which they also can respond, might be expected to have a growth or survival advantage *in vivo* as well as in culture (1, 23).

The presence of NGF on the surface of human melanoma cells is of further interest in view of the known immunogenicity of this tumor and the associated melanoma antigens (28–32). Certain of these tumor antigens have recently been partially characterized (33–35). For example, Stuhlmiller and associates (35) have reported several melanoma tumor antigens that appear to lack crossreactivity with both HLA and fetal antigens, the smallest of which has an apparent molecular weight of approximately 13,000. One can speculate that human NGF, or some component thereof, may, in fact, be a melanoma tumor-associated antigen. Regardless of whether NGF, the NGF receptor, or the hormone–receptor complex represents a melanoma tumor antigen, NGF production by human melanoma cells as detected by immunofluorescence may be an extremely useful differentiated property of melanomas. Measurement of NGF production and NGF receptor levels on human melanomas might allow correlation between production by the tumor and the clinical course of a patient with melanoma, and might therefore be useful in the clinical staging of the disease.

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