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Nerve Growth Factor (NGF) Induces Neuronal Differentiation in Neuroblastoma Cells Transfected with the NGF Receptor cDNA

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Human nerve growth factor (NGF) receptor (NGFR) cDNA was transfected into a neuroblastoma cell line (HTLA 230) which does not express a functional NGF-NGFR signal transduction cascade. Short-term treatment of stably transfected cells (98-3) expressing membrane-bound NGF receptor molecules resulted in a cell cycle-dependent, transient expression of the c-*fos* gene upon treatment with NGF, suggesting the presence of functional high-affinity NGFR. Extensive outgrowth of neurites and cessation of DNA synthesis occurred in transfectants grown on an extracellular matrix after long-term treatment with NGF, suggesting terminal differentiation. Our data support the idea that introduction of a constitutively expressed NGFR cDNA into cells with neuronal background results in the assembly of a functional NGF-NGFR signal cascade in a permissive extracellular environment.

Human nerve growth factor (NGF) is an important neurotrophic factor responsible for the growth, differentiation, and survival of sympathetic and neural crest-derived sensory neurons (33, 41, 47). NGF acts by binding to a cell membrane-bound receptor, NGFR, which has an approximate molecular mass of 70 to 75 kilodaltons (20). The receptor binds the ligand with low or high affinity (39, 44), and the biological response of NGF binding has been thought to be mediated only through the high-affinity receptor (16, 43). Few NGF-responsive cell lines, such as the PC12 line and some neuroblastoma (NB) cell lines, which have high-affinity NGFR, have been isolated (19, 42).

Recently, the genes encoding the human and rat NGFR have been isolated and sequenced, and only the low-affinity receptor was found when transfected into fibroblasts or melanoma cells (27, 35). Transfection of the receptor cDNA into a mutant PC12 cell line which does not express a functional NGF-NGFR cascade indicated the presence of a high-affinity binding site, but no neurite outgrowth occurred upon treatment with NGF (25). This may be due to a lack of a permissive extra- or intracellular environment as has been previously suggested (9, 15, 29). We hypothesize that constitutive expression of a cloned human NGFR cDNA in cells with neuronal background can reconstitute a functional NGF-NGFR signal cascade and that a proper extracellular environment would allow for terminal differentiation of the cells upon treatment with NGF. We have chosen NB cells grown on an in vitro biosynthetically produced extracellular matrix (ECM) to test our hypothesis.

NB is a highly malignant childhood tumor of neural crest origin. Some tumors show differentiation, and spontaneous regression can occur (11); however, the mechanisms involved are unknown. Most NB cell lines lack a functional NGF-NGFR signal transduction cascade. We have recently established a human NB cell line, HTLA 230 (2), which does not express a functional NGFR. Here, we demonstrate that transfection of the human NGFR cDNA into these cells results in the expression of high-affinity cell membrane-bound NGF receptors. Transient activation of the c-*fos* proto-oncogene followed by neurite outgrowth and cessation of DNA synthesis occurred upon NGF treatment, suggestive of terminal differentiation.

Isolation of transfectants. We electroporated (6) the PMVE1 expression vector containing the human NGFR cDNA (27) into HTLA 230 cells. Transfectants were plated onto rat smooth-muscle cell (R_{22}ClF)-derived ECM (28) and selected in 1 mg of G418 (GIBCO Laboratories, Grand Island, N.Y.) per ml for 3 weeks. Several clones expressing the NGFR were isolated by four consecutive rounds of immunological rosette assay (1) using the anti-human NGFR mouse monoclonal antibody, ME20.4 (36). Transfectants were further purified by five consecutive rounds of selection of high-NGFR-expressing cells by using the fluorescence-activated cell sorter, and the fluorescence histogram of the isolated transfectant, 98-3 cells, is shown in Fig. 1. More than 95% of the 98-3 cells showed positive NGFR staining. The level of surface expression of individual cells varied, which resulted in a broad fluorescence spectrum previously described in in vitro gene transfer experiments (22). This heterogeneity may further be due to a cell cycle-dependent expression of the NGFR as has recently been suggested (37) and was also described for other growth factor receptors (7). Heterogeneity, although to a lesser extent, was also seen with A875 melanoma cells known to have high numbers of NGF receptors (36). Incubation of the parental cell line HTLA 230 with ME 20.4 antibody followed by the fluorescein isothiocyanate (FITC)-conjugated antibody did not change the fluorescence intensity compared with that of cells incubated with the FITC-conjugated antibody alone (data not shown), suggesting that these cells do not have endogenous membrane-bound NGFR.

Northern (RNA) blot analysis was performed with RNA isolated from parental cell line HTLA 230; a human melanoma cell line, A875 (14); and various transfectants, and the results are shown in Fig. 2. A NGFR mRNA transcribed from the expression construct in transfected cells showed a slightly higher molecular weight when compared to the endogenous transcript in A875 cells, consistent with previous observations (25). The same-size transcript was also found in more than 80% of neomycin-resistent clones initially screened. We, therefore, concluded that no activation

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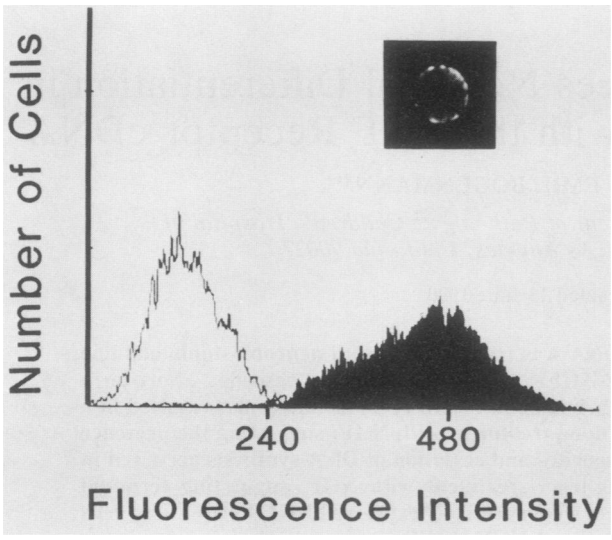

FIG. 1. FACS analysis of 98-3 cells. 98-3 cells were harvested by using phosphate-buffered saline containing 5 mM EDTA, centrifuged, and washed with phosphate-buffered saline. Cells were incubated with the ME20.4 antibody followed by incubation with a 1:40 dilution of FITC-conjugated, affinity-purified goat anti-mouse immunoglobulin G (Organon Teknika, West Chester, Pa.) (black area) or the FITC-conjugated secondary antibody alone (white area). Inset: Ring pattern of 98-3 cells after staining for the NGFR (magnification, ×400).

of the endogenous NGFR gene occurred. Several independent Northern blots demonstrated no detectable NGFR transcript in HTLA 230 parental cells. It was interesting that highly purified 98-3 cells showed a lower level of mRNA expression than the pools from which the cells were originally isolated. A possible explanation for this finding is that pool 3 and 4 represent highly heterogeneous cell populations in which we detected many clones of cells positive for the

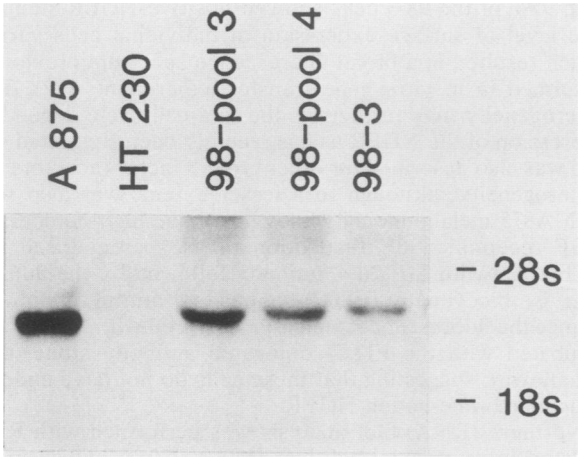

FIG. 2. Northern blot analysis of NGFR expression. Total cellular RNA from parental HTLA 230 cells, human melanoma A875 cells, and various transfectants were isolated (5) and hybridized with the 0.8-kilobase *Eco*RI fragment of the pH1-3 clone (27) of the NGFR cDNA as previously described (3). Final wash stringency of 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C was used. Rehybridization of the blot with the probe for transducin β subunit (13) showed that equal amounts of RNA (30 μg per lane) were loaded (data not shown).

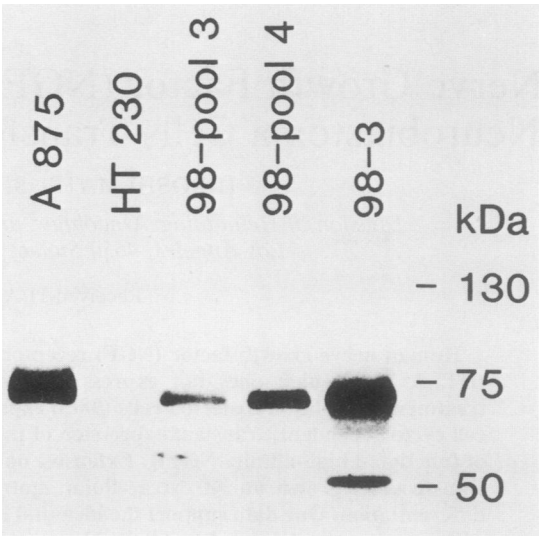

FIG. 3. Immunoprecipitation of the NGFR. Parental HTLA 230 cells, A875 melanoma cells, and three transfectant cell populations were grown in cysteine-free medium supplemented with 10% dialyzed heat-inactivated fetal bovine serum for 2 h, followed by a 16-h incubation with the same medium containing 50 μCi of [^{35}S]cysteine (ICN Pharmaceuticals Inc., Irvine, Calif.) per ml. Cells were harvested and solubilized in RIPA buffer (31) and immunoprecipitated with ME20.4 antibody. Samples were suspended in sodium dodecyl sulfate sample buffer containing 5% 2-mercaptoethanol, electrophoresed on a 10% polyacrylamide slab gel under denaturing conditions (32), and prepared for autoradiography. Note that A875 melanoma cells express high numbers of NGFR, and, therefore, we used 1×10^6 cpm per assay from melanoma cells and 20×10^6 cpm per assay from parental cells and transfectants. A 70- to 75-kilodalton protein similar to that seen in A875 cells was precipitated from transfected cells; however, no NGFR molecules were detectable in the parental HTLA 230 cell line.

transcript of the NGFR gene but negative for the surface receptor. Similar findings have also been reported for rat NGFR cDNA (35).

Immunoprecipitation (Fig. 3) was further performed to ascertain the molecular weight of the expressed NGFR protein. A receptor molecule of approximately 70 to 75 kilodaltons, similar to that found in A875 cells (20), was precipitated from lysates of transfected NB cells. Significantly more labeled NGFR protein was precipitated from 98-3 cells than from pool 3 or pool 4 cells, although equal amounts of radioactivity per lysate was used. This result is not surprising, since 98.3 cells are highly homogeneous with respect to membrane-bound NGFR, compared to pool 3 and 4 cells. Immunoprecipitation with serial dilutions of lysates from A875 cells, known to express 7×10^5 NGFR molecules per cell (12), revealed that approximately 3×10^4 receptor molecules per cell were present in transfectants. HTLA 230 cells did not show a precipitable receptor, which was consistent with previous results. A minor low-molecular-weight band was present in some lysates of 98-3 cells, possibly a contaminant or a degradation product of the receptor. The results thus far clearly demonstrated that we have isolated NB cells with stable membrane-bound NGFR expression.

Binding studies of [^{125}I]NGF with a crude membrane preparation (25) from the 98-3 cells suggested the presence of a high-affinity ($K_d = 10^{-11}$ M; 1×10^3 to 2×10^3 receptors per cell) and low-affinity ($K_d = 10^{-9}$ M; 3×10^4 receptors per cell) form of the NGF receptor (data not shown). These low numbers of receptors and the possible heterogeneous distri-

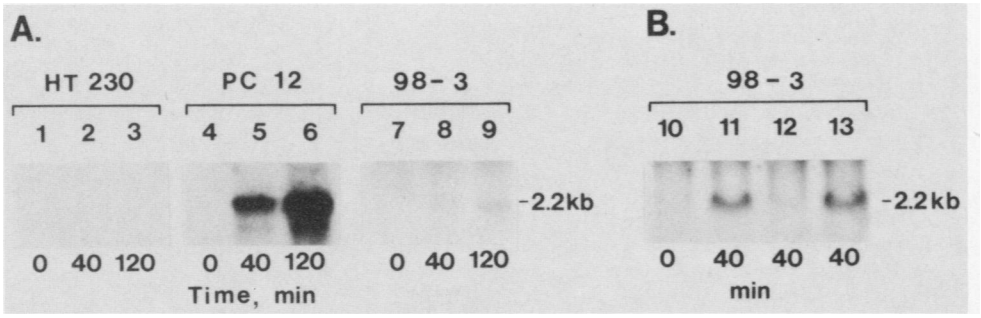

FIG. 4. C-*fos* gene expression upon NGF treatment. Total cellular RNA was isolated from cells treated with 2.5 S-NGF (Collaborative Research, Bedford, Calif.) (0.25 μg/ml) in the presence (lanes 3, 6, 9, 12, and 13) or absence (lanes 1, 2, 4, 5, 7, 8, 10, and 11) of 100 μM anisomycin at specific time points. Anisomycin was added as concentrated solutions (10 mM) (Sigma Chemical Co., St. Louis, Mo.) directly to the culture medium, and the cells were then stimulated 30 min later by the additions of NGF. In lane 12, transfectants were incubated with anisomycin only. Northern blots were hybridized with the 1.3-kilobase *Pvu*II-*Bgl*II fragment from the FBJ plasmid (8). Final wash stringency of 0.5× SSC at 60°C was used. (A) c-*fos* gene expression in exponentially grown cells treated with NGF for various time periods and long-term exposure of the Northern autoradiography. (B) c-*fos* gene expression in G1-arrested 98-3 cells 40 min after treatment with NGF and short-term exposure of the autoradiography.

bution of the receptor within the cell population make absolute determinations very difficult, and, therefore, the biochemical properties of the transfected receptor represent the best possible approximations. We, however, could unequivocally demonstrate the presence of high-affinity receptors by its biological response to NGF, since only the high-affinity form of NGFR can transiently activate the c-*fos* proto-oncogene and induce neurite extensions in NGF-responsive cells (16, 42, 43).

Expression of the c-*fos* proto oncogene in treated 98-3 cells but not HTLA 230 cells was seen 40 min after addition of NGF in long-term exposures of Northern blots (Fig. 4A). The level of the c-*fos* expression was significantly higher in 98-3 cells pretreated with anisomycin, which has been known to superinduce the c-*fos* response (17). NGF treatment of transfectants after 3 days of serum starvation, which resulted in the arrest of 80% of the cells in G1 as determined by fluorescence-activated cell sorter analysis, showed a substantial increase in c-*fos* gene transcription (Fig. 4B) when compared to that of nonsynchronized cells, indicating a cell cycle-dependent regulation of the transfected NGFR.

Since c-*fos* gene induction is a biological response not unique to NGF treatment (18, 30, 34), we also investigated the ability of the transfected cells to morphologically differentiate upon treatment with NGF. Transfectants were, therefore, grown on an ECM known to modulate differentiation abilities of various cell types (2, 40) and treated with NGF (0.5 μg/ml), which did not alter the growth rate. The cells did not show spontaneous differentiation in the absence of NGF; however, extensive neurite outgrowth was seen 5 days after treatment with NGF (Fig. 5). We further performed a clonal analysis in order to quantitate the number of cells which morphologically respond to NGF treatment. Transfected cells (98-3) were grown at clonal density (700 cells per 60-mm dish) on the ECM, and individual clones containing 20 to 30 cells were obtained within 5 days. Such cultures were subsequently treated for 9 days with NGF (0.5 μg/ml) and fixed with 2.5% glutaraldehyde, and 100 individual clones were analyzed for the presence of differentiated cells. NB cells with neurites three times the length of the cell body were considered morphologically differentiated. We were able to unequivocally identify cells with a differentiated morphology in 20% of NGF-treated clones, while no differentiation occurred in nontreated clones. None of the differentiated clones showed a uniform morphology, suggesting that only certain cells within a cloned cell population are capable of morphologically responding to NGF. Heterogeneity within cloned cell populations with regard to various biological properties is a common phenomenon (22). Moreover, primary NB lesions are often composed of morphologically heterogeneous populations of cells representing different stages of neuronal differentiation (45). Although 98-3 cells were single-cell cloned and are phenotypically homogeneous, they may be heterogeneous with regard to their stages of differentiation. NGFR is expressed and probably functional at specific stages of neuronal cell differentiation during development (4, 10). Hence, 98-3 cells, which are at specific stages of differentiation, do respond to NGF by induction of cell differentiation, while others do not, which results in a phenotypically heterogeneous cell population.

We further investigated whether morphologically differentiated cells still synthesize DNA. We, therefore, treated 98-3 cells with NGF for 7 days followed by a 24-h pulse of [methyl-^{3}H]thymidine ([^{3}H]TdR) (1 μCi/ml), and autoradiography was then performed (Fig. 6). Unlike PC12 cells treated with NGF (21), our quantitative analysis demonstrated that 95% of morphologically differentiated cells had not incorporated the label while more than 80% of non-NGF-responsive cells were labeled. Differentiated cells maintained their differentiated state upon removal of NGF, which also differs from the behavior of PC12 cells (19). We, therefore, concluded that these cells were terminally differentiated.

Our finding that 98-3 cells transiently express c-*fos* proto-oncogene and differentiate into neuronlike cells upon treatment with NGF represents the first unequivocal evidence that the human NGFR cDNA encodes a protein which participates in a functional high-affinity NGFR molecule. In contrast to a previous study (25), our investigation for the first time demonstrated that all the necessary downstream mechanisms were inducible in transfected 98-3 cells, leading to terminal differentiation when cells were grown on an in vitro biosynthetically produced ECM. Such ECMs have long been known to modulate growth and differentiation behavior of various cell types, including pediatric tumors (2, 38, 46). Our results indicate that 98-3 cells have acquired the necessary cellular mechanisms to biochemically respond to NGF, e.g., c-*fos* expression. A neuron-specific mechanism may be necessary to generate high-affinity binding sites, similar to what has been described for the interleukin-2 receptor (23,

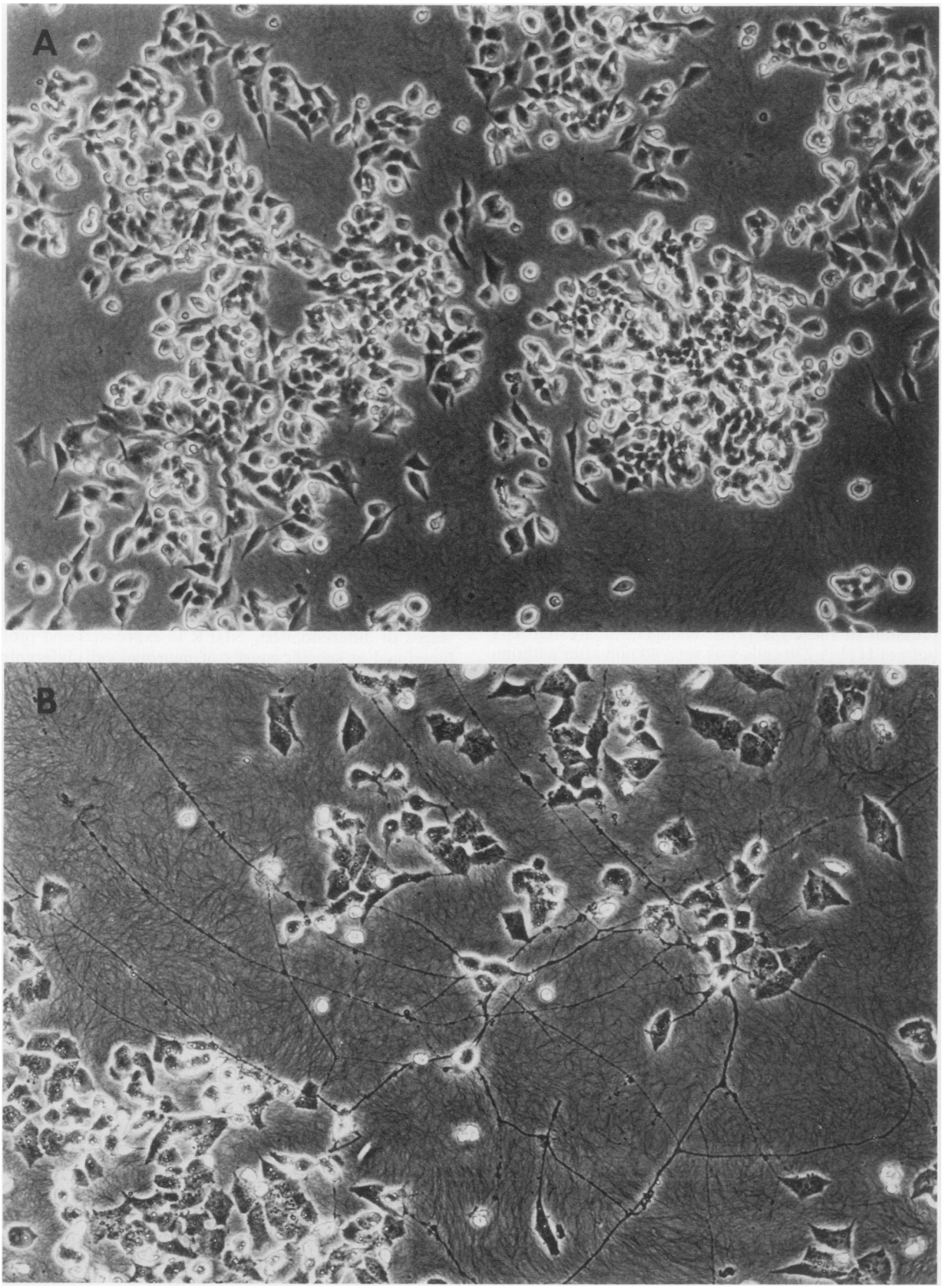

FIG. 5. Phase-contrast micrographs of 98-3 cells. 98-3 cells plated on R_{22} ClF matrices were grown in the absence (A) or presence (B) of NGF (0.5 μg/ml) for 5 days. Note that cells grown in the absence of NGF did not show any morphological features of differentiation while NGF-stimulated cells extended long neurites which connected with neurites from other differentiated cells. Most neurites extended beyond the observation field. Magnification, ×300.

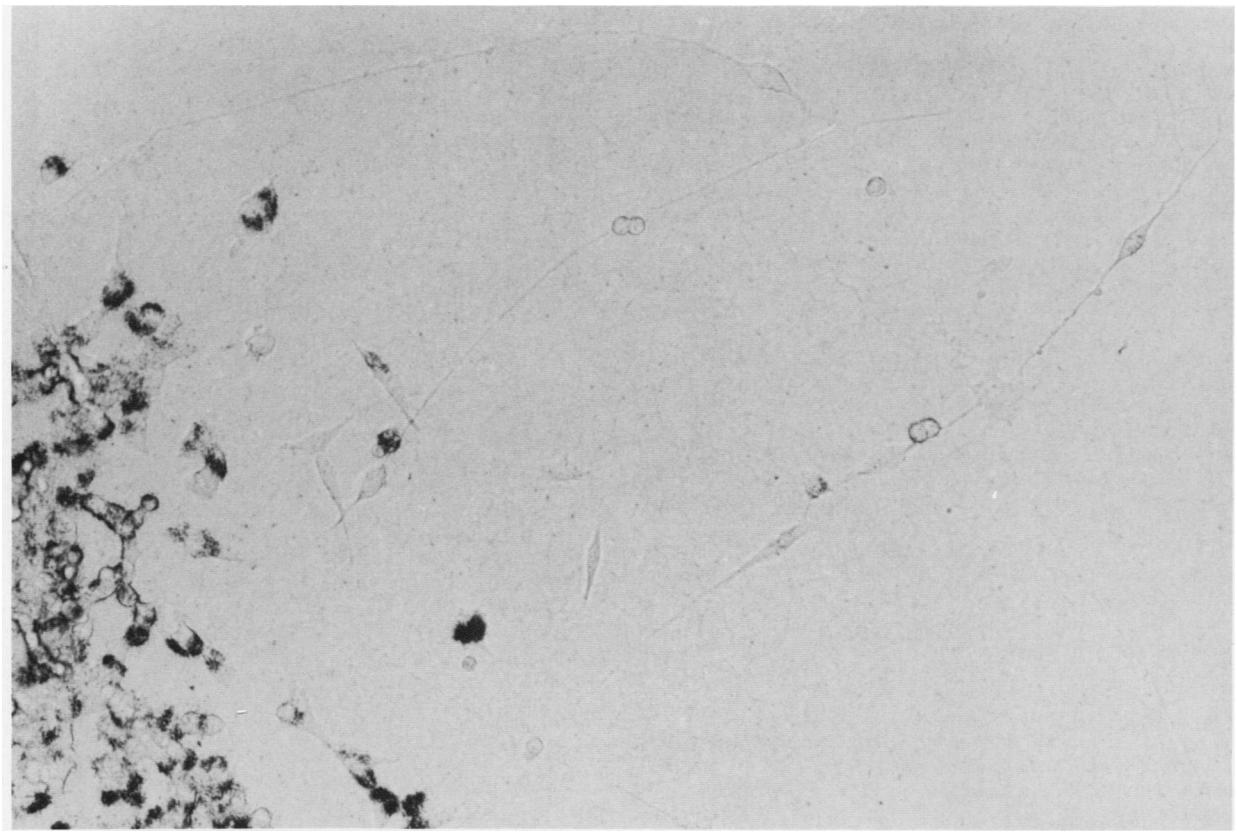

FIG. 6. Autoradiography of [^{3}H]TdR-labeled cultures. 98-3 cells were grown on R_{22}ClF matrices for 7 days in the presence of 0.5 μg of NGF per ml followed by 24 h of incubation of [^{3}H]TdR (ICN), and autoradiography was performed. No silver grains in morphologically differentiated cells were detected, while non-NGF-responsive cells showed autoradiographic labeling. Note that this photograph represents a bright field picture in which cell morphology is not easily detectable, but which allows for identification of silver grains. Magnification, ×300.

24). However, components of the ECM are required for morphological and terminal differentiation of NB cells.

Conversion of malignant cells into terminally differentiated postmitotic cells is a rare event, and only recently has it been shown that the retinoblastoma gene, when introduced into retinoblastoma cells, can suppress the tumorigenic phenotype (26). It remains to be seen whether transfected 98-3 cells, when transplanted into an immunosuppressed mouse, can be converted into nonmalignant cells by treatment with NGF. These studies will then demonstrate whether the NGF-NGFR signal transduction cascade can exert tumor suppression activity.

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