

# An Abnormal Response of Retinoblastoma Cells (Y-79) to Neurotrophins

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**PURPOSE.** To clarify the expression of neurotrophins and their receptors in retinoblastoma (Rb) cells, to elucidate their potential role in the proliferation of neuroectodermal tumor cells, and to establish conditions for Rb cell differentiation.

**METHODS.** The Rb-derived cell line Y-79 was grown in serum-free suspension or monolayer culture. Proliferating and differentiated cells were isolated and submitted to semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis, immunostaining, and flow cytometry. The proliferation rate of the cells was estimated by 5-bromo-2'-deoxyuridine (BrdU) incorporation, and the effects of neurotrophins and laminin on BrdU-incorporation, process outgrowth, or immunostaining were determined.

**RESULTS.** In contrast to previously studied normal retinal precursor cells, Y-79 cells not only express nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and p75, but also the corresponding high affinity receptors TrkA, TrkB, and TrkC. Proliferation was stimulated by exogenous and endogenous neurotrophin receptor ligands. Inhibition of protein kinase phosphorylation with K252a blocked proliferation and promoted differentiation. The effect of K252a on differentiation was enhanced by the addition of soluble laminin. After 9 days of combined treatment, the fraction of differentiated cells amounted to 30%, differentiation being characterized by improved attachment, neurite outgrowth, expression of NF-68, and a loss of glial fibrillary acidic protein (GFAP) and parvalbumin immunoreactivity. These changes were accompanied by a downregulation of TrkB and TrkC, but not TrkA or p75. Differentiated cells were isolated and further grown in the absence of K252a. However, despite the high level of TrkA expression in differentiated cells, the addition of NGF had no effect on their survival.

**CONCLUSIONS.** A mitogenic action of neurotrophins could contribute to retinal tumor growth. (*Invest Ophthalmol Vis Sci.* 2000;41:1932-1939)

In the retina, cell lineage studies have long ago established that postmitotic precursor cells exhibit the potential to differentiate into any of the various phenotypes that compose the final configuration of this organ.<sup>1,2</sup> This fact implies that environmental factors—namely, the extracellular matrix proteins and growth factors—can influence the cellular composition of the mature retina. Alterations in the responsiveness of retinal precursor cells to environmental signals may be the basis for a malignant transformation. It is therefore important

to find out under which conditions signaling pathways identified during normal retinal development can be reproduced in ectodermal tumor cells under defined growth conditions.

We have studied the effects of neurotrophins and laminin-1 on retinoblastoma (Rb) cells. Rb is a highly malignant childhood neoplasm<sup>3</sup> that results from the inactivation of a gene encoding the Rb tumor suppressor protein (pRb).<sup>4,5</sup> Mutation of both alleles of the pRb gene inevitably leads to tumor development. Several cell lines are now available for experimental studies on Rb cells. These include the human Rb cell line Y-79<sup>6</sup> that resembles pluripotent embryonic stem cells and their immature neuronal and glial progeny.<sup>7</sup> Although numerous attempts have already been made to induce Y-79 cells to differentiate, most of the investigated agents, including laminin<sup>8,9</sup> and cyclic adenosine monophosphate (cAMP) analogues,<sup>10</sup> failed to produce the full set of features expected from normally developed retinal cells. However, Rb cells were not yet tested for the effects of neurotrophins.

Neurotrophins constitute a family of growth factors with diverse functions during central nervous system (CNS) development (reviewed in References 11 and 12). The wide range of responses to nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3) are based on

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their capacity to govern multiple signaling pathways (reviewed in Reference 13). All neurotrophins activate two distinct types of plasma membrane receptors: a common low-affinity receptor, p75,<sup>14,15</sup> and ligand-specific high-affinity receptors with tyrosine kinase activity, TrkA,<sup>16</sup> TrkB,<sup>17,18</sup> and TrkC.<sup>19</sup> In the proliferating retina, neurotrophins have been found to play a major role in controlling the number and differentiation of postmitotic migrating precursor cells. In the absence of TrkA, NGF induces cell death by binding to the p75 receptor.<sup>20</sup> Conversely, BDNF and NT-3 promote the survival of retinal precursor cells that would otherwise die before reaching their final destinations.<sup>21,22</sup> In addition, BDNF and NT-3 contribute to the differentiation and functional maturation of retinal ganglion cells.<sup>21,23,24</sup>

Neurotrophins are also known as mitogens, but in the CNS their mitogenic action seems to be confined to nonneuronal precursor cells.<sup>25</sup> The proliferation rate of normal neuron precursor cells of cortical,<sup>26</sup> cerebellar,<sup>27</sup> and retinal origin<sup>21</sup> was not affected by neurotrophins. Even in transformed precursor cells of the CNS the predominant result of neurotrophin treatment was apoptosis.<sup>28,29</sup> In some neuroectodermal tumors, a more favorable outcome was associated with high expression levels of TrkA or TrkC, because the latter increased apoptosis.<sup>28,30-32</sup> However, Trk receptors were not encountered in neuronal precursors of the normal retina (see the Discussion section).

How a given cell population reacts to neurotrophins or other soluble factors may be influenced by the capacity of these factors to regulate the cellular response to extracellular matrix ECM proteins. Experiments with normal neuroepithelial cells of retinal origin showed that the acquisition of neuronal properties is stimulated by laminin-1.<sup>33</sup> However, the expression of the laminin-1 receptor subunit  $\alpha 6$  requires the presence of an insulin-like growth factor.<sup>34</sup> In neuroblastoma cells, differentiation induced by retinoic acid is associated with upregulation of the expression of integrin  $\alpha 1 \beta 1$ .<sup>35</sup> In contrast, rapid proliferation of neuroblastoma cells as a consequence of N-myc overexpression was reflected in greatly reduced levels of integrin  $\alpha 2$  and  $\alpha 3$  mRNAs.<sup>36</sup> The possible link between responsiveness to laminin and neurotrophin receptor activation has not yet been investigated in retinal tissues.

Thus, the main objective of the present study was to determine the neurotrophins and neurotrophin receptors expressed by Rb cells, to elucidate the potential role of neurotrophins in the proliferation of neuroectodermal tumor cells, and to establish in vitro conditions for the differentiation of retinal tumor cells that could be compared with previously described results from normal retinal precursors.

A preliminary account of this work has already appeared.<sup>37</sup>

## METHODS

### Cell Culture

Stocks of Y-79 human Rb cells (American Type Tissue Culture, Rockville, MD) were maintained in suspension culture at 37°C (95% air, 5% CO<sub>2</sub>) in RPMI 1640 medium, with 2 mM glutamine, 15% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (all obtained from Gibco, Eggenstein, Germany). Before plating, cells were transferred to the same, but serum-free, medium and kept for at least 1 week in suspension

culture. For attachment cultures, cells were seeded onto 0.1% poly-D-lysine-coated (Sigma, Deisenhofen, Germany) glass coverslips at a density of  $3 \times 10^4$  cells/cm<sup>2</sup> and maintained in serum-free medium. The latter was half-changed three times weekly. Cultures were tested for the effects of NGF (Alomone, Jerusalem, Israel; 50 ng/ml), K252a (Kamiya, Thousand Oaks, CA; 100 nM), or laminin from mouse Engelbreth-Holm-Swarm tumor (20 µg/ml; Becton Dickinson, Heidelberg, Germany).

### Proliferation Assay

After a 30-minute exposure to 5-bromo-2'-deoxyuridine (BrdU) for incorporation into the DNA of replicating cells, proliferating cells were detected using a BrdU staining kit (Boehringer-Mannheim, Mannheim, Germany). The label was visualized with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP).

### Immunostaining

Monolayer cultures were fixed with 1.5% paraformaldehyde and 1% glutaraldehyde (Sigma). The cells were permeabilized, and endogenous peroxidases were blocked in a solution of 3% H<sub>2</sub>O<sub>2</sub> in methanol (1:4) for 5 minutes, washed, and incubated with the primary antibody for 16 hours at 4°C. The following primary antibodies were used: monoclonal anti-parvalbumin mouse IgG1 (clone PA-235; Sigma; 1:500 in phosphate-buffered saline [PBS] containing 5% normal goat serum), monoclonal anti-calbindin mouse IgG1 (clone CL-300; Sigma; 1:300 in PBS containing 5% normal goat serum), and polyclonal anti-calretinin (Chemicon, Temecula, CA; 1:2500 in PBS containing 5% normal rabbit serum). This was followed by incubation with a biotinylated secondary antibody (goat anti-mouse; Sigma; 1:200 in PBS + 1% bovine serum albumin [BSA], for parvalbumin or calbindin staining, or rabbit anti-goat; Dianova, Hamburg, Germany; 1:200 + 1% bovine serum albumin for calretinin staining) and application of streptavidin-peroxidase (Sigma). The latter was visualized with diaminobenzidine and hydrogen peroxide (Sigma). For negative controls, primary antibodies were replaced with normal serum. No significant labeling was seen under these conditions.

### Flow Cytometry

To obtain cultures enriched with differentiated Y-79 cells for subsequent flow cytometry, monolayer cultures were subjected to an extensive washing procedure. After complete removal of the cell culture medium, the glass coverslips were dipped three times into a vessel with PBS. This resulted in a decrease in the total cell number from  $7023 \pm 183$  to  $5672 \pm 356$  cells/cm<sup>2</sup> and enhanced the fraction of process-bearing cells from  $28.6\% \pm 1.8\%$  to  $60.9\% \pm 3.4\%$  ( $n = 3$ ). Cells were then treated with ice-cold 0.1 M EDTA-PBS solution and removed from the glass coverslips by a cell scraper. Undifferentiated Y-79 cells were directly taken from suspension cultures. Both samples were washed with PBS containing 5% BSA and 0.1% NaN<sub>3</sub>, fixed with 3% paraformaldehyde, and incubated for 3 hours at 4°C with either a glial fibrillary acidic protein (GFAP) antibody (Sigma; 1:500) or an NF 68 antibody (Sigma; 1:150). After repeated washes, cells were incubated for 30 minutes in a fluorescein isothiocyanate-conjugated secondary antibody (Dianova), washed again, and analyzed in a flow cytometer (FACS Trak; Becton Dickinson, Heidelberg, Germany).

## Reverse Transcription–Polymerase Chain Reaction

For reverse transcription–polymerase chain reaction (RT-PCR) cells were processed as for flow cytometry. Total RNA was prepared from cells using Trizol reagent (Gibco). RNA was dissolved in diethyl pyrocarbonate (DEPC)-H<sub>2</sub>O and first-strand cDNA synthesis was performed using Superscript II reverse transcriptase (Gibco) and an oligo-dT primer. PCR reactions were performed in a thermal cycler (Biometra, Göttingen, Germany) under the following conditions: denaturation at 94°C for 45 seconds, annealing at 58°C for 45 seconds, and extension at 72°C for 45 seconds. Twenty-nine cycles of amplification were performed. The primers used are given in Table 1.  $\beta$ -actin amplification was used for semiquantitative analysis of Trk expression. For negative controls, cDNA was replaced by bidistilled water. PCR products were analyzed on 0.8% agarose gels stained with ethidium bromide.

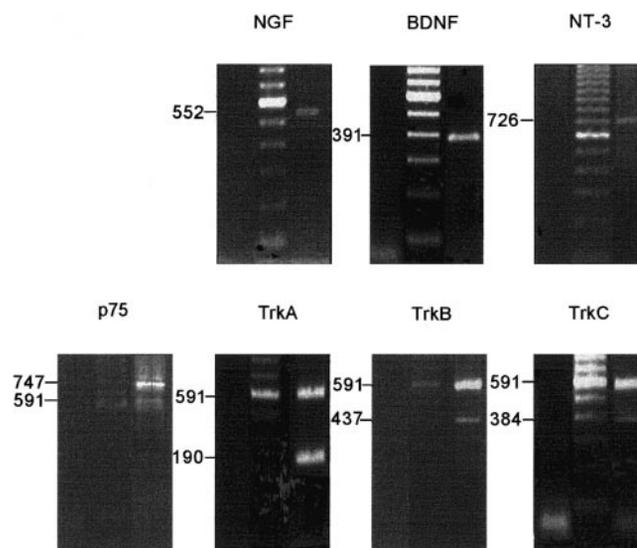
## Cell Counts and Statistics

Cells in unfixed monolayer cultures were counted under phase-contrast illumination ( $\times 200$ ). The cell density on day in vitro (DIV) 1 refers to cells in one focal plane only, 3 hours after plating. Counts were performed visually (cells with processes, parvalbumin immunostaining), or automatically, by fluorescence-activated cell sorting (GFAP, NF 68). In the former case, each data point represents the average from 15 fields (0.418 mm<sup>2</sup>) in two different dishes and at least three different experiments.

All data are represented as mean  $\pm$  SD. Significance levels for the differences between the mean values were determined by an unpaired Student's *t*-test and are shown in each graph by asterisks.

**TABLE 1.** Sequences of Oligonucleotide Primers and Predicted Sizes of RT-PCR Products

Gene	Orientation	5'–3' Sequence	Predicted Sizes of Amplified Fragments
NGF	Sense	TTCTGATCGGCATACAGGCG	552 bp
	Antisense	TGCTTTGAGTCAATGCCCCG	
BDNF	Sense	TTGGTTGCATGAAGGCTGCC	391 bp
	Antisense	ACTGTCACACAGCTCAGCT	
NT3	Sense	TCGCTTATCTCCGTGGCATC	726 bp
	Antisense	CTCGACAAGGCACACACACA	
TrkA	Sense	ATTTTGTGCACCGGGACCTG	190 bp
	Antisense	CGCTCTCGGTGGTGAACCTA	
TrkB	Sense	TGGATAAGGTGGCATGGACC	437 bp
	Antisense	TGGATTGCCACCAGGATCA	
TrkC	Sense	CAACTGCAGCTGTGACATCC	384 bp
	Antisense	AGTGTTGATGGACTGCAGCC	
p75	Sense	CAGACAGCTCAGATGTGCTGC	747 bp
	Antisense	TCTCTGCAGGCACAAGGGCTT	
$\beta$ -actin	Sense	TTCTACAATGAGCTGCGTGTG	591 bp
	Antisense	CGTCACACTTCATGATGGAGT	



**FIGURE 1.** RT-PCR analysis indicates that Y-79 cells express neurotrophins and neurotrophin receptors. *Top:* Ethidium bromide-stained agarose gels of the PCR products amplified with primers specific for NGF, NT3, and BDNF. *Bottom:* Ethidium bromide-stained gels of the PCR products amplified with primers specific for p75, TrkA, TrkB, and TrkC and, in all cases,  $\beta$ -actin (591-bp PCR product). Molecular weight markers are shown in the lanes on the left, and the numbers indicate the predicted size of the specific PCR products (in bp). *Left lanes:* negative controls, with bidistilled water instead of cDNA in the PCR reaction ( $n = 3$  in all cases).

## RESULTS

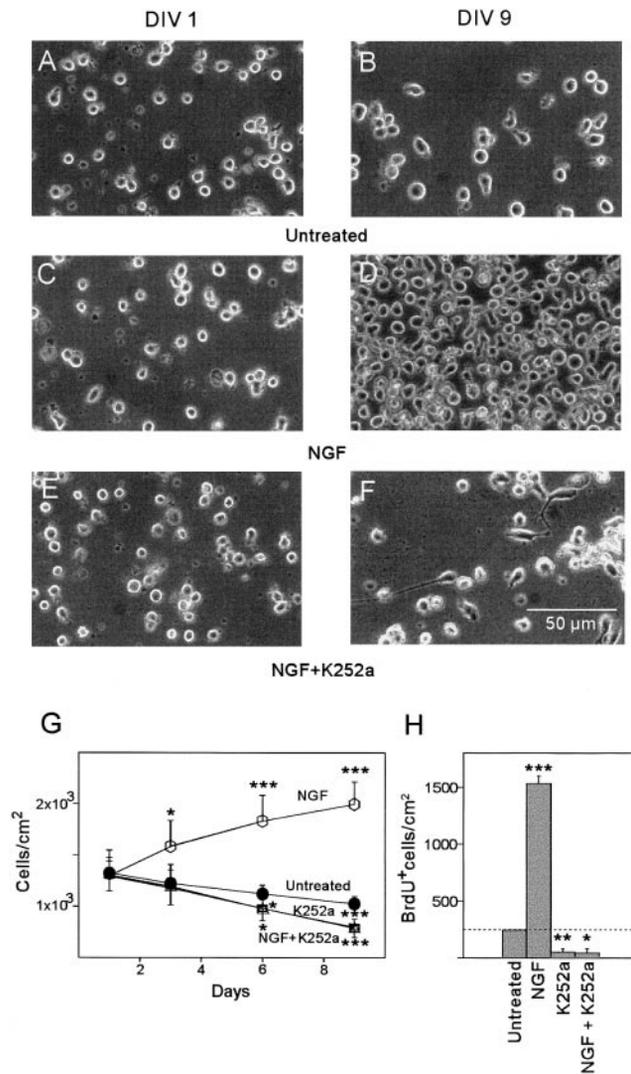
### Neurotrophins and Their Receptors in Proliferating Y-79 Cells

Samples of Y-79 cells were obtained from serum-free suspension cultures. The expression of mRNA for the neurotrophins NGF, BDNF, NT-3, and their respective receptors TrkA, TrkB, and TrkC, as well as the p75 receptor, was analyzed by RT-PCR (Fig. 1). The messages for all three neurotrophins and each of the four receptors were detected in proliferating tumor cells, a situation not encountered in neuronal precursor cells (see the Discussion section). We therefore decided to examine the effects that the exposure to neurotrophins may have on Rb cells grown in serum-free monolayer cultures.

### Proliferative Effects of Neurotrophins

Neurotrophins were added at concentrations of 50 ng/ml (NGF) or 10 ng/ml (BDNF or NT-3). After 9 days in vitro, a clear difference was observed between control and test cultures (Figs. 2B, 2D). Neurotrophin-treated cultures were denser and exclusively composed of round or pleomorphic cells, some of them forming colonies. Cell counts revealed that neurotrophins stimulated proliferation at any time after plating. After 9 days in the presence of NGF, the average cell density nearly doubled (Fig. 2G). Similar effects were found with both BDNF and NT-3 ( $n = 6$ , results not shown).

It is very likely that the proliferative response to exogenous neurotrophins was mediated by Trk receptors, because simultaneous treatment with the tyrosine kinase inhibitor K252a (100 nM) prevented the neurotrophin-induced increase in cell density (compare Figs. 2D and 2F). At DIV9 the density



**FIGURE 2.** NGF stimulates proliferation of Y-79 cells by Trk receptor activation. Photomicrographs of cultured Y-79 cells after 3 hours (A, C, and E) and 9 days in monolayer culture (B, D, and F). (A, B) Cells in defined medium without additions. (C, D) Cells treated with 50 ng/ml NGF. (E, F) Cells treated with 50 ng/ml NGF and 100 nM K252a ( $n = 6$ ). Note the strong increase in the cell number in cultures treated with NGF (E) and the presence of processes in cells treated with the Trk receptor inhibitor (F). (G) Plot of cell density versus time in monolayer culture under different culture conditions ( $n = 6$ ). (H) Number of cells/cm<sup>2</sup> incorporating BrdU at DIV 9 ( $n = 3$ ). Asterisks show results of Student's *t*-test. In this and Figures 3 through 6: \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ .

of K252a- and NGF-treated cultures decreased to 77% of the control value at DIV1 (Fig. 2G, compare filled circles with open squares and triangles). This density was similar to that of cultures treated with K252a alone, suggesting that proliferation could in part be maintained by endogenous Trk receptor ligands.

To estimate the rate of proliferation in Y-79 cultures more directly, we determined the fraction of BrdU-incorporating cells under various conditions. Cells were labeled at DIV9 by a single 30-minute exposure to BrdU, and cultures treated with NGF, K252a, and NGF + K252a were compared with untreated control cultures (Fig. 2H). Treatment with NGF in-

duced a 2.7-fold increase in the fraction of BrdU-labeled cells, whereas K252a decreased the fraction of BrdU-labeled cells to 36.6% (in the absence of NGF) and to 32.8% (in the presence of NGF). Because the K252a concentration was sufficient to block all Trk receptors,<sup>38,39</sup> we conclude that, in the absence of added NGF, proliferation was largely dependent on Trk receptor activation by ligands derived from the tumor cells through an autocrine or paracrine route.

**Differentiating Effects of Neurotrophin Receptor Blockade**

A characteristic feature of the cultures in which neurotrophin-induced proliferation had been blocked for 9 days with K252a was the presence of cells that had processes longer than one cell diameter (see Fig. 2F). This indicated that some degree of differentiation had occurred in these cultures. To provide molecular evidence that the process-bearing cells were in fact differentiated, Y-79 cultures were characterized by immunocytochemistry. Cells possessing these long processes downregulated the expression of the calcium-binding protein parvalbumin (Figs. 3A, 3B, arrowheads), whereas round cells without processes were strongly stained. Process outgrowth and loss of parvalbumin immunostaining were regarded as evidence of Y-79 cell differentiation. Flow cytometry confirmed that both criteria actually reflect the acquisition of a neuronlike phenotype (described later).

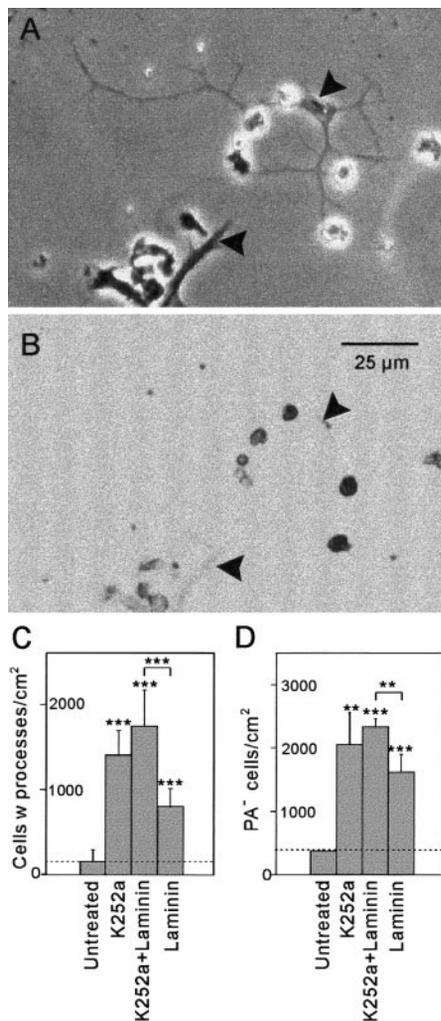
It should be mentioned that immunocytochemical investigation of two other calcium-binding proteins, calbindin and calretinin, did not reveal significant differences between round cells without processes and process-bearing cells. In untreated control cultures, 96% ± 1% and 94% ± 3% of the cells were immunoreactive for calbindin and calretinin, respectively. In treated monolayer cultures, the fraction of calbindin- and calretinin-immunoreactive cells amounted to 92% ± 3% and 91% ± 1%, respectively.

The criteria process outgrowth and parvalbumin immunostaining were used to estimate the increase in the number of differentiated cells in the presence of K252a, laminin, or both (Figs. 3C, 3D). K252a increased the density of process-bearing or parvalbumin-negative cells 6.8-fold and 5.5-fold, respectively.

Treatment with laminin has been reported to produce a degree of morphologic differentiation in Y-79 cultures.<sup>8</sup> Under our culture conditions, the addition of laminin in soluble form increased the density of differentiated Y-79 cells 4.4-fold (process outgrowth) or 4.3-fold (parvalbumin immunostaining), whereas the proliferation rate remained unchanged (three experiments with BrdU incorporation, as in Fig. 2H). The effects of both K252a and of laminin were further augmented when both treatments were combined. Therefore, to obtain a maximal differentiation response, Y-79 cultures were treated for 9 days with both K252a (100 nM) and laminin-1 (20 μg/ml). With this standard protocol, nearly one third (30%) of the Y-79 cells were considered to be differentiated. This figure should be compared with only 3% of differentiated cells in the untreated controls in serum-free monolayer culture.

**Neuronlike Phenotype of Differentiated Y-79 Cells**

In other pediatric brain tumors, increased GFAP expression has been associated with less mature cell types and correlated with



**FIGURE 3.** Laminin and K252a increase the number of differentiated and parvalbumin-negative Y-79 cells. (A, B) Photomicrographs of differentiated Y-79 cells under phase-contrast (A) and bright-field illumination (B). (C) Number of cells with processes per square centimeter at DIV 9 after different treatment ( $n = 6$ ). (D) Number of parvalbumin-immunonegative cells per square centimeter ( $n = 5$ ). Same conditions as in (C). Student's *t*-tests were performed to reveal significant differences between treated and untreated cultures (asterisks) and laminin-treated cultures in the presence and absence of K252a (horizontal bar).

a poorer prognosis.<sup>40</sup> It could therefore be expected that, in cultures treated with K252a and laminin, differentiated Y-79 cells lose GFAP expression. We tested this possibility by flow cytometry. Differentiated cells were obtained by submitting treated Y-79 monolayer cultures to three strong washes with PBS. This removed the nonadherent cells and created a bias for differentiated neuronlike cells. Proliferating cells were obtained from serum-free suspension cultures. Differentiated and proliferating Y-79 cells were incubated with antibodies against GFAP and NF 68 as markers for glial and neuronal cells, respectively. Flow cytometric analysis (Fig. 4) revealed that differentiated cells were indeed devoid of GFAP but expressed the neuronal marker NF 68. In contrast, proliferating Y-79 cells showed both markers. We thus concluded that differentiated Y-79 cells acquire a neuronlike phenotype.

### Persistence of TrkA Receptors in Differentiated Neuronlike Y-79 Cells

To find out whether differentiation changed the expression of neurotrophin receptors, semiquantitative RT-PCR analysis was performed in differentiated and proliferating Y-79 cells, prepared as for flow cytometry. The expression of both TrkA and p75 clearly persisted in the differentiated cell population, whereas TrkB and TrkC expression decreased to below detection level (Fig 5).

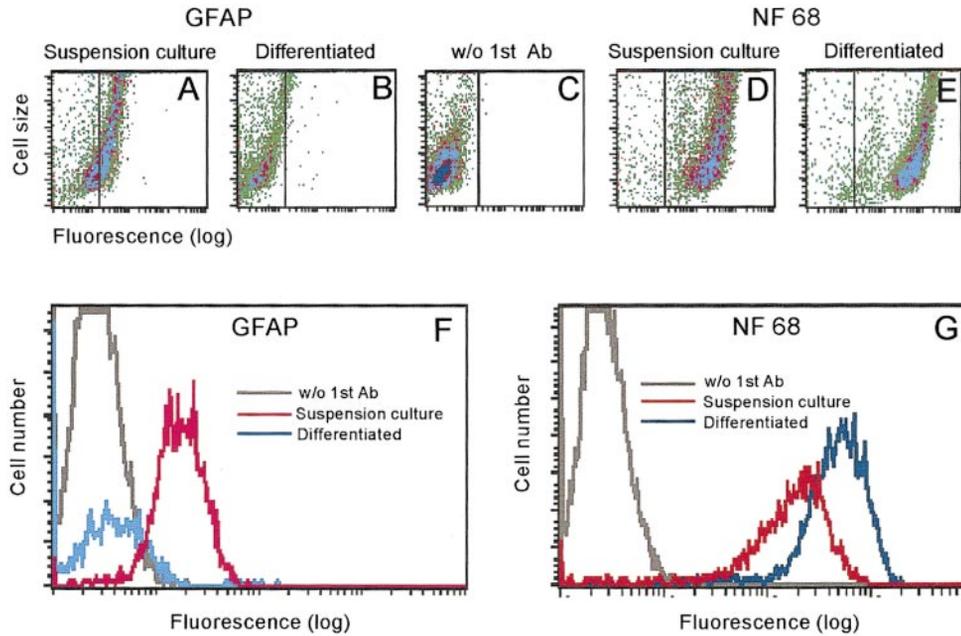
To address what might be the function of TrkA and p75 in differentiated Y-79 cells we considered the possibility that these receptors control, as in neurons, survival. To clarify this point, differentiated cells were again isolated by washing off the nonadhering cells. This procedure also removed the remnants of K252a, facilitating the response of newly expressed neurotrophin receptors to exogenous NGF (50 and 100 ng/ml). However, an effect of NGF on the survival of Y-79 cells was not observed (Fig. 6). Both in the presence and absence of NGF, cell density decreased within 4 days to approximately 50%. Thus, the likely fate of differentiated neuronlike Y-79 cells was to die, and NGF could neither prevent nor facilitate this process.

### DISCUSSION

The results of the present study led us to conclude that neurotrophins can contribute to the progression of Rb. Y-79 cells express mRNA for NGF, BDNF, NT-3, and the corresponding receptors TrkA, TrkB, and TrkC, and p75. Even under conditions of serum-free monolayer culture, the number of Y-79 cells significantly increased in the presence of added neurotrophins, and decreased when protein kinases were inhibited. Thus, proliferation of Rb cells was stimulated by exogenous as well as endogenous neurotrophins. Correspondingly, differentiation could be initiated by neurotrophin receptor block.

The present tumor cell differentiation model is based on the use of K252a<sup>38,41</sup> which has already been applied in other tumor models.<sup>42,43</sup> The essential point here is that K252a not only acts as an antiproliferative but also as a differentiating agent. It is, however, possible that the double antiproliferative, as well as differentiating effect of K252a reflects the inhibition of several tyrosine kinases. Rubin et al.<sup>44</sup> identified and characterized a mitogenic factor released by Y-79 cells, the so-called Rb-derived growth factor (RDGF). Y-79 cells were found to exhibit significant protein tyrosine kinase activity, and tyrosine phosphorylation was stimulated in the presence of RDGF.<sup>45</sup>

To what extent the antiproliferative effect of K252a is a prerequisite for the subsequent differentiation of Y-79 cells cannot be answered with certainty. The experiments with laminin were undertaken to satisfy the potential need for additional signals. Laminin-1 stimulates neuron differentiation inducing neurite outgrowth (reviewed in Reference 46). In the present experiments, added laminin indeed increased the fraction of differentiated neurons beyond the level achieved with K252a alone. But in contrast to normal retinal precursor cells in vitro,<sup>33</sup> the presence of laminin-1 was not sufficient to drive all the cells into differentiation. Moreover, differentiated Y-79 cells had similar neuronlike properties when growing on another adhesive substrate. This could, however, be related to the low levels of expression of the corresponding integrin



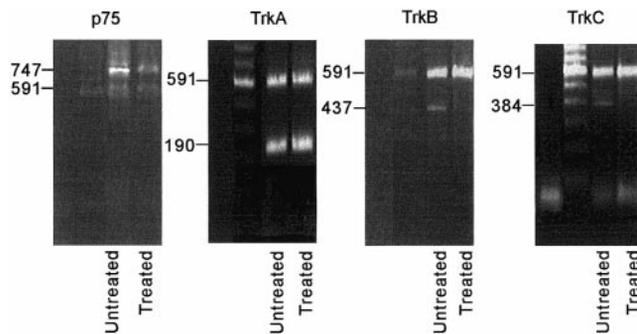
**FIGURE 4.** Flow cytometric analysis of GFAP and NF 68 immunofluorescence in treated and untreated Y-79 cells. (A through E) Size and fluorescence of immunostained Y-79 cells. (A, C, and D) Y-79 cells from serum-free suspension cultures. (B, E) Y-79 cells at DIV 9 after standard treatment with K252a and laminin. (C) Cells after staining without primary antibody, serving as control for (A, B, D, E). (A, B, and F) After GFAP immunostaining. (D, E, and G) After NF 68 immunostaining. (F, G) Overlay histograms of GFAP and NF 68 immunofluorescence. Note the decrease of GFAP and increase of NF 68 immunofluorescence in differentiated Y-79 cells.

receptor subunit  $\alpha 6^{34}$  or other changes in integrin receptor expression, which in turn would require other ECM components to induce neurite outgrowth. Indeed, a recent study identified an epitope on the internal domain of integrin  $\alpha 3$  in medulloblastoma cells that has not been observed in normal brain tissue.<sup>47</sup>

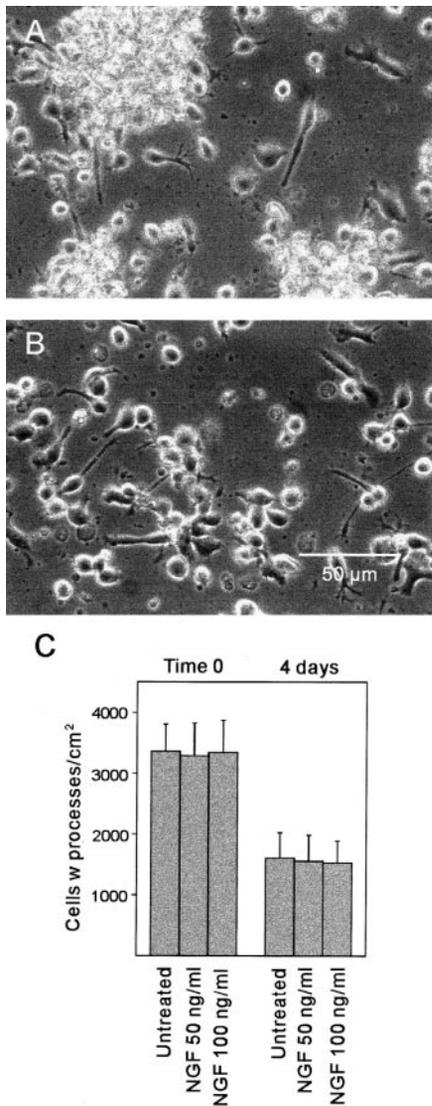
Although normal retinal precursors<sup>1,2</sup> as well as Y-79 cells<sup>7</sup> have been regarded as pluripotential, nearly all postmi-

totic Y-79 cells acquired a neuronlike phenotype. Further attempts to clarify the identity of the neuronlike Y-79 cells revealed some similarities with horizontal cells. Differentiated cells stained for  $\gamma$ -aminobutyric acid (GABA), had no axons and failed to generate action potentials. They also showed no immunoreactivity to Thy-1, a marker of ganglion cells; 3BA8, a marker of amacrine cells; and 4F3, a marker of Müller cells (Offermann and Grantyn, unpublished data, 1999). However, the absence of parvalbumin is not consistent with the assumption that differentiated Y-79 cells are horizontal cells.<sup>48,49</sup> We therefore conclude that postmitotic Y-79 cells did not completely reproduce any of the known retinal cell types.

How a given tissue responds to environmental factors depends on the available receptor forms and the signaling pathways recruited on receptor activation. With regard to NGF, it is already known that the final outcome of NGF treatment depends on the relative levels of p75 and TrkA expression. For instance, oligodendrocytes could be rescued from p75-mediated cell death by inducing the expression of TrkA.<sup>50</sup> In the developing retina, NGF promotes apoptosis only in cells that express p75 and not TrkA.<sup>20</sup> This expression pattern is characteristic of neuron precursor cells at the earliest stages of retinal development. It is very likely that these cells also have no TrkB and TrkC (Rodríguez-Tébar, unpublished data, 1989). Undifferentiated Y-79 cells, in contrast, clearly expressed all three Trk receptors, and an apoptotic effect of NGF, if present, was at least not obvious. Thus, the difference in the responses of normal retinal precursor cells and neuroectodermal tumor cells may be explained by qualitative and/or quantitative differences in the expression of p75 and Trk receptors. Whereas normal precursor cells preferentially express p75, tumor cells express Trk receptors and perhaps smaller amounts of p75. A quantitative analysis of p75 and Trk expression in human Rb



**FIGURE 5.** Downregulation of TrkB and TrkC, but not TrkA, in differentiated Y-79 cells revealed by semiquantitative RT-PCR. Ethidium bromide-stained agarose gels of the PCR products amplified with primers specific for p75, TrkA, TrkB, and TrkC. In all cases,  $\beta$ -actin expression was used as an internal standard. Molecular weight markers are shown in the lanes on the far left; numbers indicate the predicted size of the specific PCR products (in base pairs). Untreated cultures were maintained for 9 days in serum-free RPMI 1640 with N-2 supplement. Treated cultures received in addition K252a (100 nM) and laminin (20  $\mu$ g/ml). Left lanes: negative controls, with bidistilled water instead of cDNA in the PCR reaction ( $n = 3$  in all cases).



**FIGURE 6.** Isolation of differentiated cells and test for survival-promoting effect of NGF on these cells. (A) Photomicrograph of a culture at DIV9 after standard treatment with K252a and laminin. (B) Same culture after three strong washes in PBS (time 0). This procedure produces a bias for differentiated cells. (C) Counts of cells with neurites longer than soma diameter at time 0 and after 4 days of treatment with NGF at the indicated concentrations. Increased survival was not observed ( $n = 3$ ).

cells and retinal precursor cells is needed to validate this hypothesis. This in turn may lead to a better understanding of the role of p75 in the development of CNS tumors whereby an imbalance between p75 and Trk expression may underlie the transition from a normally proliferating to a transformed state of the tissue. It has already been shown in epithelial tumors of the prostate that a downregulation of p75 marks the beginning of a malignant state of the cells.<sup>51</sup> Moreover, in mouse sensory ganglion cells, Rb gene inactivation resulted in a decreased expression of p75<sup>52</sup> and the WT1 tumor-suppressor protein downregulated p75 promoter activity in kidney cells.<sup>53</sup> Taken together, these observations point to the possibility that p75 could act as a target of tumor-suppressor factors, first of all pRb. Inactivation of the latter in Y-79 cells could result in low expression levels of p75.

In contrast to the developmental increase in the overall expression of Trk receptors in the normal retina,<sup>22,54,55</sup> post-mitotic Y-79 cells lost the message for TrkB and TrkC. The mechanism of this process is not yet clear, but it may be a sign of reversed malignancy. In any case, it was associated with a downregulation of parvalbumin immunoreactivity, thereby supporting previous observations that Trk receptor activation controls the expression of calcium-binding proteins. Interestingly, a suppression of TrkB expression in the developing rat retina by antisense oligonucleotides was also accompanied by a decrease in parvalbumin staining.<sup>56</sup>

In conclusion, the present experimental model seems to be well suited to study the mechanisms underlying the control of proliferation as well as various aspects of neuron differentiation. The proliferative response to neurotrophins in malignant cells without pRb was tentatively explained by their unusually high expression of Trk receptors when compared with normal neuroectodermal cells. Finally, it seems very likely that endogenous neurotrophins contribute to the malignant growth of Rb.

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