Journal of Cellular Biochemistry 91:206–215 (2004)

ARTICLE

p75 Neurotrophin Receptor Functions as a Survival Receptor in Brain-Metastatic Melanoma Cells

Dario Marchetti,* Rebecca Aucoin, Jason Blust, Brian Murry, and Andrea Greiter-Wilke

Department of Comparative Biomedical Sciences, School of Veterinary Medicine, Louisiana State University at Baton Rouge, Baton Rouge, Louisiana 70803

Abstract The p75 neurotrophin receptor ($p75^{NTR}$), a common receptor for members of the neurotrophins (NT) family, was previously identified as a molecular determinant of brain metastasis. We have also reported that NT treatment of murine and human brain-metastatic melanoma cells affects their invasive capacities and increases the production of heparanase, an important and unique extracellular matrix (ECM) degradative enzyme. Neurotrophism can be a survival-support mechanism for brain-metastatic cells and a survival assay was devised to mimic the growth limiting conditions of rapidly expanding metastatic tumors prior to neoangiogenesis. We report that $p75^{NTR}$ promoted the survival of brain-metastatic melanoma cells but not melanocytes in stress cultures conditions. Secondly, melanoma cells fluorescently sorted for high $p75^{NTR}$ expression ($p75^{NTR\text{-}H}$ cells) had an up to a 15-fold greater survival than those sorted for low $p75^{NTR}$ expression ($p75^{NTR\text{-}L}$ cells). Thirdly, cells overexpressing $p75^{NTR}$ associated with the growth fraction and provided these cells with an inherent growth advantage. Finally, we observed an increased survival of sorted $p75^{NTR\text{-}L}$ cells, dependent upon treatment of NT members whose functional receptors are present on these cells. Together, these results delineate that $p75^{NTR}$-mediated trophic support profoundly affects competitive melanoma-cell survival when the tumor cell microenvironment becomes growth limiting. J. Cell. Biochem. 91: 206–215, 2004.

Key words: $p75^{NTR}$; survival; neurotrophins; brain-metastatic melanoma

Melanoma frequency is augmenting at alarming levels and more than any other human cancer malignancy, with the unfortunate distinction of possessing the highest increase of cases among young adults. The brain is often a primary target of melanoma metastasis, brain-metastatic melanoma being of biological and clinical relevance. However, mechanisms responsible for malignant melanoma progression to highly aggressive brain-metastatic disease are not completely understood [Marchetti, 2002]. The progression of malignant melanoma cells involves a deviation from normal cell activity to uncontrolled cell survival, growth, and proliferation, depending on an enhanced responsiveness to either autocrine or paracrine growth factors [Nicolson, 1993]. It is known that highly malignant cell subpopulations and their progenitors may become clonally dominant within a tumor [Nowell, 1976]. However, when conditions within the neoplastic microenvironment become growth limiting, malignant cells like melanoma cells, may be forced to utilize other mechanisms in order to survive. Trophic factors, which support these cells in a state of growth arrest, suppression, or dormancy, can have profound effects on cell survival under these conditions. As a consequence, cells responsive to trophic factors may be selected for further diversification to become clonally dominant.

Neurotrophins (NT) are one of the best examples of neurotrophic substances [Bibel and Barde, 2000] and brain-metastatic murine and human melanoma cells respond to select NT members [Marchetti et al., 1993; Marchetti et al., 1995; Marchetti et al., 1998]. Mammalian NT include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) [Chao and Bothwell, 2002; Chao, 2003]. All NT

Grant sponsor: NIH (to D.M.); Grant number: CA61832.

*Correspondence to: Dr. Dario Marchetti, Department of Comparative Biomedical Sciences, Room 2522-SVM, Skip Bertman Drive, Louisiana State University at Baton Rouge, Baton Rouge, LA 70803.
E-mail: dmarchetti@vetmed.lsu.edu

Received 24 July 2003; Accepted 29 July 2003
DOI 10.1002/jcb.10649

bind to the low-affinity p75 neurotrophin receptor (p75^{NTR}) [Chao and Bothwell, 2002; Chao, 2003] but selectively to the high-affinity Trk family of tyrosine kinase receptors: mainly, NGF binds TrkA, BDNF and NT-4/5 interact with TrkB, while TrkC is the putative receptor for NT-3 [Barbacid, 1993; Kaplan and Miller, 2000; Chao and Bothwell, 2002].

Melanoma cells that overexpress NT receptors (NTR) are expected to compete more successfully than cells that express low NTR levels, particularly when NT are in limited supply. Binding of NGF, the prototypic NT, has been associated with an uncharacterized survival advantage [Fabricant et al., 1977] with p75^{NTR} frequently overexpressed by aggressive malignant melanoma cells [Brocker et al., 1991; Herrmann et al., 1993; Mattei et al., 1994]. We have previously observed that NT responsiveness, regulation of extracellular matrix (ECM) degradative enzymes, i.e., heparanase, and cell invasion correlated with augmented p75^{NTR} levels in brain-colonizing melanoma cells [Marchetti et al., 1993; Marchetti and Nicolson, 2001]. Active penetration of ECM by brain invasive melanoma cells can therefore involve mechanisms that are similar to NT-stimulated invasion of neurites toward peripheral neuronal target tissues, however, there have been no reports relating p75^{NTR} to melanoma cell survival.

In this study, we show that (1) p75^{NTR} promoted the survival of brain-metastatic melanoma cells in stress cultures conditions; (2) survival of p75^{NTR}-generated variants derived from melanoma lineages by cell sorting correlated with their p75^{NTR} levels; (3) high p75^{NTR} expression associated with an increased cell growth fraction by cell cycle analysis; and (4) survival of p75^{NTR}-sorted melanoma cells is NT-dependent.

MATERIALS AND METHODS

Tumor Cells and Cell Culture Conditions

The human melanoma MeWo cell line and its wheat germ agglutinin-selected sublines, the poorly metastatic 3S5 and the highly brain-metastatic 70W [Ishikawa et al., 1988], were maintained as previously described [Marchetti et al., 1993]. The human melanoma A875 cell line was provided by Dr. D. Djakiew (Georgetown University Medical Center, Washington, DC). It was originally isolated from a brain metastasis in the right frontal lobe of a 38-year-old female [Giard et al., 1973]. Briefly, early-passage melanoma cells were grown as monolayer cultures in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium (DMEM/F-12) supplemented with 5% (v/v) fetal bovine serum (FBS). Neonatal melanocytes were obtained from Clonetics (Santa Rosa, CA) and grown in melanocyte growth medium (Clonetics). Cells were subcultured using 2 mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) and were grown without the addition of antibiotics. Cells were regularly screened for possible mycoplasma contamination using a GEN-PROBE[3] (San Diego, CA) [^{3}H]-single-stranded DNA detection procedure and were found to be mycoplasma-free.

Immunofluorescence Analysis

Cell lines were grown in 8-well chamber slides (Lab-Tek, Fisher Scientific, Pittsburgh, PA) and washed four times with *N*-2-hydroxyethylpiperazine-*N*′2-ethane-sulfonic acid (HEPES)-buffer saline (HBS: 129 mM NaCl, 5 mM KCl, 0.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1 mM $NaHCO_3$, 5 mM glycine, 25 mM HEPES, pH 7.4). Immunofluorescence analysis for p75^{NTR} was performed by incubating the cells (1–2 h at 25°C) with a 1:250 dilution of ME20.4 monoclonal antibody (MAb) to p75^{NTR} (1 μg/μl stock; Santa Cruz Biotechnology, Santa Cruz, CA) in HBS–gelatin. Parallel controls consisted of wells without ME20.4 MAb as primary antibody. The cells were then fixed in 1% paraformaldehyde in PBS for 15 min and then washed as above with 1% gelatin as blocking agent. Cells were then again washed free of unbound primary antibody and incubated with a 1:250 dilution of phycoerythrin (PE)-conjugated rabbit anti-mouse $IgG_{(H+L)}$ antibody (1 μg/μl stock in HBS–gelatin; Zymed Laboratories, South San Francisco, CA). No background staining was observed with this secondary antibody preparation. Digital images were produced on an axioplan fluorescent microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY) with advanced spot imaging program (Bio-Rad Laboratories, Hercules, CA) using identical conditions for all photographs and antibodies used.

Fluorescence-Activated Cell Sorting

Exponentially growing 70W and A875 human melanoma cells were incubated with a 1:250

dilution of anti-$p75^{NTR}$ ME20.4 MAb (1 μg/μl stock) for 1 h at 37°C. The cells were washed three times to remove unbound ME20.4 and then incubated on ice with a 1:250 dilution of secondary PE-conjugated rabbit anti-mouse $IgG_{(H+L)}$ (1 μg/μl stock solution; Zymed Laboratories) for 30 min. The cells were then detached using 2 mM EDTA and washed three times to remove unbound PE-secondary antibody. Cells were permeabilized with 0.1% Triton X-100 and resuspended in PBS containing 10 μg/ml propidium iodide (PI) and 10 μg/ml RNase A (Sigma Chemical Co., St. Louis, MO). Following a 30 min incubation on ice, cells were analyzed by flow cytometry on a FACStar Plus fluorescence-activated cell sorter (Becton Dickinson Immunocytochemistry Systems, Mountanview, CA).

Selection of High and Low $p75^{NTR}$ Expressing Cells

Sterile FACS sorts of exponential-growth-phase 70W and A875 melanoma cells with anti-$p75^{NTR}$ ME20.4 MAb were performed to obtain high ($p75^{NTR\text{-}H}$) and low ($p75^{NTR\text{-}L}$) expressor sublines. These sublines were resorted for re-expression of $p75^{NTR}$ after 2 weeks of in vitro culture, to obtain additional $p75^{NTR}$ populations ($p75^{L\text{-}L}$, $p75^{L\text{-}H}$, $p75^{H\text{-}L}$, and $p75^{H\text{-}H}$, respectively). Growth fractions were analyzed by sorting of Triton X-100 permeabilized cells stained with PI and analyzed for DNA content. Cell cycle analysis was performed using a fluorescence-activated cell sorter equipped with the ModFit cell cycle analysis program (Verity Software House, Topsham, ME). The percentage of cells in G_0-G_1 phase and in the growth fraction ($S+G_2+M$) was analyzed for DNA content by PI staining.

Survival Assays and Colony Counting

We devised tissue culture conditions (stress cultures) to mimic the selective microenvironment of a rapidly growing tumor at the brain-metastatic site. This was done in an attempt to recreate zones of marginal or inadequate blood supply prior to or in absence of neoangiogenesis. In stress cultures, melanoma cells were grown to high density without changing the culture medium for a period of 3–4 weeks. The cells that survived gradually utilized cellular debris and formed colonies, slowly repopulating the tissue culture plate. Colony counts and statistical analysis on surviving colonies were performed after washing, fixation in PBS containing 2.5% glutaraldehyde, and staining with Giemsa according to manufacturer instructions (Sigma Chemical Co., St. Louis, MO). In some experiments, the colonies that formed were rescued with fresh medium to demonstrate their ability to rapidly recolonize the tissue culture plate.

NT Effects on Cell Survival

Melanoma cells were plated in 24-well plates at first observable signs of conditional death and cultured in serum-free conditions without additional components except by supplementing biologically active human NT (50–200 ng/ml; Promega, Madison, WI) for 0–48 h. As above, surviving colonies were fixed, stained, and counted. Live- and dead-cell assays were performed by incubating tissue-culture plates with 5 μM calcein-AM (Molecular Probes, Inc., Eugene, OR), which is cleaved by intracellular esterases and retained only in cells with intact plasma membranes, and 5 μM ethidium homodimer (Molecular Probes, Inc.), a dye that intercalates into nuclear DNA in cells that lose plasma membrane integrity [Marchetti et al., 1993].

RESULTS

$p75^{NTR}$ Is Expressed in Melanoma Cells Depending Upon Their Brain-Metastatic Propensities

We have examined $p75^{NTR}$ cell-surface expression in a series of melanoma cells possessing a progressive brain-metastatic phenotype: parental MeWo, its two variants—poorly metastatic 3S5 and highly brain-metastatic 70W cells—and A875 cells. The A875 melanoma line, is known to possess elevated levels of $p75^{NTR}$ [Fabricant et al., 1977]. We analyzed $p75^{NTR}$ levels in these cells and human melanocytes using the well-characterized monoclonal antibody $p75^{NTR}$, ME20.4 [Ross et al., 1984]. Melanocytes did not possess appreciable amounts of $p75^{NTR}$ and low-levels of $p75^{NTR}$ were found in 3S5 cells which are refractory to metastasis formation (Fig. 1). $P75^{NTR}$ levels increased in parental MeWo, which are lung—but not brain—metastatic [Ishikawa et al., 1988], and reached highest levels in brain-metastatic 70W and A875 cells (Fig. 1).

$p75^{NTR}$ Promotes the Survival of Brain-Metastatic Melanoma Cells

Our previous studies have demonstrated that the pattern of $p75^{NTR}$ expression in melanoma

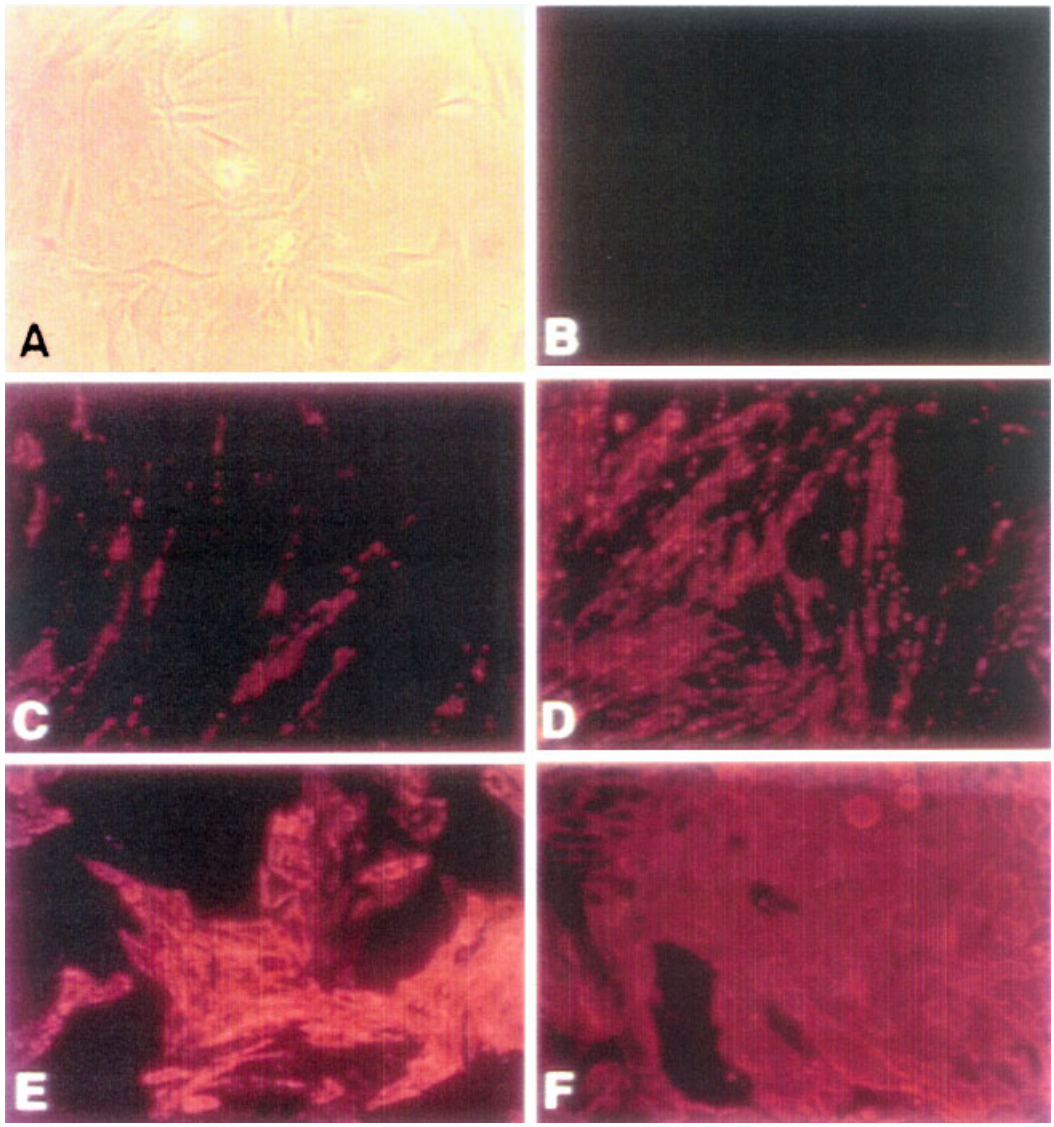

Fig. 1. Immunofluorescence analysis of p75^{NTR} expression in human melanocytes and melanoma cells derived from sequential stages of tumor progression. Each cell line was examined by using p75^{NTR} ME20.4 MAb followed by phycoerythrin-conjugated secondary antibody (red staining positivity) and cell fixation. Human melanocytic cultures (**A**, phase contrast) were negative for p75^{NTR} staining (**B**). P75^{NTR} was expressed on preconfluent melanoma variants to varying degrees (**Panels C**, **D**, **E**, **F**): 3S5 cells expressed very little p75^{NTR} (C) compared to increasingly higher levels in MeWo (D), and, of relevance, in brain-metastatic melanoma 70W (E), and A875 cells (F). Negative controls consisted of cell preparations incubated without ME20.4 MAb. [Color figure can be viewed in the online issue, which is available at www.interscience.com.]

correlated with their NT responsiveness, invasive behavior, heparanase production, and ability to form brain tumor colonies [Ishikawa et al., 1988; Herrmann et al., 1993; Marchetti et al., 1993, 1995].

To assess the contribution of p75^{NTR} to cell survival, we performed sterile fluorescence activated cell sorting (FACS) to characterize p75^{NTR} expression in melanoma cells established from spontaneous tumor lineages. Preconfluent 70W and A875 cells were sorted for high and low p75^{NTR} (no detectable cell-surface immunofluorescence) expression and high/low p75^{NTR} expressor subpopulations (p75$^{NTR\text{-}H}$ and p75$^{NTR\text{-}L}$, respectively) were derived (Fig. 2). Differential p75^{NTR} levels in p75$^{NTR\text{-}H}$ versus p75$^{NTR\text{-}L}$ sublines were confirmed by immunoblotting experiments using ME20.4 MAb. A representative Western blotting analysis using 70W cells is shown (Fig. 3).

Melanocyte and melanoma cell survival characteristics were determined by growing these

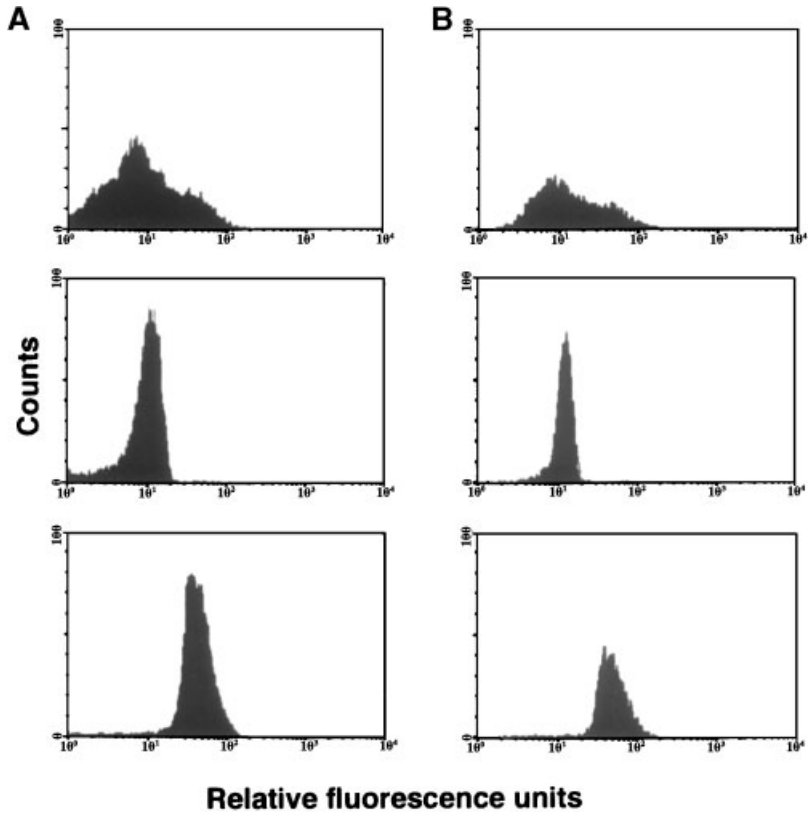

Fig. 2. FACS analysis of brain-metastatic 70W (**A**) and A875 (**B**) melanoma cells for $p75^{NTR}$ content. The broad distribution of $p75^{NTR}$ by 70W and A875 (**top panels**) enabled us to sort them for low $p75^{NTR}$ expression ($p75^{NTR-L}$; **middle panels** and high $p75^{NTR}$ ($p75^{NTR-H}$; **bottom panels**), respectively.

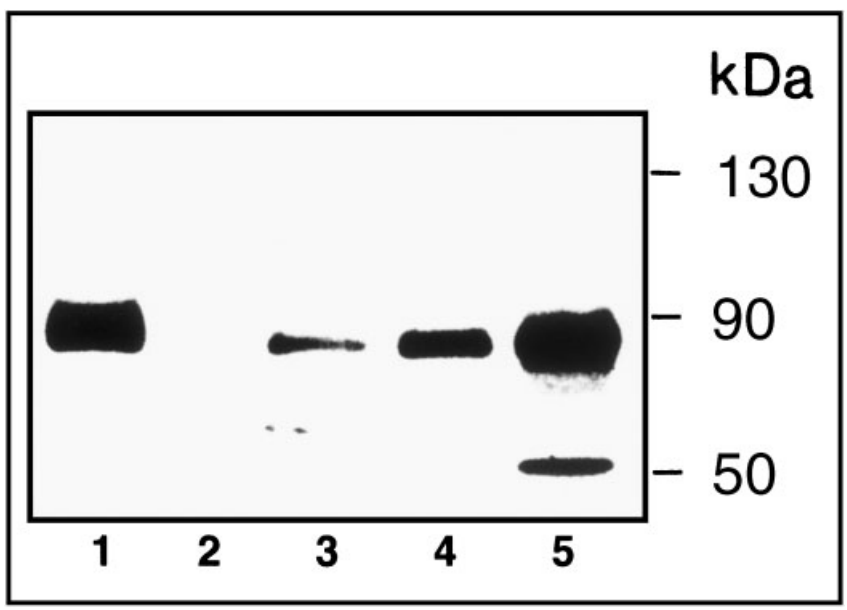

Fig. 3. $p75^{NTR}$ protein content in cell-sorted 70W variants. 70W cells were sorted to obtain high- and low-$p75^{NTR}$ expressors ($p75^{NTR-H}$ and $p75^{NTR-L}$, respectively; see also ''Materials and Methods''). Cells were immunoprecipitated using ME20.4 MAb and Western blotting analysis was performed as previously reported [Marchetti et al., 1998]. Differences in cell-surface protein levels of $p75^{NTR}$ are shown as follows: **Lane 1**: A875 (positive control); **Lane 2**: 3S5 (negative control); **Lane 3**: $p75^{NTR-L}$ 70W; **Lane 4**: Unsorted 70W; and **Lane 5**: $p75^{NTR-H}$ 70W.

cells in stress-culture conditions. Stress cultures consisted of highly confluent cells carried in tissue culture medium that remained unchanged for a period of 3–4 weeks. Melanocytes did not express p75^{NTR} at significant levels, they grew very slowly, and exited the cell cycle at confluence. As a result, melanocytes did not overstress the tissue culture medium and failed to rapidly create stress culture conditions surviving in stressed cultures for approximately 60 days (data not shown).

Secondly, 3S5 cells which do not express p75^{NTR} (Fig. 1) [Marchetti et al., 1998] failed to survive in stress cultures. Conversely, all melanoma cells expressing p75^{NTR} to some degree, grew rapidly, failed to exit the cell cycle at confluence, and created stress culture conditions by over-utilizing the tissue culture medium. For example, MeWo cells expressing low levels of p75^{NTR} (Fig. 1), survived as single cells or small colonies of less than 10 cells. 70W cells and A875 cells expressing high amounts of p75^{NTR} (Fig. 1), survived as colonies of greater than 25 cells (Table I) and were able to recolonize a tissue culture plate 6–8 days after a fresh medium exchange (data not shown).

Importantly, to determine if elevated p75^{NTR} expression provided a survival advantage to brain-metastatic melanoma cells, we subjected p75$^{NTR\text{-}H}$ and p75$^{NTR\text{-}L}$ cells to stress culture conditions. When examined for cell survival, p75$^{NTR\text{-}H}$ sublines had an up to 15-fold greater number of surviving colonies compared to p75$^{NTR\text{-}L}$ cells (Table II).

High p75^{NTR} Expression Is Associated With an Increased Growth Fraction

Cell-cycle analysis was performed on unstressed cultures. It indicated that p75$^{NTR\text{-}L}$ and p75$^{NTR\text{-}H}$ sorted populations are comprised of approximately 73% and 52% G_0–G_1 phase cells, respectively (Fig. 4). During analysis of unstressed cell cultures, the p75$^{NTR\text{-}L}$ subline was found to exhibit a 25% forward drift toward re-expression of p75^{NTR}, compared with only 7.5% backward drift in p75^{NTR} loss by p75$^{NTR\text{-}H}$ cells. Similar analysis performed on the p75$^{L\text{-}L}$ subline, which was resorted from p75$^{NTR\text{-}L}$ cells

TABLE I. Time-Dependent Survival of p75^{NTR} Expression Cell Variants

	Surviving colonies[a]	
Cell type	0 h	72 h
MeWo	N.D.[b]	N.D.
3S5	N.D.	N.D.
70W	$2.5 \times 10^2 \pm 5.0 \times 10$	$1.7 \times 10^3 \pm 3.5 \times 10^2$
A875	$2.9 \times 10^3 \pm 7.0 \times 10^2$	$4.3 \times 10^3 \pm 1.0 \times 10^2$
	n = 4[c]	

[a]Cells were plated in 100 mm dishes at equal densities and cultured under stress conditions (see "Materials and Methods"). Colonies greater than 25 cells were counted following glutaraldehyde fixation and Giemsa staining. Left panel represents initial (0 h) surviving colonies; right panel represents colony counts 72 h after rescue with fresh medium.
[b]N.D. = not detectable by conditions used.
[c]Mean ± SD of four independent experiments.

TABLE II. Survival and NGF Response of p75^{NTR} Expression Variants

	Surviving colonies[a]	
Cell type	−NGF	+NGF
p75$^{NTR\text{-}L}$ 70W	$5.3 \times 10 \pm 3.0 \times 10$	$3.3 \times 10^2 \pm 5.8 \times 10$
p75$^{NTR\text{-}L}$ A875	$7.8 \times 10 \pm 3.2 \times 10$	$4.1 \times 10^2 \pm 9.7 \times 10$
p75$^{NTR\text{-}H}$ 70W	$8.0 \times 10^2 \pm 1.2 \times 10$	$5.2 \times 10^3 \pm 1.8 \times 10^2$
p75$^{NTR\text{-}H}$ A875	$6.9 \times 10^2 \pm 5.1 \times 10$	$4.7 \times 10^3 \pm 9.0 \times 10$
	n = 4[b]	

[a]Survival potential of p75^{NTR} sorted brain-metastatic melanoma cells (70W, A875) was determined by colony counting after 4 weeks in culture. The p75$^{NTR\text{-}H}$ and p75$^{NTR\text{-}L}$ cells were plated and later treated using glutaraldehyde fixation and Giemsa staining (see also "Materials and Methods"). To evaluate NGF responsiveness, selected lines were plated containing serum-free medium or serum-free medium containing NGF (50 ng/ml).
[b]Mean ± SD of four independent experiments.

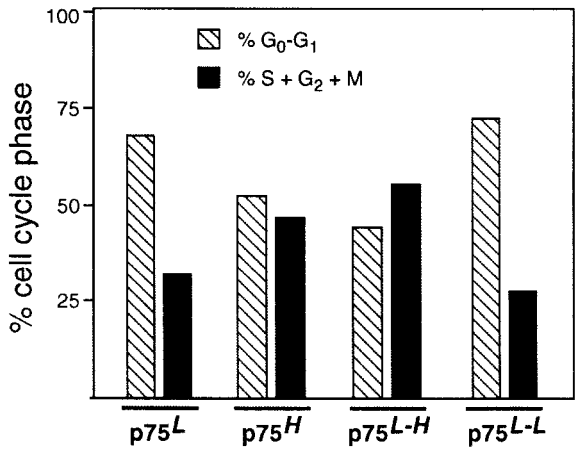

Fig. 4. High $p75^{NTR}$ expression associates with an increased growth fraction. Cell cycle analysis was performed on $p75^{NTR}$ sorted 70W cells by FACS. After 2 weeks of in vitro culture, $p75^{NTR-L}$ and $p75^{NTR-H}$ sublines were resorted for re-expression of $p75^{NTR}$ and four subpopulations were obtained ($p75^{L-L}$, $p75^{H-L}$, $p75^{L-H}$, and $p75^{H-H}$, respectively). The growth fraction for each set of these subclones was analyzed for DNA content using propidium iodide (PI). $P75^{NTR}$ distribution in relation to percentage of cell cycle phase ($S + G_2 + M/G_0–G_1$) are shown.

for low $p75^{NTR}$ expression, indicated that approximately 75% of these cells were in $G_0–G_1$ phase which corresponded to approximately 50% of the growth fraction of the $p75^{L-H}$ subline which was resorted from the $p75^{L}$ for high $p75^{NTR}$ expression. The forward drift toward elevated $p75^{NTR}$ by $p75^{NTR-L}$ cells demonstrates the importance of reestablishing original NT response characteristics that were present prior to $p75^{NTR-L}$ cell population sorting. In contrast, the increase in growth fraction associated with elevated $p75^{NTR}$ following primary and secondary sorts ($p75^{H}$ and $p75^{L-H}$, respectively) illustrates the inherent growth advantage provided by $p75^{NTR}$ presence (Fig. 4).

Surviving Melanoma Cells—$p75^{NTR}$ Expressing—Respond to NT

The survival advantage exhibited by $p75^{NTR-H}$ cells prompted us to examine the responsiveness of sorted brain-metastatic melanoma cells to NT. The greatest difference in NT responsiveness was exhibited by $p75^{NTR-L}$ cells. Supplementing growth medium with human NGF to serum-free stress cultures of $p75^{NTR-L}$ cells enhanced colony survival (Table II). The survival advantage exhibited by $p75^{NTR-L}$ cells following NGF treatment may result from the selective pressure exerted by stress culture conditions.

We expanded these results to include NT other than NGF. Melanoma cells were incubated with purified preparations of biologically active BDNF, NT-3, and NT4/5 at optimal concentrations to saturate NTR. Of these, only NT-3 (although at a lesser extent than NGF) enhanced survival in 70W and A875 cells (Fig. 5). These results are consistent with presence of TrkC to form $p75^{NTR}$–TrkC complexes in these cells (Fig. 5) [Herrmann et al., 1993; Marchetti et al., 1998].

DISCUSSION

An undeveloped area of tumor metastasis research is studying trophic properties that promote cell survival in suboptimal microenvironments. Trophic support can be particularly important when proliferation of malignant cells is limited by competition for factors necessary to support their rapid growth. Neurotrophism in the developing embryo is the best example of trophic behavior [Raff, 1992]. Neuronal cells that are overproduced in the embryo must compete for limited supplies of target tissue-derived NT. Hence, only a small number of successful neurons survive and innervate the target tissue, whereas the majority of neurons die by apoptosis (neurotrophic theory) [Oppenheim, 1991]. NGF, the prototypic NT, was first demonstrated to rescue NGF-dependent sympathetic and sensory neurons in developing embryos [Hamburger et al., 1981]. Definitive confirmation of these early experiments was provided by analysis of null-allele mice that were subsequently generated by

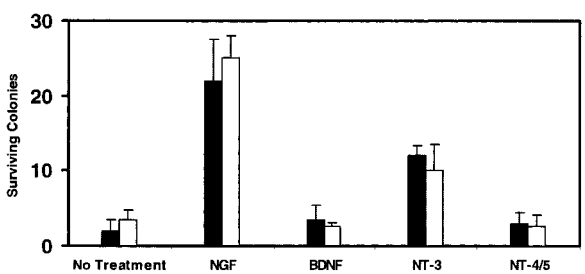

Fig. 5. NT-dependent survival of $p75^{NTR-L}$ subpopulations. Low $p75^{NTR}$ subpopulations ($p75^{NTR-L}$) from sorted melanoma cells (black bars: 70W; white bars: A875) were plated into 24-well plates containing only serum-free medium or serum-free medium containing either NGF, BDNF, NT-3, or NT-4/5 (50 ng/ml or approximately 2 nM). Exogenous human NGF and NT-3 but not BDNF, or NT4/5 enhanced survival of $p75^{NTR-L}$ in serum-free medium. Data represent the means (±SD) from four independent experiments. Similar results were obtained using higher NT concentrations (up to 200 ng/ml).

NGF-gene targeting [Crowley et al., 1994]. The exogenous addition of various NT supports specific developing neurons in vitro and in vivo, and the same subset of neurons will die in null allele mice if the corresponding NT are subjected to gene targeting [Lee et al., 1992; Raff, 1992; Klein et al., 1993; Lee et al., 1994; Smeyne et al., 1994; Escandon et al., 1994; Snider, 1994; Chao, 2003].

Applying the concept of trophism to rapidly growing tumors or to the large number of metastatic cells in the circulation may, in part, account for their survival under growth limiting conditions. This may be particularly true of NT effects on tumors that have a neuroectodermal developmental origin like malignant melanoma [Nicolson et al., 1994].

There are a number of important differences between melanocyte and melanoma cell growth. Melanocytes in culture grow very slowly compared to tumor cells and do not over-tax the tissue culture medium compared to melanoma cells that instead create a stress culture environment by over utilizing it. Secondly, intercellular contact between melanocytes causes these cells to exit the cell cycle normally and become quiescent, whereas melanoma cells fail to exit the cell cycle at confluence and overpopulate the tissue culture dish. Finally, melanocytes do not express p75^{NTR} at significant levels, yet remain alive in a quiescent state for extended periods of time without tissue culture medium exchange. In addition, melanocytes in these conditions undergo only a limited number of cell doublings before becoming senescent and when they begin to die they are unable to recover. By comparison, melanoma cells undergo repeated cycles of growth-apoptosis-survival and p75^{NTR} turnover in stress cultures leading to colony formation and repopulation of the tissue culture dish. These are important differences between immortalized melanoma cells and the normal melanocyte phenotype supporting the notion of p75^{NTR} involvement in survival under growth limiting conditions.

Accordingly, we propose that in order for malignant melanoma cells to survive, they must maintain high levels of p75^{NTR} expression by avoiding or remaining unresponsive to cell–cell contact. In NT-rich microenvironments, p75^{NTR} expression on melanoma cells, even in the presence of cell–cell contact, may become stabilized or increased on the cell surface, enabling them to survive. In light of these observations, we propose that neurotrophic support may profoundly influence the survival and clonal dominance of NT responsive melanoma cells.

It is known that p75^{NTR} have alternative functions depending on the cellular context in which it is expressed. p75^{NTR} provide retrograde transport to certain neuronal cell types [Verdi et al., 1994] in addition to triggering apoptosis in certain virally transformed neuronal cells [Rabizadeh et al., 1993] or survival when expressed in neutrophils [Kannan et al., 1992]. Findings have also been reported demonstrating that p75^{NTR} is required for NGF-mediated survival of sensory neurons [Barrett and Bartlett, 1994]. In these studies, p75^{NTR} promoted either survival or death of sensory neurons depending on their stage of development. Certain properties of p75^{NTR} may therefore allow it to function in regulating the death [Tabassum et al., 2003] or survival of tumor cells. In this regard, p75^{NTR} is analogous to the other members of the tumor necrosis factor (TNF) superfamily of receptors which includes, besides p75^{NTR}, Fas (APO I), TNF receptors I and II, and the B-cell antigen CD40 [Beutler and van Huffel, 1994; Smith et al., 1994].

We have demonstrated that p75^{NTR} in certain human malignant melanoma cells, can function independently of TrkA to signal invasion of ECM and ECM-degrading heparanase production [Herrmann et al., 1993; Marchetti et al., 1993]. Although TrkC is also present in these cells, the reported experiments indicate that p75^{NTR} can play a direct and active role in NT signaling to promote invasion of melanoma cells [Marchetti et al., 1998]. Furthermore, p75^{NTR} is the only NT receptor identified in these melanoma cells whose expression levels correlate with functional and behavioral properties [Herrmann et al., 1993]. Supplementation of the growth medium with exogenous recombinant NGF and NT-3, but not BDNF or NT-4/5, to serum-free stressed cultures of p75$^{NTR\text{-}L}$ cells enhanced colony survival. The survival advantage exhibited by p75$^{NTR\text{-}L}$ cells following NGF and NT-3 treatment may result from the selective pressure exerted by stress cultures to promote the survival of p75$^{NTR\text{-}L}$ cell subpopulations. Nonetheless, and besides p75^{NTR} dimer formation/activation in these cells, the biological potential for p75^{NTR}–TrkC interaction exists and is important as demonstrated by (1) NT-3 affecting the survival of p75$^{NTR\text{-}L}$ cells, consistent with presence of TrkC, and (2) BDNF

and NT-4/5 failing to promote survival, likely due to TrkB absence in these cells.

Neurotrophic support for brain-metastastic melanoma cells can have a significant influence on the formation of these types of metastasis. There is a high frequency of brain metastasis formation by malignant melanoma. This behavior may reflect specialized melanoma-brain tissue interactions that are related to their neural-crest origin [Nicolson, 1993; Nicolson et al., 1994]. Using clinical specimens and immunohistochemistry, we have observed elevated NT levels in brain tissue adjacent to the brain invasive melanoma front [Marchetti et al., 1995]. These results suggest that the NT-rich microenvironment of the brain is a likely target for NT-responsive metastatic cells. They also explain the tendency of highly malignant melanoma cells to express $p75^{NTR}$ in relation to tumor cell survival and metastasis to the brain. The pattern of melanoma cell survival observed in association with $p75^{NTR}$ expression can therefore be a mechanism for ensuring that certain cells remain responsive to NT and continue to survive and grow in growth-limiting conditions.

The broad implications of trophic support as a tumor cell survival mechanism are certainly far reaching. Much research in cancer biology has focused on cell proliferative responses to growth factors, primarily because of the easily definable increase or decrease of cell number that results from growth-factor treatment. Survival assays tend to require longer time periods and are more difficult to accomplish technically. As such, trophic biological effects can remain unobserved. Although NT are one of the best examples of trophic substances, growth factors not typically thought of as trophic molecules may actually support cell survival of certain tumor cells in absence of proliferation. Similarly, trophic substances may exist that are as yet undescribed or whose effects are yet to be defined. Critical instances of trophic survival mechanisms can be tumor dormancy, cell survival in the region of necrotic tumors, cryptic survival of micrometastases, and organ targeting. The implications of these undescribed phenomena can be particularly profound for the tumor biologist, clinician, and importantly, the cancer patient.

ACKNOWLEDGMENTS

We thank Ms. Jeannie Tyler for her excellent secretarial assistance and Ms. Jennifer Galjour for her editorial assistance.

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