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An anti-apoptotic role for NGF receptors in human rhabdomyosarcoma

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Abstract

The expression and biological function of Nerve Growth Factor (NGF) receptors was studied in a panel of rhabdomyosarcoma cell lines derived from embryonal and alveolar histotype. All the cell lines expressed both the high affinity receptor TrkA and the low affinity receptor $p75^{NTR}$. Treatment with exogenous NGF did not considerably alter rhabdomyosarcoma cell growth or differentiation, but significantly inhibited spontaneous apoptosis as well as apoptosis, and induced by serum starvation or apoptosis induced by treatment with cycloheximide (CHX). Rhabdomyosarcoma cell lines expressed NGF and other neurotrophins and trace amounts of NGF protein were found in the supernatants of rhabdomyosarcoma cell cultures. Blocking the putative autocrine loop with an anti-NGF antibody resulted in an increase in apoptosis compared with control cultures. These data suggest that the simultaneous presence of both high and low affinity NGF receptors engaged by endogenous or exogenous NGF might contribute to the escape from apoptosis exhibited by the rhabdomyosarcoma cells. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: NGF; $p75^{NTR}$; TrkA; Human rhabdomyosarcoma; Apoptosis

1. Introduction

Nerve Growth Factor (NGF), as well as other neurotrophins (Brain-Derived Neurotrophic Factor (BDNF); Neurotrophin-3 (NT-3); Neurotrophin 4/5 (NT-4/5)), plays an important role in the development, maintenance and repair of the nervous system, influencing several pathways including cell survival, differentiation and apoptosis [1].

The effects of NGF are mediated by two different receptors: the high affinity receptor TrkA, a member of the TRK family of tyrosine kinase receptors, is specific for NGF, while the low affinity receptor ($p75^{NTR}$) binds to the other neurotrophins as well [2]. Current studies suggest that different responses to NGF are almost completely dependent on the relative amount of the two receptors: while in the absence of a TrkA receptor, NGF binding to $p75^{NTR}$ triggers apoptosis, the simultaneous expression of the two receptors increases high affinity NGF binding and signals cell survival [2–4].

The importance of the neurotrophin receptors in tumours originating in the nervous system has been reported [5,6] while, apart from hints as to the role of these receptors in the progression of malignant melanoma [7], there is little data regarding tumours not originating from the nervous system. In particular, no functional study has been carried out on tumours of musculo-skeletal origin, like rhabdomyosarcoma. A few immunohistochemical studies on the expression of the NGF receptors in human rhabdomyosarcoma have been reported [8,9].

In the present study we analysed the expression and function of high and low affinity NGF receptors in four cell lines derived from human embryonal and alveolar rhabdomyosarcomas, and examined the possibility of an anti-apoptotic role for NGF receptors in these tumours.

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2. Materials and methods

2.1. Cell culture and induction of differentiation

Four cell lines derived from human rhabdomyosarcomas of different histotypes were used: RD/18 (a clone derived from the RD cell line) [10] and CCA [11] of embryonal histotype; RMZ-RC2 [12] and RH30 [13] derived from alveolar rhabdomyosarcomas. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum (FBS) (Life Technologies, Milan, Italy).

Proliferation and differentiation studies were performed as reported in Refs. [14,15]. Cells were seeded on day 0 at 30 000 cells/cm^2 in DMEM + 10% FBS referred to as growth medium and switched on day 1 to a differentiation-inducing medium (DMEM + 2% horse serum) with a lower content of growth factors [16]. Cells were harvested on days 1, 4 and 7, counted and cytocentrifuged at 400*g* onto glass slides and fixed with ethanol/acetone (3:7) at −20°C. Centrifuged slides were stained in an indirect immunofluorescence assay with the primary BF-G6 monoclonal antibody recognising embryonic myosin [17] and the secondary fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Kierkegaard & Perry Laboratories, Kpl, Gaithersburg, MD, USA). At least two spots for each sample were made, and at least 400 cell elements for each spot were scored.

2.2. Expression of NGF receptors

Cells were harvested with 5 mM ethyleneglycol-bis(beta-aminoethyl ether)-N,N,N′,N′-tetra acetic acid (EGTA) in Ca^{2+}- and Mg^{2+}-free Phosphate-Buffered Saline (PBS) and stained in an indirect immunofluorescence assay with the primary antibodies anti-TrkA clone 6G10 (Oncogene Research Products, Cambridge, MA, USA) or anti-p75^{NTR} clone 8211 (Boehringer Mannheim, Milan). Cells were subsequently stained with a secondary FITC-conjugated goat anti-mouse immunoglobulin (Kpl) and resuspended in 1 μg/ml ethidium bromide PBS solution to gate out the dead cells. Cells were analysed with a FACScan flow cytometer (Beckton Dickinson, Mountain View, CA, USA).

As positive controls for the expression of TrkA and p75^{NTR}, we used the HL-60 promyelocytic leukaemia cell line (as suggested in the antibody data sheet), and SK-N-MC, a high expressor of p75^{NTR}, respectively [18].

2.3. Evaluation of apoptosis

Basal apoptosis was evaluated on cells seeded in growth medium in 24-well plates (Costar, Cambridge, MA, USA) with or without recombinant human NGF (Boehringer Mannheim), at a final concentration of 50 ng/ml. To evaluate apoptosis induced by serum starvation, cells were seeded in growth medium and shifted after 24 h to serum-free medium with or without NGF. In the experiments with cycloheximide (CHX) (Sigma, Milan, Italy), rhabdomyosarcoma cells were seeded in complete medium with or without CHX 50 μg/ml and NGF 50 ng/ml. After 24 h treatment, cells were harvested and apoptosis was evaluated either morphologically and by TdT-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) assay.

Apoptotic bodies showing typical nuclear fragmentation and condensation were counted in a Leica DM microscope on cells stained with Hoechst 33342 (Merck, Milan, Italy). For the time course experiment, the evaluation of apoptosis was performed 24, 48 and 72 h after seeding.

The TUNEL assay was performed according to the manufacturer's instructions (*In situ* cell death detection kit, Roche Diagnostics GmbH, Mannheim, Germany): briefly, harvested cells were fixed, permeabilised and stained by treatment with terminal deoxynucleotidyl transferase and fluorescein-labelled nucleotides.

At least three experiments were carried out and at least 400 cell elements were counted for each sample. In some experiments, apoptosis was evaluated in the presence of a mouse monoclonal anti-human NGF-β antibody at final concentrations of 1–10 μg/ml, or in the presence of the same concentrations of an isotype-matched irrelevant antibody (MOPC 21, Sigma).

2.4. Neurotrophin expression

Total RNA was extracted from cultured cells and from fresh normal muscle tissue obtained from surgical specimen with Tri-Zol reagent (Life-Technologies). One microgram of RNA was reverse-transcribed using M-MLV reverse transcriptase in presence of oligo dT and deoxynucleoside triphosphates (dNTPs) (Life Technologies). One microlitre of cDNA was amplified in a total volume of 25 μl with specific primer pairs for *NGF* [19], *BDNF* and *NT-3* [5] and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Clontech, Palo Alto, CA, USA). Reverse transcription polymerase chain reactions (RT-PCR) were performed with an annealing temperature of 60°C and were standardised over a wide range of cycles (20–40) to avoid plateau effects and to allow a semi-quantitative comparison.

Amplified products (*GAPDH*, 452 bp; *NGF*, 432 bp; *BDNF*, 670 bp; *NT-3*, 640 bp) were visualised in ethidium bromide-stained agarose gel and subjected to densitometric analysis. RNA was extracted from at least three independent experiments and cDNAs were amplified at least twice.

To evaluate NGF production, cells were seeded in growth medium and shifted to Roswell Park Memorial Institute medium (RPMI) + 0.5% FBS on the fourth day of culture. Supernatants were collected after 4 days, supplemented with aprotinin (Sigma) at a final con-

centration of 2 μg/ml, and were concentrated approximately 100 times by centrifugation at 1500*g* on Ultrafree-15 centrifugal filter devices (Millipore, Milan, Italy) with a molecular cut-off of 5 KDa. Boehringer Mannheim's NGF enzyme-linked immunosorbent assay (ELISA) reagents were used following the manufacturer's instructions. NGF concentration in the samples was calculated by interpolation from the standard curve.

3. Results

3.1. Expression of TrkA and p75^{NTR} by human rhabdomyosarcoma cells

We analysed the expression of the high and low affinity NGF receptors on four cell lines of human rhabdomyosarcoma using cytofluorometric analysis (Fig. 1). The high affinity receptor (TrkA) was expressed at levels comparable to the positive control HL-60 and the low affinity p75^{NTR} receptor was strongly expressed by all the rhabdomyosarcoma cell lines analysed. HL-60 and SK-N-MC profiles are shown as positive controls for TrkA and p75^{NTR} expression, respectively.

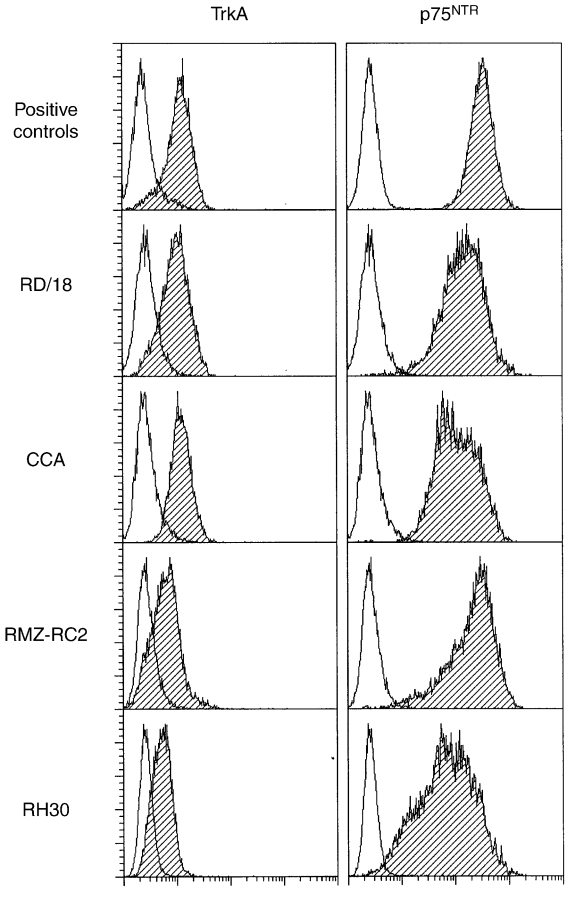

Fig. 1. Expression of high and low affinity nerve growth factor (NGF) receptors (TrkA and p75^{NTR}) evaluated by flow cytometry. Open profiles: fluorescence intensity of cells stained only with the secondary antibody. Shaded profiles: cells stained with anti-TrkA or anti-p75^{NTR}. The abscissa represents fluorescence intensity on a logarithmic scale, and the ordinate represents the number of cells. Positive controls (HL-60 for TrkA and SK-N-MC for p75^{NTR}) have been included for comparison.

3.2. Effect of NGF on rhabdomyosarcoma cells

The expression of both NGF receptors suggested that human rhabdomyosarcoma could respond to some of the multiple biological effects of NGF, ranging from cell growth to differentiation and apoptosis. Fig. 2 shows the kinetics of growth and differentiation in NGF-containing medium of RMZ-RC2 cells, which showed

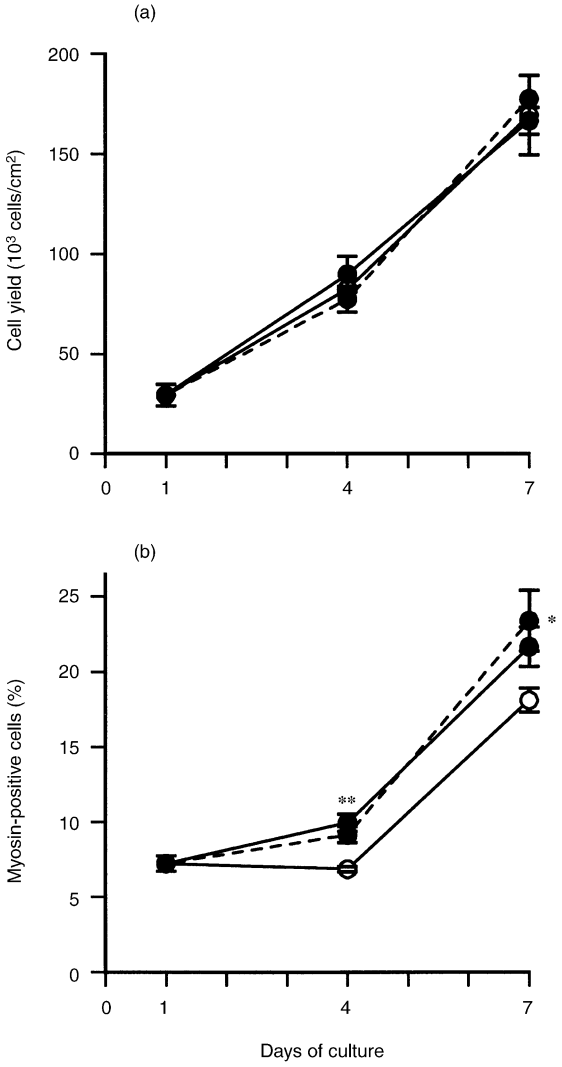

Fig. 2. Effect of nerve growth factor (NGF) at a concentration of 5 ng/ml (solid symbol, solid line) or 50 ng/ml (solid symbol, dashed line) with respect to control (open symbol) on cell growth (a) and differentiation (b) of RMZ-RC2 cells. Myogenic differentiation was evaluated on cytocentrifuged samples by staining with a monoclonal antibody directed against embryonic myosin. Each point represents the mean ± standard error of the mean (SEM), of three experiments. A significant difference in myogenic differentiation was seen on day 4 (**$P<0.01$) and day 7 (*$P<0.05$) of culture (Student's *t*-test).

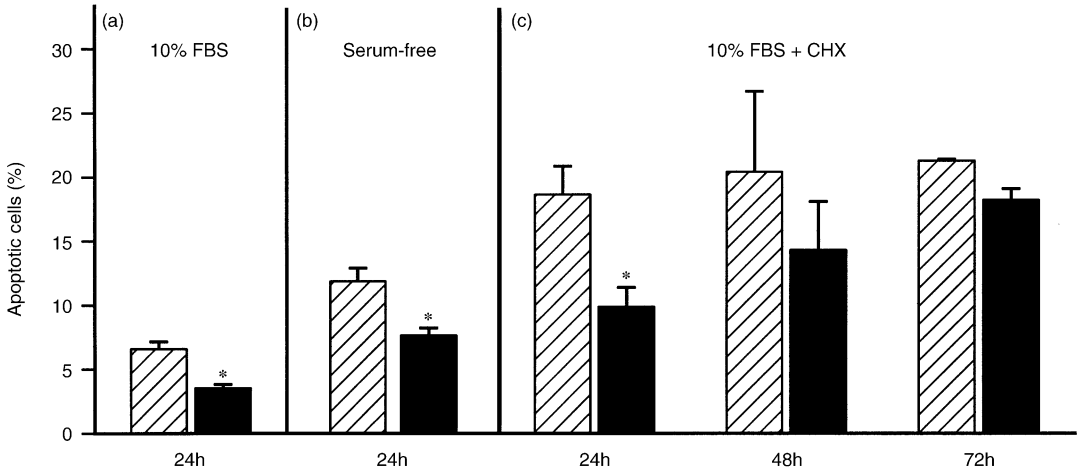

Fig. 3. Effect of nerve growth factor (NGF) on the percentage of RMZ-RC2 cells undergoing apoptosis. Hatched bars: control cells; solid bars: NGF-treated cells. Effect of NGF (50 ng/ml) on apoptosis of RMZ-RC2 cells evaluated by morphological features: (a) basal conditions (10% fetal bovine serum (FBS)), (b) serum-free medium, (c) cycloheximide (CHX) treatment (50 μg/ml) on a time course of 24, 48 and 72 h. Significance: $*P<0.05$; Student's *t*-test.

the highest expression of the low affinity receptor. While the growth of RMZ-RC2 cells was not affected by NGF, the percentage of terminally-differentiated cells expressing embryonic myosin was slightly (but significantly) increased by NGF treatment.

The proportion of apoptotic cells found in the RMZ-RC2 cells was very low under normal culture conditions (approximately 5%) and was increased by serum starvation up to approximately 10% (Fig. 3a and b). In both conditions, a 24-h treatment with NGF significantly decreased by approximately 40–50% the proportion of apoptotic cells. When cells were subjected to the apoptotic inducer CHX, the proportion of apoptotic RMZ-RC2 cells reached approximately 20% in the absence of NGF; again a 24 h treatment with NGF resulted in an approximately 50% reduction in the percentage of apoptotic cells (Fig. 3c). The time course study of apoptosis induction by CHX showed that the anti-apoptotic effect of NGF progressively decreased with time and in the 72 h cell cultures similar proportions of apoptotic cells were observed with or without NGF (Fig. 3c). Therefore, the NGF could only delay, but not prevent, CHX-induced apoptosis.

This anti-apoptotic effect of NGF was then evaluated in the other rhabdomyosarcoma cell lines, using CHX to induce apoptosis. In the CCA and RH30 cells, similar effects were observed to those in the RMZ-RC2 cell line, i.e. NGF treatment significantly reduced the proportion of apoptotic cells (Fig. 4a). The rhabdomyosarcoma cell line CCA is derived from a tumour of an embryonal histotype, thus showing that the anti-apoptotic effect of NGF is not peculiar to the alveolar histotype. The percentage of apoptotic cells induced by CHX in the RD/18 cells was very low (reaching a maximum of 6–8%) and no effect on this percentage by NGF treatment was observed (data not shown).

The anti-apoptotic effect of NGF was also studied using the TUNEL apoptosis assay in the RMZ-RC2, CCA and RH30 cells treated with CHX as above

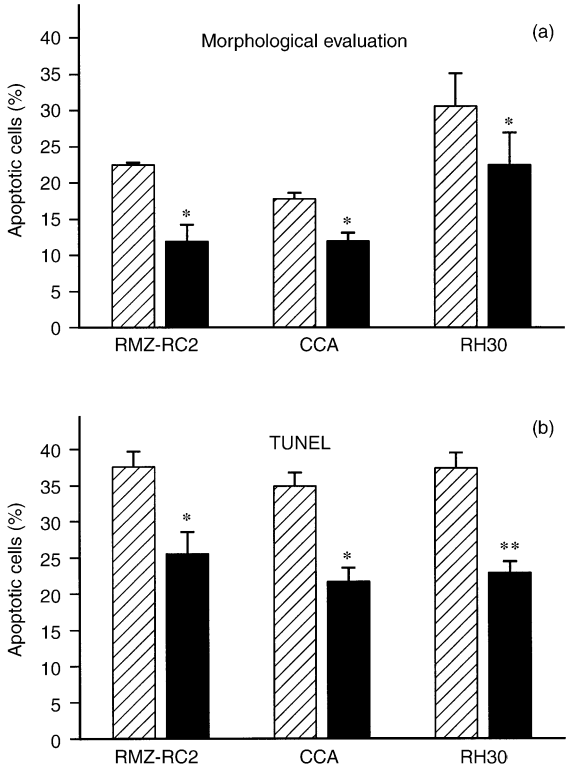

Fig. 4. Effect of nerve growth factor (NGF) (50 ng/ml) on the percentage of rhabdomyosarcoma cells undergoing apoptosis induced by a 24 h treatment with CHX (50 μg/ml). Hatched bars: control cells; solid bars: NGF-treated cells. The percentage of apoptotic cells was evaluated by: (a) morphological features (Hoechst 33342 staining); (b) TdT-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL assay). Statistical significance was evaluated by Student's *t*-test, or paired *t*-test for RH30 in panel a ($*P<0.05$; $**P<0.01$).

(Fig. 4b). TUNEL analysis confirmed the results obtained by morphological evaluation: in the three cell lines, the percentage of apoptotic cells was significantly reduced by NGF treatment. Thus, morphological evaluation and the TUNEL assay yielded comparable results, even if the overall percentage of apoptosis detected by TUNEL assay was slightly higher, possibly due to the higher sensitivity of this technique.

3.3. Autocrine expression of neurotrophins by rhabdomyosarcoma cells

Since rhabdomyosarcoma is a tumour showing multiple autocrine loops, we investigated the possibility that rhabdomyosarcoma cells could express neurotrophins in an autocrine fashion. The expression of *NGF* and other neurotrophins, such as *NT-3* and *BDNF* (all of which bind to the low affinity receptor), was studied by means of semiquantitative RT-PCR in the rhabdomyosarcoma cells and compared with the expression of normal muscle (Fig. 5). All the rhabdomyosarcoma cell lines expressed the neurotrophins studied, with minor differences in the expression levels. In particular, NGF was expressed in rhabdomyosarcoma cells at a level comparable to that found in normal muscle with the exception of RD/18 cells which showed a lower expression level.

The production of NGF by rhabdomyosarcoma cell lines was also evaluated by means of an ELISA test performed on the concentrated supernatants. Trace amounts of NGF protein (1–2 pg/10^6 cells) were observed.

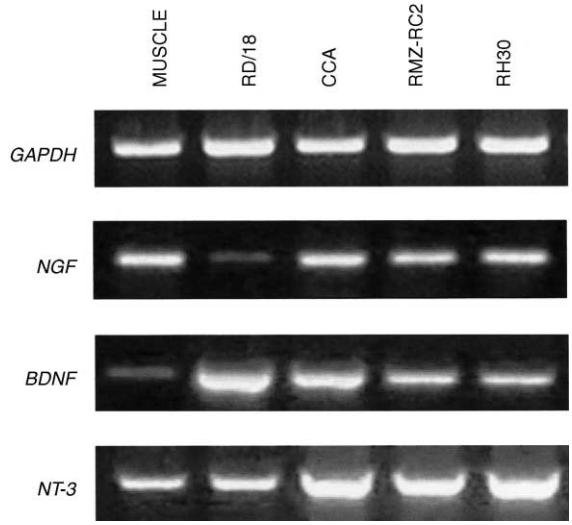

Fig. 5. Expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neutrophin-3 (NT-3) in rhabdomyosarcoma cells and normal muscle evaluated by means of reverse transcriptase-polymerase chain reaction (RT-PCR) (20 cycles of amplification for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), 40 for *NGF*, 35 for *BDNF* and *NT-3*). *GAPDH* is included to show that comparable amounts of cDNAs were obtained.

To investigate the possibility that autocrine NGF could rescue the rhabdomyosarcoma cells from apoptosis, we evaluated the percentage entity of apoptosis in the presence of an anti-NGF blocking antibody. Apoptosis induced by serum starvation was significantly increased after treatment of RMZ-RC2 cells with the anti-NGF antibody compared with untreated cultures (Fig. 6a). Even if a slight increase of apoptosis was found after treatment with the highest dose of an isotype-matched irrelevant antibody compared with untreated cultures, at both doses tested (1 and 10 μg/ml) treatment with anti-NGF antibody caused an approximately 40% increase in the number of apoptotic cells compared with cells treated with the irrelevant antibody. Anti-NGF blocking antibody was also able to significantly increase CHX-induced apoptosis of rhab-

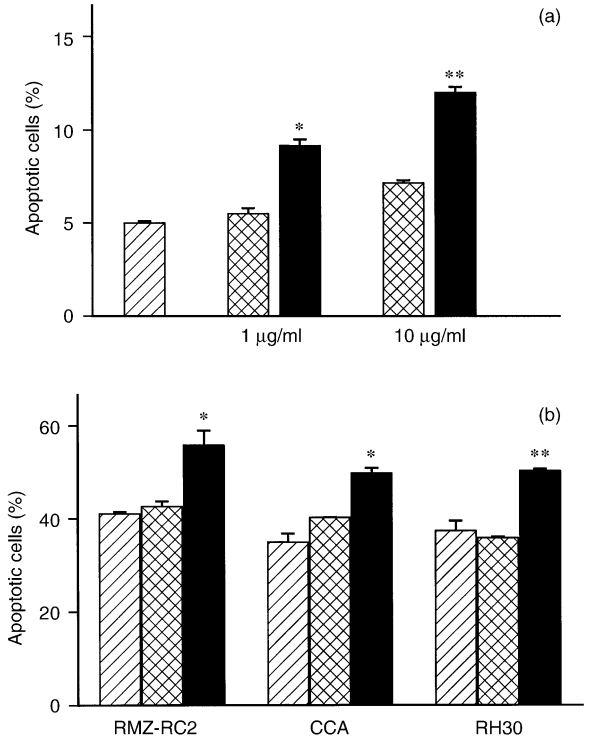

Fig. 6. Effect of a neutralising anti-nerve growth factor (NGF) antibody on the percentage of apoptosis of rhabdomyosarcoma cells. (a) effect of the anti-NGF antibody on apoptosis induced by serum starvation of RMZ-RC2 cells. Anti-NGF antibody or the isotype-matched irrelevant antibody (MOPC 21) were used at a final concentration of 1 or 10 μg/ml, and apoptosis was evaluated by Hoechst 33342 staining after 24 h of treatment. (b) Effect of the anti-NGF antibody on cycloheximide (CHX)-induced apoptosis (50 μg/ml) of rhabdomyosarcoma cells. Anti-NGF and MOPC 21 were used at a final concentration of 10 μg/ml, and apoptosis was evaluated by the TdT-mediated deoxyuridine triphosphate (dUTP) nick-end labelling (TUNEL) assay after 24 h treatment. Hatched bars: control cells; cross-hatched bars: cells treated with an irrelevant antibody; solid bars: anti-NGF-treated cells. The increase in apoptosis due to anti-NGF treatment is statistically significant (Student's *t*-test: $*P<0.05$; $**P<0.01$) both compared with the control cells and the irrelevant antibody-treated cells.

domyosarcoma cell lines, compared with cells treated with the irrelevant antibody (Fig. 6b).

4. Discussion

The expression of NGF receptors in four human rhabdomyosarcoma cell lines was assessed by means of a cytofluorometric analysis. We found that all the cell lines expressed both receptors and that the low-affinity $p75^{NTR}$ is strongly expressed. No data was previously available on the expression and function of these receptors in rhabdomyosarcoma cells. We found that NGF was able to rescue 30–50% of rhabdomyosarcoma cells from spontaneous apoptosis as well as from apoptosis, induced by serum starvation or by a conventional apoptosis inducer such as CHX. A decrease of CHX-induced apoptosis was also reported as a NGF-TrkA-dependent pathway in Rat-1 fibroblasts transfected with TrkA: in this model, NGF was able to delay, but not abolish, CHX-induced cell death [20]. A similar effect was found in the present study in rhabdomyosarcoma cells.

We detected the anti-apoptotic effect of NGF in three out of four rhabdomyosarcoma cell lines analysed, including cells from both alveolar and embryonal histotypes.

An anti-apoptotic effect of endogenously-produced NGF was suggested by the increased apoptosis found in rhabdomyosarcoma cells treated with anti-NGF blocking antibody. This effect was present in all the three NGF-sensitive rhabdomyosarcoma cell lines, which in fact produce and secrete trace amounts of NGF into the culture medium, supporting the idea of an autocrine loop, even if NGF produced by other sources could also be active in rhabdomyosarcoma cells. A slight increase in the percentage of apoptotic cells could also be found in cells treated with an irrelevant antibody at the highest dose (10 μg/ml) and thus could reflect some toxicity associated with this treatment; however, anti-NGF antibody always caused a significant increase of apoptosis when compared with cells treated with the irrelevant antibody.

The only rhabdomyosarcoma cell line that did not show any response to NGF was RD/18. This could be due to the activation of other anti-apoptotic mechanisms by RD/18 cells, which are particularly resistant to the apoptosis inducer CHX. In fact, the almost complete lack of expression of NGF suggests that this anti-apoptotic pathway does not contribute to the apoptotic-resistant phenotype of this cell line.

The involvement of NGF receptors in either inducing or protecting cells from apoptosis is well established, and whether NGF determines apoptosis or survival depends on the relative presence of the two receptors: most studies point toward a model in which NGF binding to $p75^{NTR}$ is pro-apoptotic in the absence of TrkA, while the simultaneous presence of the two receptors signals cell survival [2,3]. The anti-apoptotic effect of NGF that we observed in rhabdomyosarcoma cells could therefore be mediated by the simultaneous engagement of the two receptors, which determines high affinity binding and promotes cell survival. Anti-apoptotic effects mediated by both NGF receptors have been reported in other non-neural models, such as macrophages or keratinocytes [21,22].

NGF has been reported as being active on cells of the myogenic lineage at a very early stage of differentiation, supporting the idea that neurotrophins are not only target-derived factors for developing neurons. Dual effects of autocrine NGF on cells of the myogenic lineage are likely to occur: while it is reported that NGF promotes the early stages of differentiation of normal myoblasts via either $p75^{NTR}$ or TrkA [23–26], our results suggest that malignant cell lines of myogenic origin, like rhabdomyosarcoma, have almost completely lost the responsiveness to the NGF-driven differentiation signal, while responding to NGF with a survival signal.

It has been reported that normal adult non-neural tissues (including skeletal muscle) express at very low levels the mRNAs of *p75*^{NTR} and *TrkA* [27]. This implies that the level of expression at the adult stage should be very low, if at all present. In fact it is reported that, while fetal muscle shows a high immunohistochemical staining for $p75^{NTR}$, no protein is detectable in normal adult muscle [28]. The high expression of $p75^{NTR}$ in rhabdomyosarcomas is emerging as a diagnostic feature of this kind of tumour: a study with microarray technology on gene expression profiling of tumour cell lines of different origin showed that $p75^{NTR}$ is specifically overexpressed in alveolar rhabdomyosarcoma compared with other tumours of neuroepithelial and mesenchymal origin [29]. This feature seems not to be a consequence of *in vitro* culture, since NGF receptor expression has been reported also on biopsies of human rhabdomyosarcoma [8]. An involvement of $p75^{NTR}$ in the aetiology of rhabdomyosarcoma could be suggested based on its reported PAX3-mediated induction [30]. PAX3 is involved in the aetiology of alveolar rhabdomyosarcoma (which is characterised by the presence of the chimeric fusion protein PAX3 or PAX7-FKHR), but a role for PAX3 and PAX7 has also been suggested for embryonal rhabdomyosarcoma which overexpresses either of these transcription factors [31,32].

The anti-apoptotic effect of NGF on rhabdomyosarcoma cell lines suggests that trying to interfere with the NGF survival signal might be a useful therapeutic strategy in these malignancies; however data regarding the expression of NGF and its receptors in primary tumour specimens are still scarce [8,9,28]: quantitation of receptor expression and additional data on the func-

tional significance of TrkA or $p75^{NTR}$ expression in rhabdomyosarcoma will be necessary to evaluate neurotrophins as potential therapeutic targets.

Acknowledgements

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