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Nerve growth factor potentiated the sodium butyrate- and PMA-induced megakaryocytic differentiation of K562 leukemia cells

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Abstract

We have recently reported that retinoic acid (RA) induced the expression of *trk*A, the high affinity receptor for nerve growth factor (NGF), in human chronic myelogenous leukemia K562 cells. In this paper, we examined the ability of several other differentiation inducers to regulate the expression of *trk*A and NGF in K562 cells. We found that the expression of *trk*A was dramatically induced by the two megakaryocyte lineage inducers sodium butyrate (NaBut) and phorbol 12-myristate 13-acetate (PMA), but not by the two erythrocyte lineage inducers hemin or 1-β-D-arabinofuranosyl cytosine (Ara-C). Furthermore, activation of the up-regulated *trk*A receptor by exogenous NGF potentiated the megakaryocytic differentiation of K562 cells induced by NaBut and PMA. Our results demonstrated that *trk*A is one of the essential genes that are up-regulated and involved in the megakaryocytic differentiation of K562 leukemia cells triggered by these differentiation inducers. Our findings suggest that NGF, in addition to its pivotal roles in the nervous system, may also play important roles in hematopoietic differentiation.
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Keywords: NGF; *trk*A; Sodium butyrate; Phorbol 12-myristate 13-acetate; K562; Megakaryocytic differentiation

1. Introduction

The human chronic myelogenous leukemia cell line K562 is a well-established model system for the study of leukemia differentiation [1]. K562 cell line was established from the pleural effusion of a patient with chronic myelogenous leukemia in blast crisis [2]. It has been extensively demonstrated that K562 cells can be induced to differentiate towards erythroid and megakaryocyte lineages by various differentiation inducers [3,4]. Hemin and 1-β-D-arabinofuranosyl cytosine (Ara-C) induced K562 cells to differentiate towards the erythroid lineage, while phorbol 12-myristate 13-acetate (PMA) induced K562 cells to differentiate towards the megakaryocyte lineage [1,5–8]. In contrast, sodium butyrate (NaBut) induced the characteristics of both the erythroid and megakaryocyte lineages in K562 cells [6,8–12]. Retinoic acid (RA) alone did not induce the obvious differentiation, but RA enhanced the hemin-induced erythroid differentiation in K562 cells [13]. Although the differentiating effects of various differentiation inducers on K562 cells have been well-documented, the molecular mechanisms underlying such differentiation induction are still unclear.

We have previously demonstrated that RA induced the expression of *trk*A, the high affinity receptor for nerve growth factor (NGF), in K562 cells [14]. NGF was firstly described as a target-derived trophic factor for neurons and is the prototype of the neurotrophin family [15]. It has been established that NGF plays pivotal roles in both the peripheral and central nervous system [15,16]. The biological effects of NGF are mediated by a receptor tyrosine kinase, known as *trk*A, which resembles the receptor tyrosine kinases used by

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traditional growth factors such as epidermal growth factor or platelet-derived growth factor [17]. Although it was originally thought that the effects of NGF were restricted to the nervous system, increasing amount of evidence indicate that NGF also plays important roles outside of the nervous system, particularly in the hematopoietic and immune systems [18–21]. Consistent with this notion, our recent evidence indicated that constitutive expression of NGF mRNA could be detected, while the mRNA and protein expression of *trk*A was significantly induced by a potent differentiation inducer, RA, in K562 leukemia cells [14]. These findings suggest that NGF/*trk*A signaling may be involved in the differentiation of K562 leukemia cells. To further evaluate this possibility, we examined the ability of several other differentiation inducers, including hemin, Ara-C, NaBut and PMA, to regulate the expression of *trk*A in K562 cells. The combined effects of exogenous NGF and these inducers on the differentiation of K562 cells were subsequently determined.

In this paper, we report that the expression of *trk*A was dramatically induced by the two megakaryocyte lineage inducers NaBut and PMA, but not significantly changed by the two erythroid lineage inducers hemin and Ara-C in K562 cells. Furthermore, activation of the up-regulated *trk*A receptor by exogenous NGF potentiated the differentiating effects of NaBut and PMA, as evidenced by the increase in the expression level of the megakaryocyte markers CD41a, CD61 and α-naphthyl acetate esterase. Our findings suggest that NGF/*trk*A signaling may be involved in the regulation of the megakaryocytic differentiation of K562 cells.

2. Material and methods

2.1. Cell culture and treatment

Human chronic myelogenous leukemia K562 cells (obtained from American Type Culture Collection, ATCC, MD) were cultured in RPMI 1640 medium (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GIBCO-BRL). The cell cultures were incubated in humidified air with 5% CO_2 at 37°C. Cells were treated with control solvent, 1 μM all-*trans* RA (Sigma, St. Louis, MO), 1 μM 9-*cis* RA (Sigma), 5 μM hemin (Sigma), 1 μM Ara-C (Sigma), 1 mM NaBut (Sigma), 10 nM PMA (Sigma), 50 ng/ml NGF (Upstate Biotech., Inc., Lake Placid, NY, USA), anti-human NGF neutralizing monoclonal antibody (1:100 or 1:500, Wako Pure Chemical Industries, Japan), alone or in combination.

2.2. Northern blot analysis

Total cellular RNA was prepared according to the method of Chomczynski and Sacchi [22] at day 3 and 6 after treatment. Equal amounts of RNA (20 μg) were separated by gel electrophoresis using 1% agarose–formaldehyde gel. The RNA was transferred onto a nylon membrane (Micron Separations Inc., Westborough, MA) and crosslinked by UV irradiation (Stratalinker, Stratagene, CA). The RNA blots were hybridized with ^{32}P-labelled cDNA probes, including partial cDNA fragment of trkA extracellular domain [23] and full-length cDNA fragment of NGF, neurotrophin-3 (NT-3) and NT-4/5 [24,25]. The cDNA probes was gel-purified by using GENECLEAN II Kit (BIO 101, La Jolla, CA), and labelled by random priming using Megaprime Labelling Kit (Amersham, UK). Hybridization was carried out in 0.5 M sodium phosphate buffer (PBS, pH7.4) containing 7% SDS, 1% BSA, 1 mM EDTA, and 40 μg/ml salmon sperm DNA at 65°C. The hybridized membranes were washed in 2 × SSC/0.1% SDS at 65°C for 3 × 30 min, then exposed to X-ray films (Kodak, Eastman Kodak Company, Rochester, NY) with intensifying screens (C.B.S. Scientific Co., CA) at −80°C.

2.3. Immunophenotyping analysis

Leukemia cells (1×10^6 cells/sample) were collected and washed with PBS twice, then stained with phycoerythin (PE)- or fluorescein-5-isothiocyanate (FITC)-conjugated antibodies (with 1:10 dilution) against specific cell surface markers at room temperature for 30 min. The cell surface markers examined include the myeloid lineage marker CD15 (Becton Dickinson, San Jose, CA, USA), the megakaryocyte lineage markers CD41a and CD61 (PharMingen, San Diego, CA, USA), and the erythroid lineage markers CD71 and Glycophorin A (PharMingen). The stained cells were subsequently washed with PBS, fixed with ice-cold 1% paraformaldehyde and stored at 4°C. The fluorescence signal of the stained cells was recorded by using a flow cytometer (Becton Dickinson). The mean of fluorescence intensity and the percentage of positively stained cells were analyzed by using Lysis II software (Becton Dickinson).

2.4. α-Naphthyl acetate esterase staining analysis

The expression of cytoplasmic α-naphthyl acetate esterases was detected by staining with an α-Naphthyl Acetate Esterase Kit (Sigma) according to the manufacturer's procedure. Briefly, leukemia cells were collected onto a glass slide using a Cytospin II (Shandon Scientific Limited, UK) centrifuge, then fixed in fixative solution. The fixed cells were subsequently stained in

α-naphthyl acetate esterase staining solution at 37°C for 30 min. The stained slides were washed thoroughly in deionized water, air-dried and mounted with Airvol mounting media (Air Products and Chemicals, Inc., Allentown, PA). The morphology of the stained cells was examined using Zeiss Axiophot microscope (Zeiss, Germany) equipped with a 40 × objective.

3. Results

3.1. Induction of the expression of trkA by RA, NaBut and PMA

Total cellular RNA was extracted from K562 cells after treatment with various differentiation inducers for 3 and 6 days. The mRNA expression of trkA, NGF, NT-3 and NT-4/5 was determined by Northern blot analysis. The expression of trkA was dramatically induced by 1 μM all-*trans* RA, 1 μM 9-*cis* RA, 1 mM NaBut, and 10 nM PMA, but not significantly changed by 5 μM hemin or 1 μM Ara-C (Fig. 1). In contrast, constitutive expression of NT-3 and NT-4/5 was detected in K562 cells, but the expression level of these neurotrophins was not significantly induced by any differentiation inducer examined in this study. The mRNA expression of NGF could not be detected in control K562 cells or after treatment with these differentiation inducers by Northern blot analysis (data not shown).

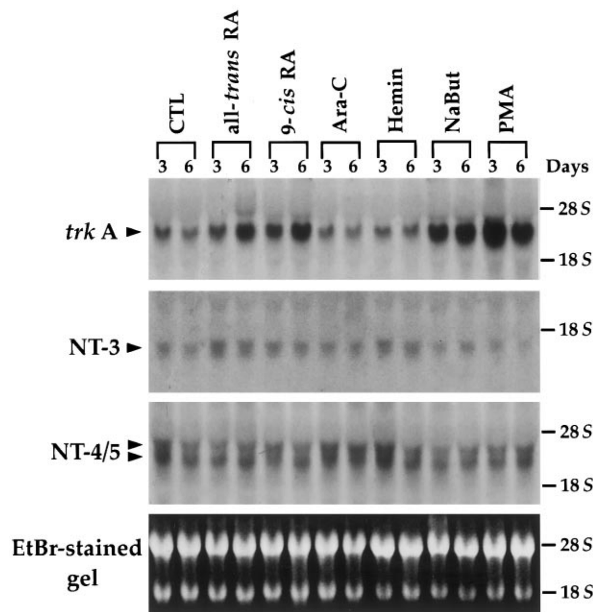

Fig. 1. The expression of *trk*A, NT-3 and NT-4/5 mRNA in K562 cells following treatment with various differentiation inducers. K562 cells were treated with control solvent (CTL), 1 μM all-*trans* RA, 1 μM 9-*cis* RA, 1 μM Ara-C, 5 μM hemin, 1 mM NaBut, or 10 nM PMA. RNA samples were prepared on day 3 and 6 after treatment. The mRNA expression for *trk*A, NT-3 and NT-4/5 was examined by Northern blot analysis using specific cDNA probes. Ethidium bromide (EtBr)-stained gel was shown to indicate that equal amount of RNA was loaded (20 μg per sample). Ribosomal RNA bands (18*S* and 28*S*) are indicated on the right, and the probes used are indicated on the left.

3.2. Inhibition of the erythroid differentiation by RA and NGF

The growth curves of K562 cells after treatment with 50 ng/ml NGF and 1 μM all-*trans* RA alone or in combination were determined. No significant combined effect of NGF and RA on cellular proliferation was detected in K562 cells (data not shown). The effects of RA and NGF on the differentiation of K562 cells were examined by immunophenotyping analysis. The expression level of the myeloid lineage marker CD15 and the erythroid lineage marker Glycophorin A was dose-dependently inhibited by RA (Fig. 2A). No significant combined effect between RA (1 μM) and NGF (50 ng/ml) on the expression of CD15, CD41a, CD61 and CD71 was detected after treatment for 3, 4, 6, 8 or 10 days (data not shown). In contrast, the expression of the erythroid lineage marker Glycophorin A was inhibited by all-*trans* RA and NGF in an additive manner (Fig. 2B). The decrease in the expression level of Glycophorin A caused by 1 μM all-*trans* RA and 50 ng/ml NGF was similar to that achieved by 10 μM RA alone.

3.3. Synergistic induction of the megakaryocytic differentiation by NaBut and NGF

The growth curves of K562 cells after treatment with 1 mM NaBut and 50 ng/ml NGF alone or in combination were determined. The addition of NGF together with NaBut caused a slight further inhibition of the proliferation of K562 cells when compared to NaBut alone (data not shown). The effects of NaBut and NGF on the differentiation of K562 cells were determined by immunophenotyping analysis. Fig. 3A shows that NaBut induced the expression of CD61 in a dose-dependent manner, while the expression of Glycophrin A was increased by lower doses of NaBut (0.5 and 1 mM), but decreased by higher doses of NaBut (5 and 10 mM). The combined effects of NGF (50 ng/ml) and NaBut (0.5, 1, 2.5, 5, and 10 mM) on the differentiation of K562 cells were tested after treatment for 3 or 5 days. The maximal co-operative effects between NGF and NaBut were achieved by treatment with 50 ng/ml NGF in combination with 1 mM NaBut for 5 days. The percentage of CD41a + cells was increased from about 20 to 40%, while the percentage of CD61 + cells was increased from about 40 to 65% by addition of 50 ng/ml NGF to 1 mM NaBut (Fig. 3B). A similar extent of increase in the percentage of CD61 + cells could only be achieved by 5 mM NaBut alone. The expres-

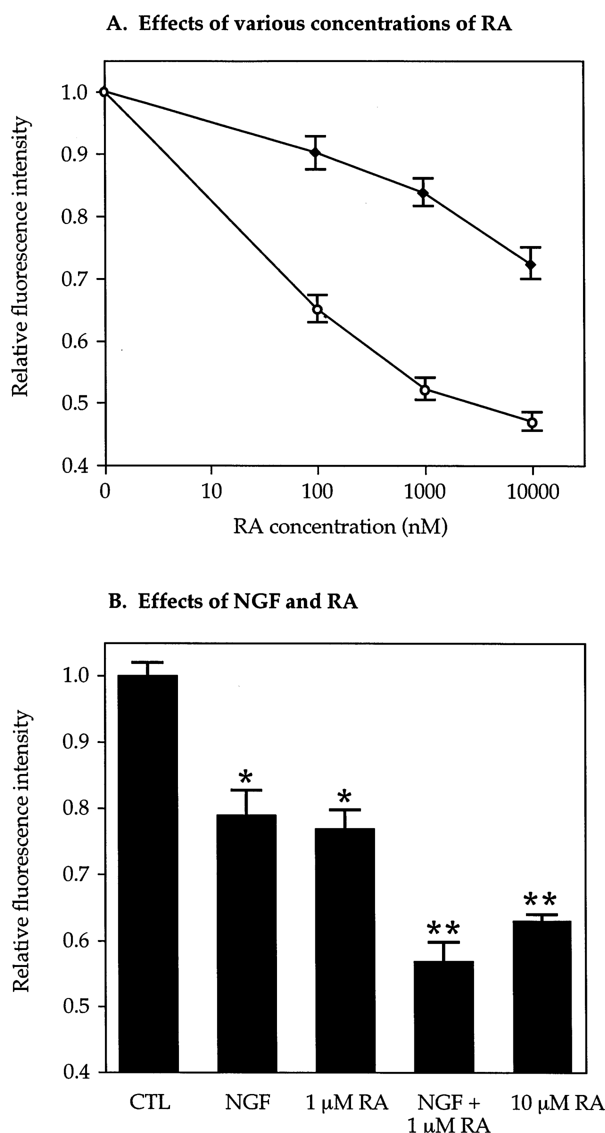

Fig. 2. Effects of RA and NGF on K562 differentiation. (A) Effects of various concentrations of RA. K562 cells were grown in RPMI 1640 medium containing 10% FBS and treated with control solvent, or various concentrations of all-*trans* RA (100 nM, 1 or 10 μM). The immunophenotyping analysis was performed on day 6 after treatment. Cell surface markers examined include CD15 (–○–) and Glycophorin A (–◆–). Data represents the mean of two experiments ± SD. In each experiment, each sample had duplicates. (B) The relative expression level of the erythroid lineage marker Glycophorin A in K562 cells after treatment with NGF and RA. Cells were grown in RPMI 1640 medium containing 5% FBS in the presence of control solvent (CTL), 50 ng/ml NGF (NGF), 1 μM all-*trans* RA (1 μM RA), 50 ng/ml NGF in combination with 1 μM all-*trans* RA (NGF + 1 μM RA), or 10 μM all-*trans* RA (10 μM RA). The immunophenotyping analysis was performed on day 5 after treatment. The mean of fluorescence intensity was analyzed by using Lysis II software (Becton Dickinson). Data represents the mean of three experiments ± SEM. In each experiment, each sample had three independent replicates. *, significantly different from control (t-test, $P < 0.001$); **, significantly different from the * data (t-test, $P < 0.001$).

sion level (indicated by relative fluorescence intensity) of both CD41a and CD61 was also synergistically increased by 50 ng/ml NGF in combination with 1 mM NaBut (Fig. 3C). The expression level of CD61 induced by NGF plus 1 mM NaBut was almost the same as that induced by 5 mM NaBut alone. In contrast, NGF and NaBut inhibited the expression level of the myeloid lineage marker CD15 in an additive manner, while NGF slightly reduced the expression level of the two erythroid lineage markers CD71 and Glycophorin A induced by 1 mM NaBut alone (Fig. 3D). Furthermore, NGF also further increased the expression of α-naphthyl acetate esterases induced by 1 mM NaBut alone (Fig. 4).

3.4. Synergistic induction of the megakaryocytic differentiation by PMA and NGF

No significant combined effect of PMA (0.5 and 10 nM) and NGF (50 ng/ml) on cellular proliferation was detected in K562 cells (data not shown). The effects of NGF (50 ng/ml) and PMA (0.25, 0.5, 1, 2.5, 5 and 10 nM) on the differentiation of K562 cells were tested by immunophenotyping analysis after treatment for 3 or 5 days. The maximal co-operative effect was achieved by treatment with 50 ng/ml NGF in combination with 0.5 nM PMA for 5 days. NGF significantly further increased the expression level of the megakaryocyte lineage marker CD61 induced by 0.5 nM PMA alone (Fig. 5A). The expression level of CD61 induced by NGF plus 0.5 nM PMA was almost the same as that induced by 1 nM PMA. In contrast, NGF and PMA inhibited the expression level of the two erythroid lineage markers CD71 and Glycophorin A in an additive manner (Fig. 5B). Furthermore, NGF also further increased the expression of α-naphthyl acetate esterases induced by 0.5 nM PMA alone (Fig. 6).

As an attempt to block the potential endogenous NGF/*trk*A signaling, we applied an anti-NGF neutralizing antibody. The effects of the anti-NGF neutralizing antibody (1:500, 1:100) alone or in combination with 1 μM all-*trans* RA, 1 mM NaBut, 10 nM PMA, or 1 mM NaBut plus 50 ng/ml NGF were determined by immunophenotyping analysis after treatment for 3 or 5 days. The anti-NGF neutralizing antibody could not affect or reduce the differentiating effects of RA, NaBut or PMA on K562 cells. In contrast, the effects of exogenous NGF was completely abolished by the simultaneous addition of the anti-NGF neutralizing antibody (data not shown).

4. Discussion

It has been extensively reported that human chronic myelogenous leukemia K562 cells can be induced to differentiate towards the erythroid or megakaryocyte

lineage by various differentiation inducers, including hemin, Ara-C, NaBut and PMA [3,4]. However, the molecular mechanisms underlying such differentiation induction still remain unclear. We have recently demonstrated that RA, a potent differentiation inducer, significantly induced the expression of *trk*A, the high affinity receptor for NGF, at both the mRNA and protein levels in K562 cells [14]. This finding suggested that NGF/*trk*A signaling may be involved in the regulation of K562 leukemia differentiation. In the present study, we further investigated this possibility by examining the ability of several other differentiation inducers to regulate the expression of *trk*A in K562 leukemia cells. We found that, in addition to RA, the two megakaryocyte inducers NaBut and PMA also dramatically induced the expression of *trk*A in K562 cells. In contrast, the expression of *trk*A was not significantly changed by the two erythroid inducers hemin and Ara-C. Our results suggest that NGF/*trk*A signaling may be involved in the regulation of lineage-specific differentiation in K562 cells.

To explore the functional roles of the up-regulated *trk*A receptors, we examined the effects of exogenous NGF alone or in combination with these differentiation inducers on the proliferation and differentiation of K562 cells. While no significant effect of NGF on cellular proliferation could be detected, we found that NGF could regulate the differentiation of K562 cells. NGF alone slightly inhibited the expression level of the two erythroid lineage markers CD71 (about 85%) and Glycophorin A (about 75%). Moreover, NGF in combination with certain differentiation inducers significantly enhanced the differentiating effects of these inducers. For example, NGF in combination with NaBut or PMA synergistically induced the expression of the megakaryocyte lineage markers CD41a and

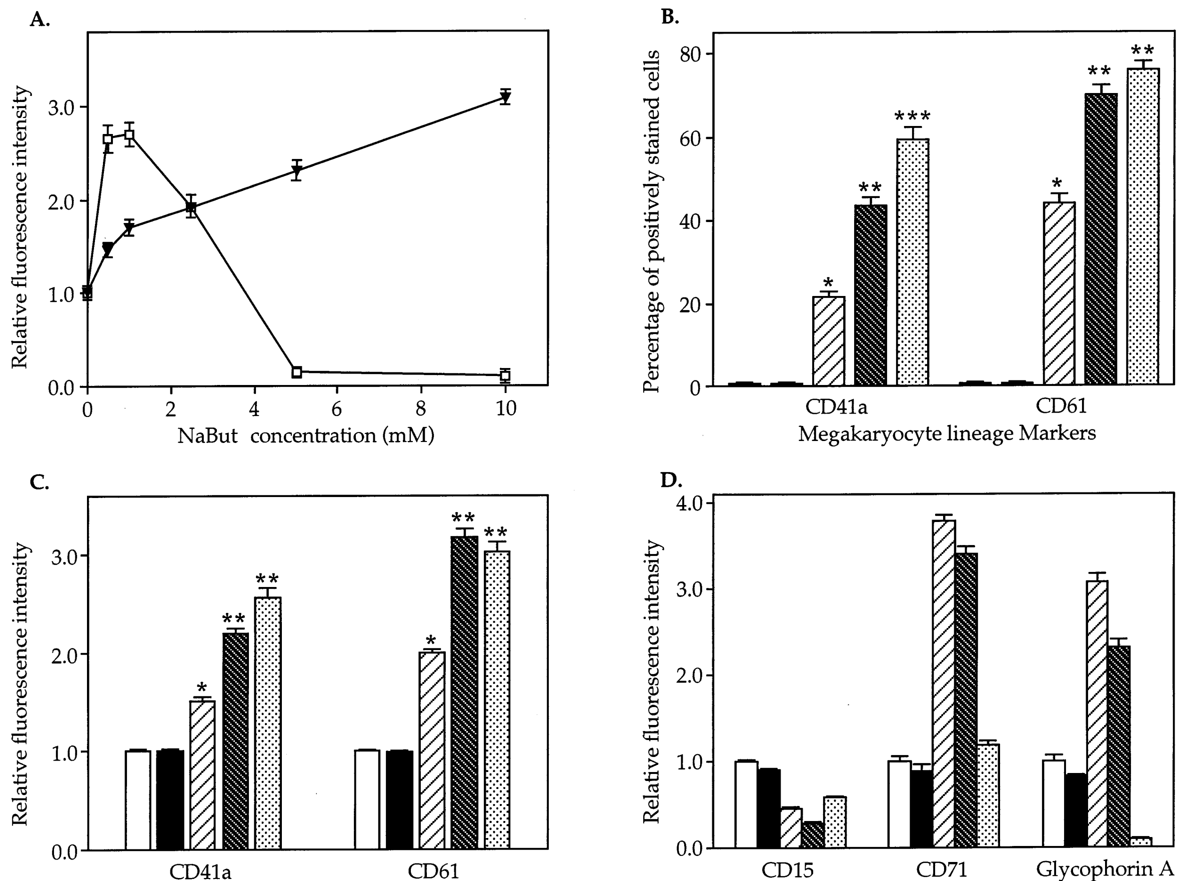

Fig. 3. Effects of NaBut and NGF on K562 differentiation. A, Effects of various concentrations of NaBut. K562 cells were grown in RPMI 1640 medium containing 10% FBS in the presence of control solvent, various concentrations of NaBut (1, 2.5, 5 or 10 mM). The immunophenotyping analysis was performed on day 4 after treatment. Cell surface markers examined included CD61 (–▼–) and Glycophorin A (–□–). Data represents the mean of two experiments ± SD. In each experiment, each sample had two independent replicates. (B, C and D), The combined effects of NGF and NaBut. K562 cells were grown in RPMI 1640 medium containing 5% FBS in the presence of control solvent (□), 50 ng/ml NGF (■), 1 mM NaBut (▨), 50 ng/ml NGF in combination with 1 mM NaBut (▧), or 5 mM NaBut (▩). The immunophenotyping analysis was performed on day 5 after treatment. The percentage of positively stained cells (B) and the mean of fluorescence intensity (C, D) was analyzed by using Lysis II software (Becton Dickinson). Data represents the mean of three experiments ± SEM. In each experiment, each sample had three independent replicates. *, significantly different from control (t-test, $P < 0.001$); **, significantly different from * data (t-test, $P < 0.001$); ***, significantly different from ** data (t-test, $P < 0.001$).

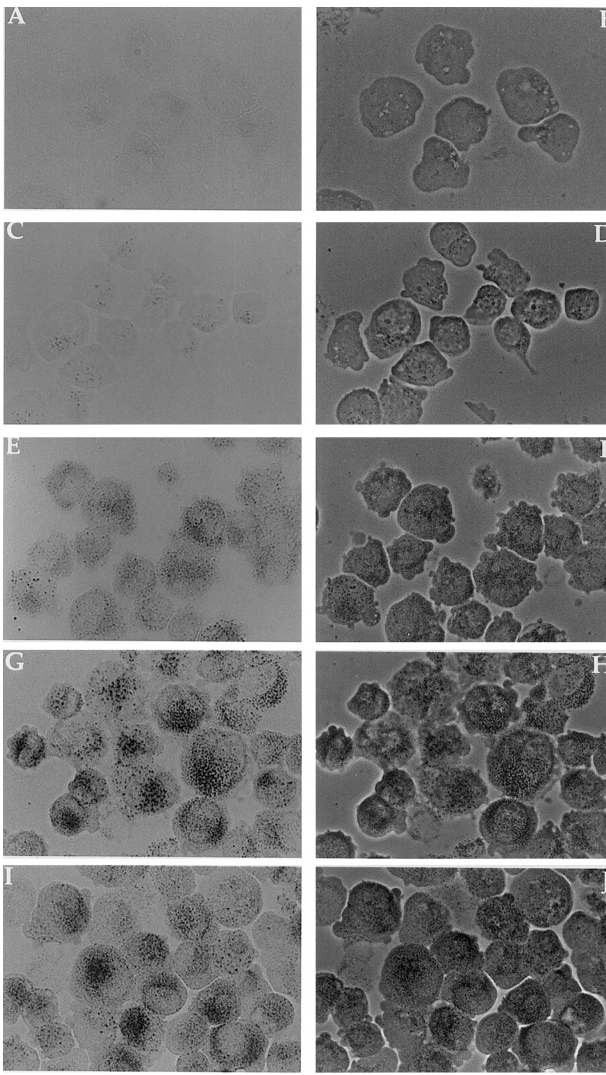

Fig. 4. Expression of α-naphthyl acetate esterase in K562 cells after treatment with NGF and NaBut. K562 cells were grown in RPMI 1640 medium containing 5% FBS in the presence of control solvent (A and B), 50 ng/ml NGF (C and D), 1 mM NaBut (E and F), 50 ng/ml NGF in combination with 1 mM NaBut (G and H), or 2 mM NaBut (I and J). The cytoplasmic expression of α-naphthyl acetate esterase was visualized by α-naphthyl acetate esterase staining after treatment for 5 days. The left panel (A, C, E, G and I) are bright-field micrographs showing the positively stained granules (black particles in the cytoplasm), while the right panel (B, D, F, H and J) are phase-contrast micrographs showing the morphology of the cells. Bar, 20 μm.

CD61 as well as α-naphthyl acetate esterases. Furthermore, NGF in combination with RA or PMA inhibited the expression level of the erythroid lineage markers CD71 and Glycophorin A in an additive manner. Our results suggest that NGF/*trk*A signaling may serve as a positive regulatory signal for the megakaryocytic differentiation, but a negative regulatory signal for the erythroid differentiation in K562 cells.

Our previous evidence has shown that constitutive expression of NGF mRNA was detected in K562 cells by RT-PCR analysis, suggesting that NGF and the up-regulated *trk*A receptors may work in an autocrine manner [14]. As an attempt to block the potential endogenous NGF/*trk*A signaling, we examined the effects of an anti-NGF neutralizing antibody. Although the anti-NGF neutralizing antibody completely abolished the effects of exogenous NGF, it could not abrogate or reduce the differentiating effects of various differentiation inducers in K562 cells. Similar ineffectiveness of neutralizing antibodies against growth factors has been reported in other studies. One suggested possibility is that endogenous growth factors may be

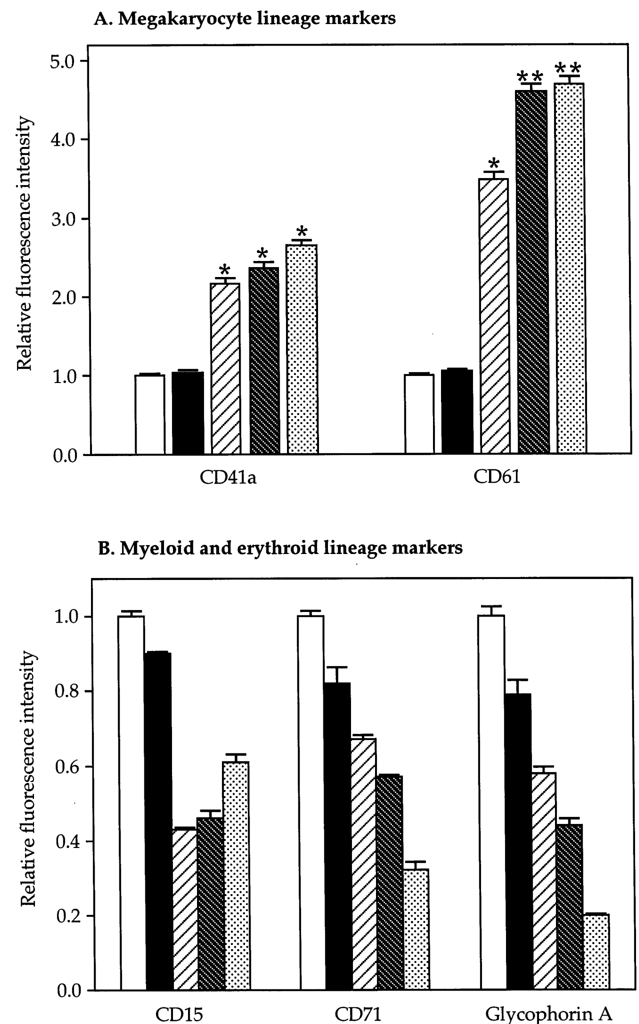

Fig. 5. Effects of NGF and PMA on K562 differentiation. K562 cells were grown in RPMI 1640 medium containing 5% FBS in the presence of control solvent (□), 50 ng/ml NGF (■), 0.5 nM PMA (▨), 50 ng/ml NGF in combination with 0.5 nM PMA (▧), or 1 nM PMA (▩). The immunophenotyping analysis was performed on day 5 after treatment. The mean of fluorescence intensity was analyzed by using Lysis II software (Becton Dickinson). Data represents the mean of three experiments ± SEM. In each experiment, each sample had three independent replicates. *, significantly different from control (t-test, $P < 0.001$); **, significantly different from the * data (t-test, $P < 0.001$).

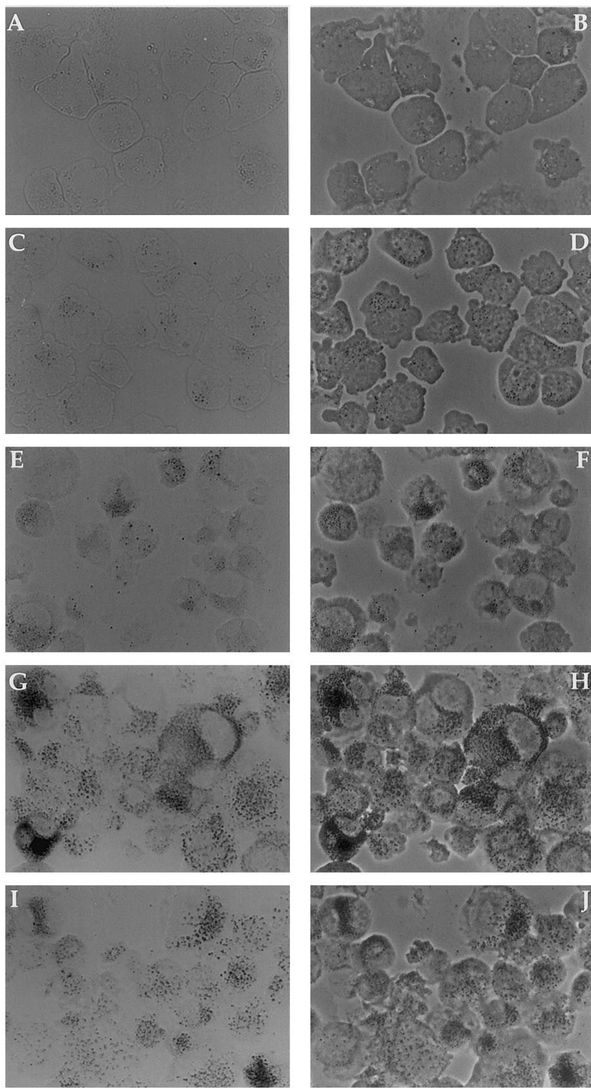

Fig. 6. Expression of α-naphthyl acetate esterase in K562 cells after treatment with NGF and PMA. K562 cells were grown in RPMI 1640 medium containing 5% FBS in the presence of control solvent (A and B), 50 ng/ml NGF (C and D), 0.5 nM PMA (E and F), 50 ng/ml NGF in combination with 0.5 nM PMA (G and H), or 1 nM PMA (I and J). The cytoplasmic expression of α-naphthyl acetate esterase was visualized by α-naphthyl acetate esterase staining after treatment for 5 days. The left panel (A, C, E, G, and I) are bright-field micrographs showing the positively stained granules (black particles in the cytoplasm), while the right panel (B, D, F, H and J) are phase-contrast micrographs showing the morphology of the cells. Bar, 20 μm.

bound to other factors or extracellular matrix, which may protect them from the recognition by neutralizing antibodies [26]. Hence, whether NGF and the up-regulated *trk*A receptor work in an autocrine manner in K562 cells still awaits for further investigation.

Because of the paucity of normal hematopoietic stem and progenitor cells, examination of the intracellular events regulating the hematopoietic differentiation requires the utilization of established leukemia cell lines as the model systems of the differentiation process [1,27,28]. Such extensive studies have led to the conclusion that leukemia cells do indeed have their counterparts in normal development and hematopoiesis [27–29]. On the other hand, increasing evidence demonstrated that both NGF and *trk*A are expressed in normal cells of the hematopoietic system. For example, the expression of NGF and *trk*A was detected in bone marrow stromal cells [30,31], while *trk*A receptors was expressed by hematopoietic progenitor cells, basophils, eosinophils, monocytes, mast cells, T lymphocytes and B lymphocytes [32–37]. Based on these previous observations, our findings obtained with the leukemia cell line K562 may reflect certain events occuring in normal hematopoiesis. It has been shown that NGF in combination with IL-3, IL-5, GM-CSF or M-CSF promoted the differentiation of myeloid progenitor cells, basophils, mast cells and B lymphocytes in vitro [37–41]; NGF stimulated the formation and release of lipid mediators by mature basophils and mast cells, suppressed the synthesis of lipid mediators in eosinophils, and enhanced the chemotaxis for polymorphonuclear neutrophil [33,36,42–44]. In the present study, our results suggest that NGF may promote the megakaryocyte differentiation, but inhibit the erythroid differentiation. Thus, while NGF was originally thought to be restricted to the nervous system, this neurotrophin actually has a broad spectrum of action in the hematopoietic system.

In summary, the present study provided the first systematic examination of the regulation of *trk*A and NGF expression by various differentiation inducers in human chronic myelogenous leukemia K562 cells. Our results demonstrated that the expression of *trk*A was dramatically induced in K562 cells by the two megakaryocyte inducers NaBut and PMA, but not by the two erythroid inducers hemin and Ara-C. Activation of the induced *trk*A by exogenous NGF promoted the megakaryocytic differentiation of K562 cells induced by NaBut and PMA. Taken together, our results indicated that *trk*A is one of the essential genes which are up-regulated by certain differentiation inducers and mediate the lineage-specific differentiating effects of these inducers in K562 cells. Our findings are consistent with the notion that NGF and its receptor *trk*A not only play crucial roles in the nervous system, but may also play important roles in hematopoietic differentiation.

Acknowledgements

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