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Increased Expression of *trk* Proto-oncogene by γ-Interferon in Human Neuroblastoma Cell Lines

Akane Shikata,[1,3] Tohru Sugimoto,[1,4] Hajime Hosoi,[1] Yasuhiro Sotozono,[1] Takuma Shikata,[1] Tadashi Sawada[1] and Luis F. Parada[2]

[1]*Department of Pediatrics, Kyoto Prefectural University of Medicine, 465 Kajii-cho, Kawaramachi, Hirokoji, Kamigyo-ku, Kyoto 602, Japan and* [2]*Molecular Embryology Section, ABL-Basic Research Program, National Cancer Institute-Frederick Cancer Research and Development Center, P.O. Box B, Frederick, Maryland 21702, USA*

Three human neuroblastoma cell lines were examined to determine the effect of recombinant γ-interferon (IFN-γ) treatment on the expression of *trk* proto-oncogene. Increased levels of *trk* proto-oncogene mRNA were observed in two neuroblastoma cell lines (KP-N-RT and KP-N-SI(FA)) after IFN-γ treatment. The levels of *trk* mRNA increased with growth inhibition and morphological change in a time- and dose-dependent manner. The decreased level of N-*myc* mRNA after IFN-γ treatment in KP-N-RT was inversely correlated with *trk* mRNA. Our results suggest that IFN-γ can modulate the signal transduction of nerve growth factor in human neuroblastoma cells.

Key words: γ-Interferon — *trk* — N-*myc* — Neuroblastoma

Neuroblastoma arises from primitive sympathetic neuroblasts, and is one of the most common malignant solid tumors in childhood. The spontaneous regression *in vivo* and the induction of differentiation by natural and chemical agents *in vitro*[1] are well known characteristics of neuroblastoma. Nerve growth factor (NGF) is one of the candidates to play a role in the differentiation of neuroblastoma cells because NGF is normally required for the survival and differentiation of developing sympathetic neuroblasts.

NGF is known to bind to both high- and low-affinity receptors. Multiple abnormalities of low-affinity NGF receptor ($p75^{LNGFR}$)[2] and an inverse relationship of gene expression between N-*myc* and LNGFR[3] have been reported in human neuroblastoma cells. However, as the function of low-affinity NGF receptor is unclear even in normal cells, the relationship between LNGFR expression and the biological behavior of neuroblastoma cells has not been identified. Recently, it was shown that transmembrane tyrosine kinase $p140^{proto\text{-}trk}$ encoded by *trk* proto-oncogene is required for high-affinity NGF binding and functional NGF signal transduction.[4] The relationship between *trk* mRNA expression and the clinical prognosis of neuroblastomas has been examined. Several investigators have shown that high expression of *trk* mRNA appears to be associated with favorable outcome in human neuroblastomas[5–8] and there was an inverse relationship with N-*myc* amplification.[5] These results also suggest that *trk* expression is associated with the differentiation of neuroblastoma cells.

γ-Interferon (IFN-γ), a secretory cellular protein, has been shown to modulate cell differentiation and expression of cell surface antigens including major histocompatibility (MHC) antigens,[9,10] growth factor receptors,[11] and cell adhesion molecules.[12] To date, the influence of IFN-γ treatment on *trk* expression has not been examined. In the present study, we examined the effect of IFN-γ on the expression of *trk* mRNA in human neuroblastoma cell lines to understand better the antiproliferative effect of IFN-γ on neuroblastoma.

Materials used were three human neuroblastoma cell lines (KP-N-RT,[13] KP-N-SI(FA),[10,14] SJ-N-KP[15]). The amplification of N-*myc* gene is 50-fold in KP-N-RT cell line, and there is no amplification in the other two cell lines. Cells were cultured in RPMI1640 medium containing penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum at 37°C in a 5% CO_2 incubator. In every experiment, cells were plated in new flasks and the medium was replaced with supplemented medium containing recombinant human IFN-γ (Kyowa Hakko Kogyo Co., Tokyo) on a three-day schedule.

To determine the expression of *trk* and N-*myc* proto-oncogenes, Northern blot analysis was performed. Total RNA was isolated from cells lysed by adding Nonidet P-40 as described by Kaufmann and Sharp.[16] The samples were denatured and electrophoresed through a 1% agarose-formaldehyde gel, transferred to nylon filters,

[3] To whom correspondence should be addressed.

[4] Present address: Department of Pediatrics, Miyazaki Medical College, 5200 Kihara, Kiyotake-cho, Miyazaki 889-16.

and hybridized to complementary DNA (cDNA) probes labeled with [α-^{32}P]dCTP using random priming (Amersham, Buckinghamshire, UK). The filters were washed three times with 0.1% sodium dodecyl sulfate and 0.2×SCC (1×SCC: 0.15 *M* NaCl/0.015 *M* sodium citrate) at 60°C for 15 min. The filters were exposed at −70°C to Hyperfilm-MP (Amersham). The following cloned cDNA fragments were used as probes: (1) *trk*, a 2.6 kb *Eco*RI fragment of pMS-11[17]; (2) N-*myc*, a 1.0 kb *Eco*RI-*Bam*HI fragment of pNB-1[18]; (3) β-actin, a 1.2 kb *Pst*I fragment of pAL41.[19] Rat pheochromocytoma cell line PC12D[20] was used as a positive control of *trk* mRNA expression.

Untreated KP-N-RT and SJ-N-KP cells showed no *trk* mRNA while KP-N-SI(FA) cells expressed a moderate level of *trk* mRNA. After 14 days of IFN-γ treatment (25 IU/ml), *trk* mRNA expression was induced in KP-N-RT cells and increased in KP-N-SI(FA) cells (Fig. 1A). However, in SJ-N-KP cells, *trk* mRNA was not detected even at 50 IU/ml IFN-γ treatment. In contrast, the levels of N-*myc* mRNA decreased in KP-N-RT cells (Fig. 1B). IFN-γ (25 IU/ml) exerted 50% growth inhibition in KP-N-RT and KP-N-SI(FA) cells at day 5–7 after treatment, but had no inhibitory effect in SJ-N-KP cells (data not shown). Morphological changes characterized as neurite outgrowth and formation of cell clusters also occurred in both KP-N-RT and KP-N-SI(FA) cells treated with IFN-γ (Fig. 2). Such morphological changes were not observed in SJ-N-KP cells.

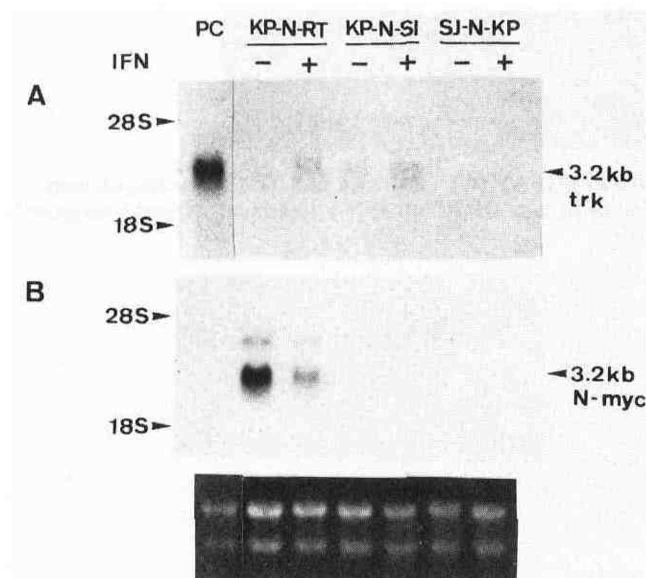

Fig. 1. Northern blot analysis of three neuroblastoma cell lines. The samples were 20 μg of total RNA extracted from KP-N-RT, KP-N-SI(FA) and SJ-N-KP cells with (+) or without (−) 14 days of IFN-γ treatment (KP-N-RT and KP-N-SI(FA): 25 IU/ml, SJ-N-KP: 50 IU/ml). The filters were hybridized to *trk* probe (A) and N-*myc* probe (B) as described in the text. Rat pheochromocytoma PC12D cell line was used as a positive control of *trk* (PC). The third panel shows the ethidium bromide staining pattern of the gel and indicates that approximately equal amounts of RNA were electrophoresed.

The time course of *trk* mRNA induction by IFN-γ was examined in KP-N-RT cells (Fig. 3). KP-N-RT cells were treated with IFN-γ (25 IU/ml) every three days, and total RNA was extracted for Northern blot analysis on days 1, 4, 7 and 13. The expression of *trk* mRNA increased while that of N-*myc* mRNA decreased with time in culture over 13 days.

We also analyzed RNA obtained from KP-N-SI(FA) cells after 7 days of treatment with increasing concentrations of IFN-γ. Cells were treated with 5, 50 and 500 IU/ml of IFN-γ every three days. The level of *trk* mRNA increased in a dose-dependent manner (Fig. 4). The greater the dosage of IFN-γ to which cells were exposed, the greater the growth inhibition and morphological changes that appeared. On day 7 at the concentration of 500 IU/ml, the majority of cells were enlarged and flattened rather than showing neuronal differentiation.

This report is the first to describe the effect of IFN-γ on the expression of *trk* mRNA in human neuroblastoma cell lines. Our results show that *trk* mRNA expression was induced in KP-N-RT cells and increased in KP-N-SI(FA) cells by IFN-γ treatment, in a time- and dose-dependent manner. *trk* mRNA expression appeared to correlate with growth inhibition and morphological changes. Thus, *trk* mRNA was not induced in SJ-N-KP cells, of which the growth and morphology were not modulated by IFN-γ. However, the correlation between *trk* expression and morphological differentiation remains uncertain, since in KP-N-RT cells *trk* mRNA expression became evident as early as one day after IFN-γ treatment, without morphological change. In addition, although 14-day treatment with polyprenoic acid (E5166) induced more marked morphological differentiation than IFN-γ treatment, with extremely extended neurites and overexpression of neurofilament mRNA in KP-N-RT cells, almost equal levels of *trk* mRNA were induced on day 14 by either agent (data not shown). However, it remains clear that *trk* expression is induced by IFN-γ *in vitro*. As IFN-γ can modulate several cell surface antigens, there is a possibility that some regulatory proteins modulated by IFN-γ induce *trk* mRNA.

N-*myc* amplification and overexpression are well known to be a poor prognostic factor in human neuroblastoma.[21] In the present study, decreased N-*myc* expression was observed in IFN-γ-treated KP-N-RT cells. Our previous investigation also showed that N-*myc* expression decreased, with morphological changes, in an

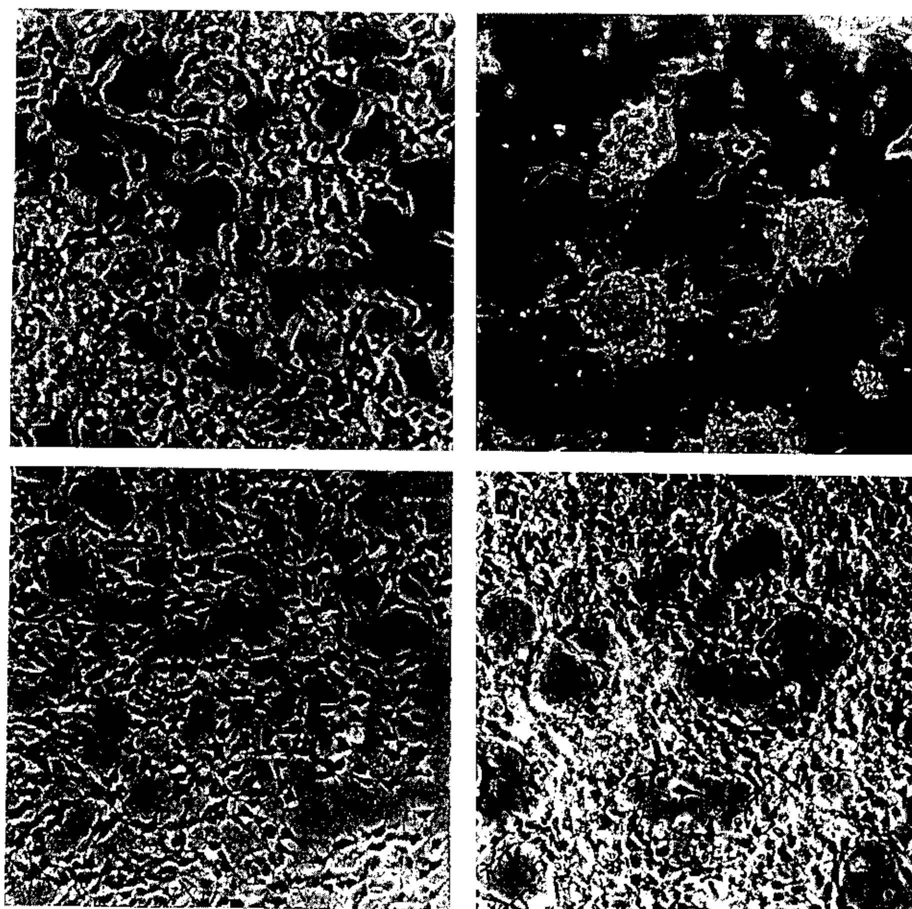

Fig. 2. Phase-contrast micrographs of KP-N-RT (A) (B), and KP-N-SI(FA) (C) (D). (A) and (C) show morphology of cells in the steady state. (B) and (D) show morphology of cells after 14 days of 25 IU/ml IFN-γ treatment. Neurite outgrowth appeared on both cells with IFN-γ treatment. Magnification: ×100.

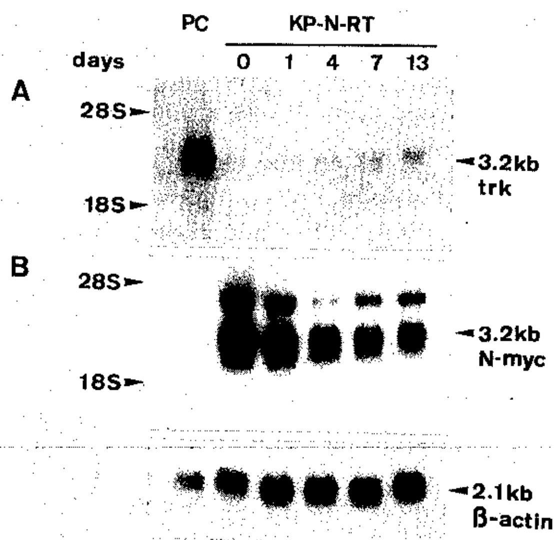

Fig. 3. The time course of induction of *trk* mRNA by IFN-γ in KP-N-RT cells. The total RNA was extracted at the steady state, on day 1, 4, 7 or 13 of 25 IU/ml IFN-γ treatment. The filters were hybridized to *trk* probe (A) and N-*myc* probe (B) as described. β-Actin expression was determined by re-hybridization of the same filters.

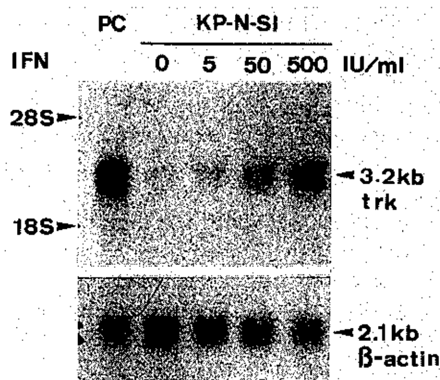

Fig. 4. The dose dependency of induction of *trk* mRNA by IFN-γ in KP-N-SI(FA) cells. KP-N-SI(FA) cells were treated with each dosage of IFN-γ for 7 days. β-Actin expression was determined by re-hybridization of the same filters.

IFN-γ-treated neuroblastoma cell line, GOTO, and it was suggested that the decreased N-*myc* expression might be related to proteins induced by IFN-γ, because it was inhibited by cycloheximide, a protein translation inhibitor.[22] Moreover, there was an inverse relationship between *trk* expression and N-*myc* expression in IFN-γ-treated KP-N-RT cells. As tumors with high *trk* and low N-*myc* have the best prognosis in neuroblastoma,[5] IFN-γ-treated, *trk*-induced cells could be useful as an *in vitro* model to clarify the biological mechanisms involved.

A recent study suggests the abnormal function of $p140^{proto\text{-}trk}$ in neuroblastoma cell lines,[23] because neither normal signal transduction nor neurite outgrowth was induced by nerve growth factor in several neuroblastoma cell lines with *trk* expression. In cell lines with IFN-γ treatment, functions such as tyrosine phosphorylation of induced $p140^{proto\text{-}trk}$ require further study.

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