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Effect of Human Interferons on Morphological Differentiation and Suppression of N-*myc* Gene Expression in Human Neuroblastoma Cells

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The activity of human interferons (HuIFNs) to induce morphological changes and the suppression of N-*myc* gene expression on human neuroblastoma cells (GOTO and KP-N-RT) was evaluated. Morphological differentiation, characterized as the extension and bifurcation of neurites, the formation of multinucleated giant cells and the formation of neurite networks, was induced by treatment with recombinant HuIFN-γ (rHuIFN-γ) and also with natural HuIFN-γ on human neuroblastoma cells (GOTO and KP-N-RT). But recombinant HuIFN-αA and recombinant HuIFN-β did not induce any changes. The rHuIFN-β and rHuIFN-γ inhibited the growth of GOTO and KP-N-RT cells more strongly than the rHuIFN-αA did. The expression of N-*myc* gene was suppressed in GOTO cells treated with rHuIFN-γ. The suppressive effect of rHuIFN-γ was dependent on the duration of the treatment. However, rHuIFN-αA and rHuIFN-β did not suppress N-*myc* gene expression. Moreover, both morphological differentiation and the suppressive effect on N-*myc* gene expression by rHuIFN-γ were inhibited in the presence of cycloheximide. These results suggest that the morphological changes and N-*myc* gene expression in neuroblastoma cells are closely related. Furthermore, this decreased N-*myc* gene expression during the morphological differentiation may be related to the proteins induced by HuIFN-γ.

Key words: Neuroblastoma — Differentiation — Human Interferon-γ — N-*myc*

Direct anti-proliferative effects and indirect effects such as stimulation of natural killer (NK) cell and macrophage activities have been considered as the action mechanisms of antitumor effect of interferons (IFNs). Furthermore, IFN is also known to induce cell differentiation. For example, Mitsui *et al.*[1] and Sariban *et al.*[2] have reported the induction of cell differentiation in promyelocytic HL-60 cells by HuIFNs, but so far cell differentiation of neuronal cells has not been examined. Also, there are only a few reports using mouse IFN[3,4] or HuIFN of low purity on the neuroblastoma cells.[5]

It is known that neuroblastoma, one of the most common malignant solid tumors in childhood, is frequently associated with the overexpression of the N-*myc* oncogene. In several studies[6–8] the morphological differentiation associated with decreased N-*myc* gene expression is induced by treatment of human neuroblastoma cells with retinoic acid or its derivatives. Therefore, N-*myc* gene expression and cell differentiation appear to be closely related in neuroblastoma cells. We studied the activity of 3 types of recombinant human IFN (rHuIFN) on morphological differentiation and N-*myc* gene expression of human neuroblastoma cells.

MATERIALS AND METHODS

Cells Two human neuroblastoma cells, GOTO and KP-N-RT cells, were cultured in RPMI1640+Eagle's minimum essential medium (1:1) (Nissui) containing kanamycin (80 μg/ml) and 10% heat-inactivated fetal calf serum (GIBCO) at 37°C in a 5% CO_2 incubator. N-*myc* DNA was amplified in GOTO and KP-N-RT cells.

IFNs Recombinant HuIFN-αA, recombinant HuIFN-β and recombinant HuIFN-γ were provided by Takeda Pharmaceutical Co., Ltd., Osaka and Kyowa Hakko Kogyo Co., Ltd., Tokyo, respectively.

Induction of morphological change by IFNs Cells (1×10^4 cells/ml) were plated into 35 mm petri dishes and cultured at 37°C in a 5% CO_2 incubator. The cells were also cultured on Lab-Tek chamber slides. After incubation for 24 h, when the cells were attached to the dish surface, the medium was replaced with the medium containing 0, 10, 100 and 1,000 IU/ml of IFNs. The cells were cultured for 6 days, and cell viability was determined by a trypan blue dye exclusion test.

RNA preparations GOTO cells were cultured with rHuIFNs (α, β and γ, 1,000 IU/ml) at 37°C in a 5% CO_2 incubator for 1, 3 or 6 days. Total RNA was extracted

from the cells (1×10^7 cells) by the guanidine isothiocyanate procedure[9] followed by CsCl centrifugation.

Northern blot analysis Sample RNA (20 μg of RNA per lane) was fractionated by electrophoresis through a 1% formaldehyde agarose gel and transferred to a nylon membrane (Gene Screen), before being hybridized with ^{32}P-labeled pNb-1 DNA,[10] which contains a 1.0 kb *Bam*HI-*Hin*dIII fragment from the second exon of human N-*myc* gene.

RESULTS

Effect of HuIFNs on morphological differentiation Morphological differentiation was observed after treat-

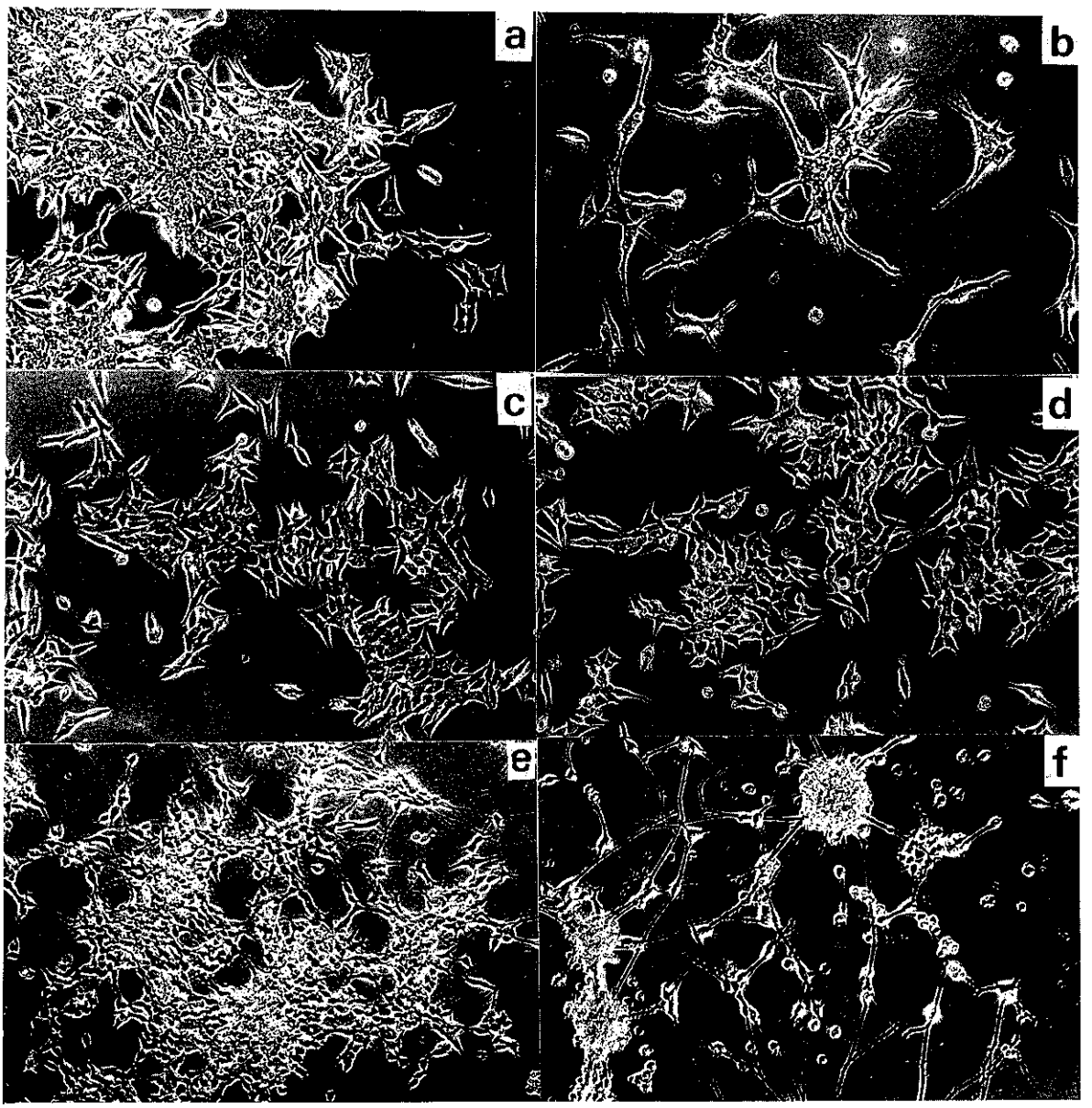

Fig. 1. Morphological changes of neuroblastoma cells treated with rHuIFN-γ. The neuroblastoma cells were treated for 6 days. Control GOTO cells (a), rHuIFN-γ (1,000 IU/ml) (b), cycloheximide (0.2 μg/ml) (c), cycloheximide (0.2 μg/ml) and rHuIFN-γ (1,000 IU/ml) (d), control KP-N-RT cells (e), rHuIFN-γ (1,000 IU/ml) (f).

ment of GOTO and KP-N-RT cells with rHuIFN-γ (more than 100 IU/ml) for 6 days. The morphological differentiation was manifested by the appearance of extension and bifurcation of neurites, the formation of multinucleated giant cells and the formation of neurite networks (Fig. 1). In contrast, rHuIFN-αA or β treatment for 6 days had no influence on the morphological differentiation even at 1,000 IU/ml (data not shown).

Effect of HuIFNs on cellular proliferation The growth inhibition of GOTO and KP-N-RT cells was examined 5 days after culture with various rHuIFNs. A dose-dependent growth-inhibition was observed following incubation of cells with HuIFNs. Cell growth was strongly inhibited 5 days after culture with 1,000 IU/ml of rHuIFN-β and -γ. On the other hand, 1,000 IU/ml of rHuIFN-αA showed about 50% growth inhibition (Fig. 2).

Effect of HuIFNs on overexpression of N-*myc* gene The N-*myc* gene expression was assessed by Northern blot analysis, performed by hybridizing N-*myc* DNA from pNb-1 with the total RNA from IFN-treated cells. A detectable inhibition in the levels of N-*myc* RNA was observed in rHuIFN-γ (1,000 IU/ml)-treated cells 3 to 6 days after treatment, whereas it was not observed in rHuIFN-αA or β (1,000 IU/ml)-treated cells 6 days after treatment (Fig. 3).

Effect of cycloheximide on morphological differentiation and expression of N-*myc* gene Treatment of GOTO cells with rHuIFN-γ in the presence of cycloheximide did not induce morphological differentiation or the suppression of N-*myc* gene expression (Figs. 1 and 4).

DISCUSSION

So far, several studies on IFN-induced morphological changes in neuroblastoma cells have been reported, but in those cases, only mouse IFN or crude HuIFN was used. We examined the induction of morphological change and the suppression of expression of N-*myc* gene on human neuroblastoma cells (GOTO and KP-N-RT) with highly purified recombinant HuIFNs (αA, β and γ), and have found that only HuIFN-γ could induce the morpholog-

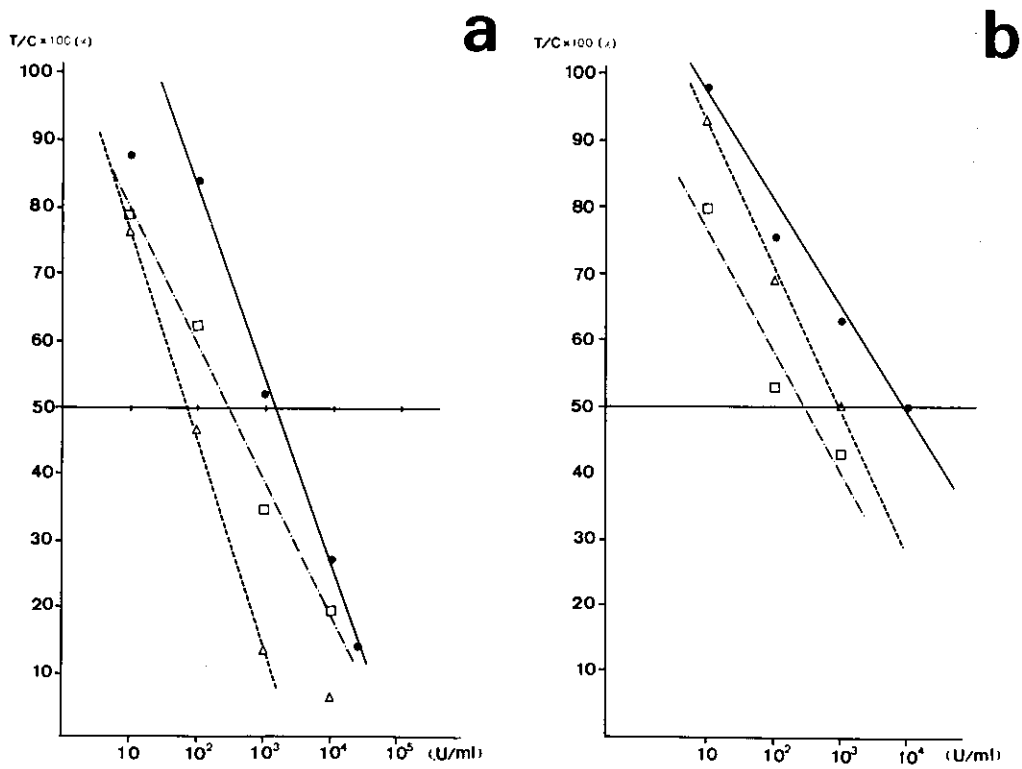

Fig. 2. Effect of HuIFNs on the growth of GOTO and KP-N-RT cells. Triplicate cultures were run for the cell growth experiment. The cells were treated with 0, 10, 100, 1,000 and 10,000 IU/ml HuIFNs for 5 days. The cell viability was determined by means of the trypan blue dye exclusion test. The vertical axis shows the ratio of the number of viable cells treated with IFNs to that of untreated cells. The horizontal axis shows the concentration of IFNs (IU/ml). ●, rHuIFN-αA; △, rHuIFN-β; □, rHuIFN-γ. (a) GOTO cells, (b) KP-N-RT cells.

ical changes and suppress the expression of N-*myc* gene.

In general, cell differentiation is related to the inhibition of cell growth, but we found that although both HuIFN-β and γ strongly inhibit the cell growth of neuroblastoma cells, only HuIFN-γ could induce morphological differentiation. The induction of morphological changes may be an intrinsic property of HuIFN-γ.

Recently, the expression of oncogenes and the development of malignant tumors have been suggested to be closely correlated. Moreover, IFN inhibited the synthesis of oncogene product[11] and the expression of oncogenes.[12-14] Kelly *et al.*[15] reported that IFN-γ strongly inhibited the cell growth of HeLa cells, but it enhanced the expression of c-*myc* oncogene.

In our study, HuIFN-γ inhibited both the cell growth and the expression of N-*myc* gene, but HuIFN-α and β influenced neither the expression of N-*myc* gene nor the induction of morphological differentiation. The suppression of N-*myc* gene expression and the induction of morphological differentiation may be closely related.

Moreover, the level of N-*myc* DNA was not changed during differentiation (data not shown). This indicates that HuIFN-γ suppressed the expression of N-*myc* gene at the transcriptional level. When GOTO cells were treated with HuIFN-γ and cycloheximide, morphological differentiation was not induced and the N-*myc* gene expression was not suppressed. The induction of morphological differentiation and the suppression of N-*myc* gene expression by HuIFN-γ may be related to the proteins induced by HuIFN-γ.

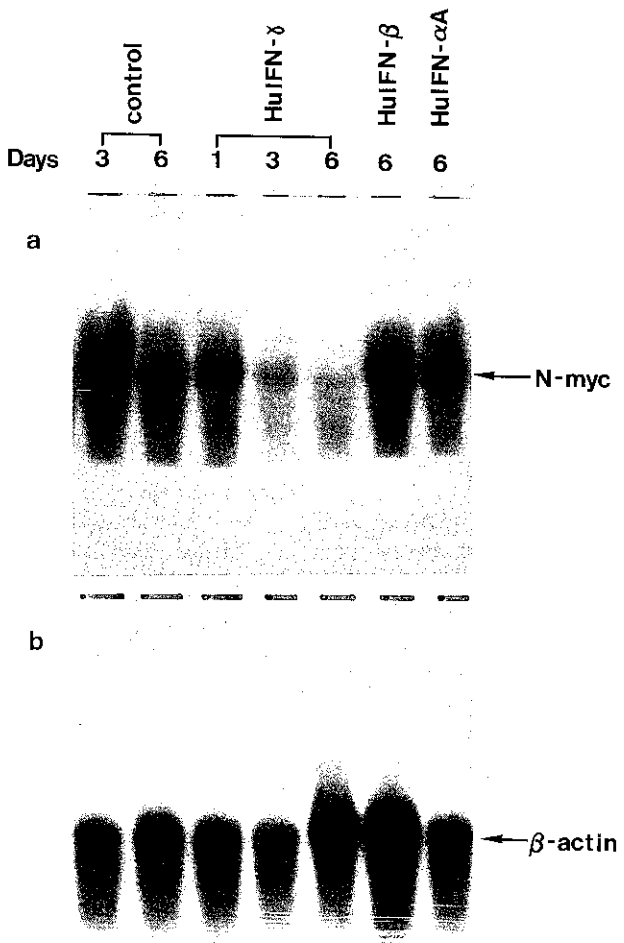

Fig. 3. Suppression of N-*myc* RNA in GOTO cells by rHuIFN-γ. The expression of N-*myc* gene was measured by Northern blotting as described in "Materials and Methods." Briefly, total RNA was extracted from GOTO cells treated with HuIFNs (1000 IU/ml) for 1, 3 or 6 days. The RNA was electrophoresed on a formaldehyde agarose gel and transferred to nylon membrane. Then the RNA was first hybridized with the ^{32}P-labeled N-*myc* DNA probe (a) and autoradiographed. After the first autoradiography, the same filter was rehybridized with ^{32}P-labeled β-actin DNA probe (b).

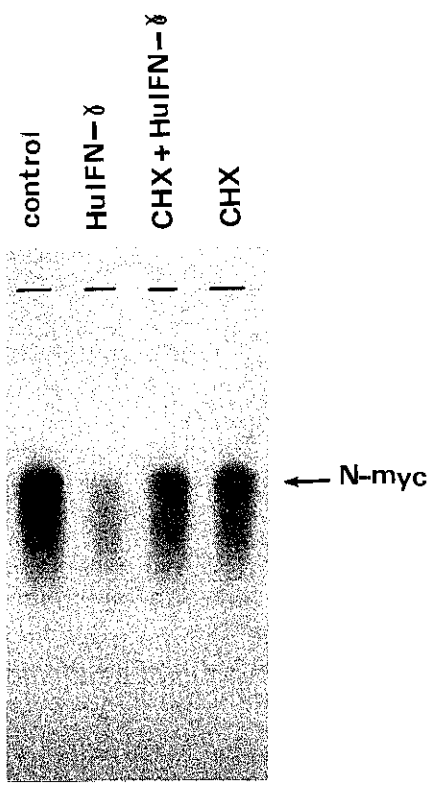

Fig. 4. Effect of cycloheximide on the suppression of expression of N-*myc* gene in GOTO cells treated with rHuIFN-γ. rHuIFN-γ (1,000 IU/ml) or CHX (cycloheximide, 0.2 μg/ml) was added alone or in combination to GOTO cell culture and incubated for 6 days. Then, RNA was extracted and analyzed on Northern blots as in Fig. 3.

Schwab *et al.*[10] and Grady-Leopardi *et al.*[16] reported that the N-*myc* gene expression is related to malignancy in tumor development, and that neuroblastoma with overexpression of N-*myc* gene has a poor prognosis. Although both HuIFN-β and HuIFN-γ have similarly strong growth-inhibitory effects assayed by trypan-blue dye exclusion assay, our results suggest that HuIFN-γ might be a possible therapeutic agent for neuroblastoma.

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