Acta Neuropathol (1989) 79:217–221

Acta Neuropathologica

Expression of proto-oncogene products during drug-induced differentiation of a neuroblastoma cell line SK-N-DZ*

M. Matsumoto[1], T. Akiyama[2], S. Miyatake[1], Y. Oda[1], H. Kikuchi[1], M. Hanaoka[1], and Y. Namba[2]

[1] Department of Neurosurgery, Kyoto University Medical School, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606, Japan
[2] Department of Pathology, Institute for Virus Research, Kyoto University, Kyoto, Japan

Summary. Four human neuroblastoma cell lines exhibited differences in their ability to differentiate into neuron-like cells in response to three different treatments, serum deprivation, or additions of dibutyryl cyclic-AMP or retinoic acid. Expression of N-myc gene product was reduced in neuroblastoma cell line SK-N-DZ differentiated by retinoic acid as compared with untreated cells. On the contrary, expression of c-src gene product, $pp60^{c\text{-}src}$, was considerably enhanced in differentiated SK-N-DZ cells. Tyrosine phosphorylation of several cellular proteins was found to be enhanced in differentiated cells. Alteration in expression of these proto-oncogene products might be important in the differentiation of neuroblastoma cells into neuron-like cells.

Key words: Neuroblastoma – Differentiation – Retinoic acid – Proto-oncogene – Tyrosine phosphorylation

Several lines of evidence implicate certain proto-oncogenes, normal cellular homologs of retroviral oncogenes, as genes that might regulate cellular differentiation. One such proto-oncogene is c-src, the cellular homolog of Rous sarcoma virus (RSV) transforming gene v-src [7]. This proto-oncogene is highly expressed in post-mitotic neurons [3]. In addition, neurons contain an additional form of $pp60^{c\text{-}src}$ which is structurally distinct from the form found in astrocytes and fibroblasts [16]. The difference involves an altered pattern of phosphorylation and the insertion of six amino acids in the N-terminal half of the molecule [6]. Moreover, rat pheochromocytoma cells can be differentiated into neuronal cells when infected with RSV [1]. Thus, $pp60^{c\text{-}src}$ seems to be closely related to the neuronal differentiation processes. N-myc gene is highly expressed in murine fetal tissues [17] and is also thought to be related to cell growth and differentiation. This gene is known to be frequently amplified in both primary tissues and human neuroblastoma cell lines [11].

In this study we examined the expression of the N-myc gene product and the c-src gene product, $pp60^{c\text{-}src}$, during in vitro differentiation of human neuroblastoma cell line SK-N-DZ. To induce differentiation, serum deprivation, or treatment with dibutyryl cyclic-adenosine 3′,5′-monophosphate (Bt_2cAMP) or retinoic acid were used, and neurite extension from treated cells was assessed for the determination of differentiation.

Materials and methods

Cell lines and culture conditions

Retinoic acid and Bt_2cAMP were purchased from Sigma. Retinoic acid and Bt_2cAMP were used at 0.5 μM and 1 μM, respectively. Human neuroblastoma cell lines SK-N-DZ [12], GOTO, NB-1 and IMR-32 were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum. Human glioblastoma cell line T98G and RSV-transformed rat fibroblast SRA-3Y1 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Determination of differentiation

Cells (3×10^5) were seeded in 35-mm dish and were grown under serum deprivation or in the presence of retinoic acid or Bt_2cAMP for 4 to 6 days at 37°C. Differentiation was assessed by the elongation of neurites as a morphological change (at least twice as long as the soma diameter). The degree of differentiation was expressed as a percentage of the cells having elongated neurites to the total number of the cells.

Immunoprecipitation

For immunoprecipitation with anti-$pp60^{c\text{-}src}$ antibody, cells were labeled for 4 h in methionine-free RPMI 1640 medium sup-

* Supported in part by Grants-in-aid for Cancer Research (63015039, 63-16) from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare of Japan

Offprint requests to: M. Matsumoto (address see above)

plemented with 5% fetal calf serum and ^{35}S-methionine (400 μCi/ml, Amersham). For immunoprecipitation with anti-phosphotyrosine antibody, cells were labeled for 2 h with ^{32}P-orthophosphate (250 μCi/ml, Amersham) in phosphate-free medium. Cells were lysed in RIPA buffer [40 mM Hepes(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)-NaOH, pH 7.4, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 0.15 M NaCl, 1 mM phenylmethylsulfonyl fluoride]. Aliquots (150 μl) of the lysates were incubated with anti-pp60$^{c\text{-}src}$ antibody or with anti-phosphotyrosine antibody for 1 h on ice. The immunocomplexes were adsorbed to protein A-Sepharose 4B (Pharmacia) and washed extensively with RIPA buffer. The immunoprecipitates were subjected to 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography.

Western blot analysis

The immunoprecipitates prepared as described above were subjected to 10% SDS-PAGE. The proteins were then transferred onto a nitrocellulose membrane for 2 h at 240 mA in a solution containing 25 mM Tris (pH 8.3), 200 mM glycine, and 20% methanol. For detection of the N-myc gene product, the membrane was incubated with anti-N-myc monoclonal antibody (×100 diluted) for 1 h at 4 °C and washed extensively in Tris-buffered solution (TBS). Then, the filter was incubated with peroxidase-labeled rabbit anti-sheep immunoglobulin G (×280 diluted) for 1 h at 4 °C. After being washed extensively, the membrane was reacted with chromogen buffer solution [0.5 mg/ml of 3,3′-diaminobenzidine HCl (Sigma), 0.05% H_2O_2, and TBS] for about 15 min at room temperature.

Results

Induction of morphological differentiation

Four human neuroblastoma cell lines were examined for their ability to differentiate into neuron-like cells in response to serum deprivation or treatment with Bt_2cAMP or retinoic acid. As summarized in Table 1, each cell line showed a different response to induction by these three methods. Morphological differentiation of IMR-32 and GOTO cells could be induced by serum deprivation, while these two cell lines responded poorly to retinoic acid treatment (Fig. 1 C, D). By contrast, the morphological differentiation of SK-N-DZ cells was efficiently induced under the influence of retinoic acid and showed mild induction with serum deprivation. In the presence of Bt_2cAMP, only SK-N-DZ cells showed differentiated morphologies. The typical morphologies of the retinoic acid-treated SK-N-DZ cells are shown in Fig. 1 A, B. While untreated SK-N-DZ cells were spindle-shaped soma, differentiated SK-N-DZ cells formed extensive networks of neurites and aggregated into clusters. Neurite formation was most evident 6 days after treatment.

Expression of the proto-oncogene products

Since SK-N-DZ cells were most efficiently induced into differentiation by retinoic acid treatment, this system was used for the analysis of proto-oncogene product expression.

First, expression of the N-myc gene product was examined by Western blot analysis. As shown in Fig. 2, expression of the N-myc product was reduced in differentiated SK-N-DZ cells as compared with undifferentiated cells.

We next examined the expression of pp60$^{c\text{-}src}$, which was high in differentiated SK-N-DZ cells as compared with that in untreated cells (Fig. 3).

Lastly, we analyzed tyrosine phosphorylation of cellular proteins in differentiated SK-N-DZ cells under retinoic acid treatment; phosphotyrosine-containing proteins were immunoprecipitated from the lysates of ^{32}P-labeled cells using antibodies which specifically recognized phosphotyrosine. Phosphorylated proteins were immunoprecipitated in both treated and untreated cells, while the intensity of phosphorylation of the proteins in treated cells was significantly enhanced as compared to those precipitated from untreated cells (Fig. 4).

Table 1. Morphological changes in human neuroblastoma cell lines, GOTO, NB-1, IMR-32, SK-N-DZ and human glioblastoma cell line T98G induced by serum deprivation, and additions of dibutyryl cyclic adenosine 3′5′-monophosphate (Bt_2cAMP; 0.5 μM) and retinoic acid (1 μM)

	Serum (−)	Bt_2cAMP	Retinoic acid
GOTO	++	−	+
NB-1	+	−	−
IMR-32	++	−	−
SK-N-DZ	+	++	+++
T98G	−	−	−

Elongation of neurites: −, none; +, slight (10% − 30%); ++, moderate (30% − 50%); +++, marked (50% − 80%)

Discussion

Human neuroblastoma, a tumor of the neuronal crest origin, is usually highly malignant in its clinical behavior in children, however some cases had been reported to mature into ganglioneuroma, a benign tumor, spontaneously or in response to treatment [4]. In our study, human neuroblastoma cell lines were found to exhibit differences in their abilities to differentiate into neuron-like cells in response to three different treatments: serum deprivation or addition of retinoic acid or Bt_2cAMP.

It has recently been reported that thrombin contained in fetal calf serum, as a supplement in culture medium, modulates the neurite extension of neuroblastoma cells [5]. Hence, serum deprivation is followed by a reduction in regulation by thrombin. Bt_2cAMP easily passes through cell membranes and

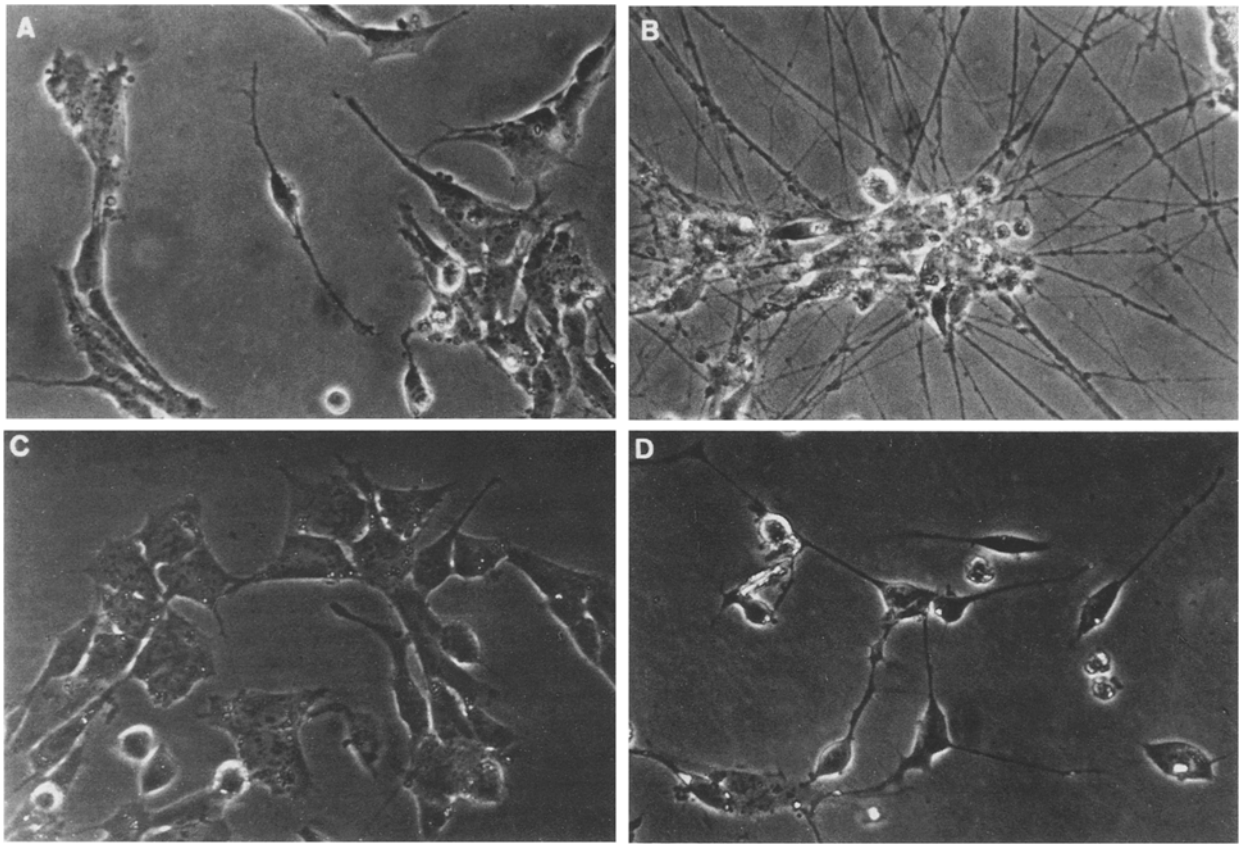

Fig. 1 A – D. Morphological alteration of neuroblastoma cells upon differentiation. **A** SK-N-DZ cells; **B** SK-N-DZ cells treated with 0.5 μM retinoic acid for 5 days; **C** GOTO cells; **D** GOTO cells treated with serum deprivation for 2 days. **A – D** ×125

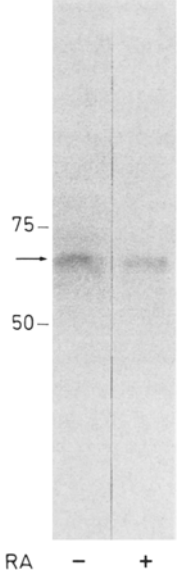

Fig. 2. Expression of N-myc gene product in retinoic acid-treated or untreated SK-N-DZ cells by Western blot analysis. Immunoprecipitates were electrophorased on 10% sodium dodecyl sulfate-polyacrylamide gel, transferred onto nitrocellulose membranes, and analyzed with anti-N-myc monoclonal antibody. *Lanes:* (–) SK-N-DZ cells; *RA* SK-N-DZ cells treated with 0.5 μM retinoic acid for 4 days

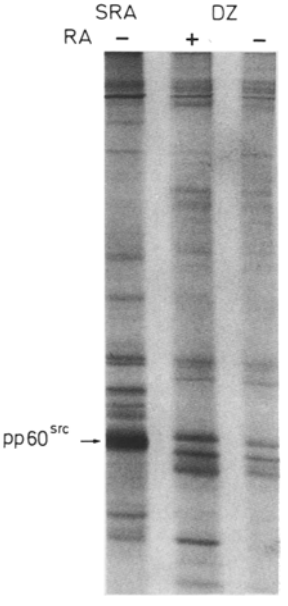

Fig. 3. Expression of $pp60^{c\text{-}src}$ in retinoic acid-treated or untreated SK-N-DZ cells by immunoprecipitation. *Lanes: DZ* (–) SK-N-DZ cells; *DZ*(+) SK-N-DZ cells treated with 0.5 μM retinoic acid for 5 days; *SRA* (–) Rous sarcoma virus-transformed rat 3Y1 cells

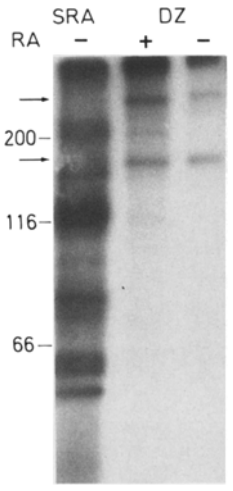

Fig. 4. Expression of tyrosine-phosphorylated proteins in retinoic acid-treated or untreated SK-N-DZ cells by immunoprecipitation. Lanes: DZ (−) SK-N-DZ cells; *DZ*(+) SK-N-DZ cells treated with 0.5 μM retinoic acid for 5 days; *SRA* (−) Rous sarcoma virus-transformed rat 3Y1 cells

activates cAMP-dependent protein kinase activity [10]. On the other hand, it has also been reported that the retinoid receptors of cells might play an initial role in the action of retinoic acid within the cells [9]. Cell line-specific differences in signal transduction machinery might cause differences in the abilities of cells to differentiate into neuron-like cells. Previous studies have suggested that proto-oncogenes, such as N-myc, c-myb and c-src, are important for the growth and differentiation of neuroblastoma cell lines [8, 14, 15]. Furthermore, the decreased expression of the N-myc gene preceded retinoic acid-induced morphological differentiation of human neuroblastoma cells [13]. In this study, we have also found decreased expression of the N-myc gene product during retinoic acid-induced differentiation of neuroblastoma cell line SK-N-DZ. On the contrary, $pp60^{c\text{-}src}$ was reported to be enhanced in retinoic acid-treated embryonal carcinoma cells [7], and $pp60^{c\text{-}src}$ tyrosine kinase has been reported to be activated in 12-*O*-tetradecanoyphorbol-13-acetate-treated human promyelocytic leukemia HL-60 cells [2]. We also detected the increased expression of $pp60^{c\text{-}src}$ during induced differentiation of neuroblastoma cell line SK-N-DZ. In retinoic acid-treated SK-N-DZ cells, which showed an extensive network of neurites, we observed an increased expression of $pp60^{c\text{-}src}$ and enhanced tyrosine phosphorylation of cellular proteins. This increased phosphotyrosine-content of proteins suggests that they are substrates for $pp60^{c\text{-}src}$, but it is also possible that they are phosphorylated by other tyrosine kinases. Of particular interest is the fact that different species of protein are tyrosine-phosphorylated in these differentiated cells as compared with those phosphorylated in RSV-transformed rat 3Y1 cells. It is interesting to speculate that differentiation-specific phosphorylation of these substrate proteins might play an important role in the events that take place in differentiated cells.

In conclusion, our study suggests that N-myc and c-src gene products may be important markers for differentiation in neuroblastoma cell lines and that the c-src gene might play a specific role via its tyrosine kinase activity in differentiation.

Acknowledgements. We thank Dr. Junkoh Yamashita and Dr. Toshiki Yamasaki for their advice.

References

1. Alema S, Casalbore P, Agostini E, Tato E (1985) Differentiation of PC12 phaeochromocytoma cells induced by v-src oncogene. Nature 316:557–559
2. Barnekow A, Gessler M (1986) Activation of the $pp60^{c\text{-}src}$ kinase during differentiation of monomyelocytic cells in vitro. EMBO J 5:701–705
3. Brugge JS, Cotton PC, Queral AE, Barrett JN, Nonner D, Keane RW (1985) Neurons express high levels of a structurally modified, activated form of $pp60^{c\text{-}src}$. Nature 316:554–557
4. Evans AE, Baum E, Chord R (1981) Do infants with stage IV-S neuroblastoma need treatment? Arch Dis Child 56: 271–274
5. Gurwitz D, Cunningham DD (1988) Thrombin modulates and reverses neuroblastoma neurite outgrowth. Proc Natl Acad Sci USA 85:3440–3444
6. Levy JB, Dorai T, Wang LH, Brugge JS (1987) The structurally distinct form of $pp60^{c\text{-}src}$ detected in neuronal cells is encoded by a unique c-src mRNA. Mol Cell Biol 7:4142–4145
7. Lynch SA, Brugge JS, Levine JM (1986) Induction of altered c-src product during neural differentiation of embryonal carcinoma cells. Science 234:873–876
8. Mellstroem K, Bjelfman C, Hammerling U, Pahlman S (1987) Expression of c-src in cultured human neuroblastoma and small cell lung carcinoma cell lines correlates with neurocrine differentiation. Mol Cell Biol 7:4178–4184
9. Petkovich M, Brand NJ, Krust A, Chambon P (1987) A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 30:444–450
10. Rupniak HT, Rein G, Powell JP, Ryder TA, Carson S, Povey S, Hill BT (1984) Characteristics of a new human neuroblastoma cell line which differentiates in response to cyclic adenosine 3′:5′-monophosphate. Cancer Res 44: 2500–2607
11. Schwab M, Ellison J, Busch M, Rosenau W, Varmus HE, Bishop JM (1984) Enhanced expression of the human gene N-myc consequent to amplification of DNA may contribute to malignant progression of neuroblastoma. Proc Natl Acad Sci USA 81:4940–4944
12. Sugimoto T, Tatsumi E, Kemshed JT, Helson, L, Green AA, Minowada J (1984) Determination of cell surface membrane antigens common to both human neuroblastoma and leukemia-lymphoma cell lines by a panel of 38 monoclonal antibodies. J Natl Cancer Inst 73:51–57

13. Thiele CJ, Reynolds J, Israel MA (1985) Decreased expression of N-myc precedes retinoic acid-induced morphological differentiation of human neuroblastoma. Nature 313:404–406
14. Thiele CJ, McKeon C, Trische R, Ross R, Reynolds C, Israel MA (1987) Differential protooncogene expression characterizes histopathologically indistinguishable tumors of the peripheral nervous system. J Clin Invest 80:804–811
15. Thiele CJ, Cohen PS, Israel MA (1988) Regulation of c-myb expression in human neuroblastoma cells during retinoic acid-induced differentiation. Mol Cell Biol 8:1677–1683
16. Wiestler OD, Walter G (1988) Developmental expression of two forms of $pp60^{c\text{-}src}$ in mouse brain. Mol Cell Biol 8:502–504
17. Zimmerman KA, Yancopoulos GD, Colum RG, Smith RK, Kohl NE, Denis KA, Nau MM, Witte ON, Allerand DT, Gee CE, Minna JD, Alt FW (1986) Differential expression of myc family genes during murine development. Nature 319:780–783

Received April 25, 1989/Revised, accepted June 27, 1989