Malignant rhabdoid tumor shows a unique neural differentiation as distinct from neuroblastoma

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Malignant rhabdoid tumors (MRT) show a multiphenotypic diversity, including a neural phenotype. To elucidate the difference in neural characteristics between MRT and neuroblastoma, we examined the expression of synapsin I, neuron-restrictive silencer factor (NRSF), neurofilament medium-size (NF-M) and chromogranin A (CGA) in five MRT cell lines (TM87-16, STM91-01, TTC549, TTC642 and YAM-RTK1) and five neuroblastoma cell lines under differentiation-induction with 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Our results showed TM87-16 and TTC642 cells, expressed synapsin I and NF-M before TPA induction, had a neural phenotype. After differentiation-induction, only TM87-16 cells expressed CGA. Among all neuroblastoma cells, expression of NF-M and CGA was stable at a high level throughout TPA-induced differentiation. In TM87-16 and TTC642 MRT cells, synapsin I mRNA promptly increased after TPA differentiation, with the peak level at 6 h, and thereafter, synapsin I mRNA rapidly decreased in a time-dependent manner. The decreased expression of synapsin I correlated with an increased expression of NRSF during differentiation-induction. In contrast, in some neuroblastoma cells, a significant up-regulation of synapsin I was observed concurrently with a down-regulation of NRSF. The inverse relationship between NRSF and synapsin I expression in TM87-16 and TTC642 MRT cells was opposite to that of neuroblastoma cells. Our results showed that the neural characteristics of these MRT cells are fairly distinct from those of neuroblastoma cells. These MRT cells appeared to have only limited capability for neural differentiation, and were still in an extremely early stage of neural differentiation. (Cancer Sci 2003; 94: 37–42)

Malignant rhabdoid tumor (MRT) is a rare, highly aggressive neoplasm specific to early childhood. MRT has an extremely poor prognosis due to a high potential for distant metastases.[1, 2] The primary sites of MRT vary greatly. A characteristic feature of MRT cells is the presence of a large eosinophilic inclusion in the cytoplasm.[3, 4] Various cellular origins have been proposed for MRT, including neuroectodermal,[3, 5] myogenic,[6, 7] histiocytic[8] and epithelial.[9–11] Recent studies of established MRT cell lines have reported multiphenotypic characteristics.[4, 12–16] Our laboratory has previously reported that certain MRT cell lines respond to various inducers of differentiation, including 12-*O*-tetradecanoylphorbol-13-acetate (TPA).[4, 16–18] Some MRT cells actively produce elongated neuritic cytoplasmic processes with multiple varicosities after treatment with some inducers.[4, 16–18] These MRT cell lines have a potential for neural differentiation and express various neural markers.[12, 16–18] However, the biological characteristics for neural differentiation of these MRT cells, possessing a neural phenotype, remain unknown.

Neuron-restrictive silencer factor (NRSF) is a silencer protein that represses a subset of neuron-specific genes in non-neural cells as well as undifferentiated neural precursors,[19–21] and suppresses the expression of neuron-specific genes such as *synapsin I*.[20, 22–24] *Synapsin I* itself is one of the established neuron-specific genes, and synaptogenesis markers.[23, 25–30] However, NRSF expression in neural cell lines is still a matter of controversy.[31, 32]

Therefore, to elucidate the neural characteristics of MRT cells, we examined the expression of synapsin I, a well known target of NRSF regulation, in conjunction with NRSF expression and expression of other neural markers (neurofilament medium-size (NF-M) and chromogranin A (CGA)) in five distinct MRT cell lines following differentiation-induction with TPA. Several methods of analyses, including reverse transcriptase-polymerase chain reaction (RT-PCR), *in situ* hybridization and immunocytochemistry, were applied in this study. We also compared the differences of the biological characteristics for neural differentiation between MRT cell lines and neuroblastoma (a well-known malignant solid tumor of neural crest origin, possessing a neural phenotype, in childhood) as models of neural differentiation in tumor cells.

Materials and Methods

Cell culture and differentiation. Five MRT cell lines and five neuroblastoma cell lines were used in this study. Four of the MRT cell lines (TM87-16, STM91-01, TTC549 and TTC642) were kindly provided by Drs. Hiroyuki Shimada and Timothy J. Triche (Childrens Hospital Los Angeles, Los Angeles, CA). The MRT cell line YAM-RTK1 was provided by Dr. Kanji Sugita (Yamanashi University, Kofu). Patients whose tumors were used to establish the cell lines had been diagnosed to have MRT in each hospital on the basis of histological studies. All MRT cells showed MRT-typical eosinophilic cytoplasmic inclusions, and electron microscopically, whorls of intermediate filaments were confirmed.[4] Cell lines were established from resected or biopsied specimens from patients. STM91-01 was established from a pulmonary metastasis of a renal MRT,[4] TM87-16 from a pleural effusion[4] and YAM-RTK1 from ascites. TTC549 and TTC642 were established from extra-renal primary tumors. The five neuroblastoma cell lines (IMR-32, NB-1, NH-12, SCCH26 and TGW) were obtained from the Health Science Research Resources Bank (Osaka). The MRT and neuroblastoma cell lines were from the 16th to 23rd passages. The cells were cultured in RPMI-1640 (Gibco BRL, Rockville, MD) supplemented with 10% fetal bovine serum (FBS: ICN Biomedicals, Inc., Costa Mesa, CA). Cellular differentiation was induced in each cell line using TPA (100 n*M*; Sigma Chemical Co., St. Louis, MO) for 8 consecutive days. The cell lines were analyzed for expression of neural marker mRNAs before and after induction with TPA. All cells were rinsed and fixed with 4% paraformaldehyde for light microscopic examination.

RNA preparation and RT-PCR. Total RNA from each cell line was isolated with TRIzol reagent (Gibco). After priming of 5 μg of total RNA with random hexadeoxynucleotide primers (TaKaRa Shuzo Co., Ltd., Shiga), reverse transcription (RT) was performed at 42°C using MMLV Reverse Transcriptase RnaseH (ReverTra Ace, Toyobo Co., Ltd., Osaka). The diluted RT solution was used as a template for each polymerase chain reaction (PCR).

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PCR primers. Forward primers (f) and reverse primers (r) were designed according to published sequence data for synapsin I,[29] NRSF,[20] NF-M[33] and CGA.[34] Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[35] served as an internal standard. All primers were obtained from Pharmacia (Tokyo) (Table 1).

PCR reaction. All PCR amplifications were carried out using *Taq*DNA polymerase (Toyobo) for 30 cycles under the following conditions; denaturing at 94°C for 60 s, annealing at different temperatures (see Table 1), and extension at 72°C for 60 s. Aliquots of PCR reaction products were electrophoresed through 2% agarose gels (Nacalai Tesque, Kyoto) containing 0.2 mg/ml ethidium bromide (Sigma). The relative quantity of expressed gene was measured by simultaneous PCR amplification of GAPDH and the target gene.

Sequencing of PCR products. PCR-amplified products were directly sequenced on an ABI Prism 310 Genetic Analyzer using a "DYEnamic" ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Inc., Piscataway, NJ).

Competitive PCR. cDNA templates were made using a competitive DNA construction kit (TaKaRa Shuzo). Analyses were routinely performed with fixed quantities of target templates and different quantities of competitor templates. Aliquots of PCR products (10 μl) were electrophoresed in 2% agarose gels. The amount of each DNA fragment was measured by quantifying the staining intensity using an AIC Epi-Light UV FA1100 (Aisin Cosmos R&D Co., Ltd, Tokyo) and the accompanying Luminous Imager software (Aisin Cosmos). The density of each band representing amplified product from both the sample and competitor was measured. Then the density ratios (sample/competitor) were calculated and normalized relative to GAPDH.

***In situ* hybridization.** The RT-PCR products of synapsin I and NRSF were cloned into the pGEM-4Z vector (Promega, Madison, WI) in two directions to obtain sense and antisense probes. These recombinant plasmids were linearized, and *in vitro* transcription was carried out in the presence of digoxigenin (DIG)-uridine 5′-triphosphate, using the T7/SP6 RNA labeling kit (Boehringer Mannheim, Mannheim, Germany). The *in situ* hybridization was performed as described in the literature.[18]

Immunocytochemistry. Cells were rinsed and fixed with 4% paraformaldehyde, 0.5% glutaraldehyde, and 0.2% picric acid in 0.1 *M* phosphate-buffered saline (PBS, pH 7.4). Primary antibodies (with their optimal dilutions) used for immunostaining were directed against the following: synapsin I (1:100, Oncogene Research Products, Cambridge, MA), NF-M (1:100, Cosmo Bio, Tokyo), and CGA (1:100, Cosmo Bio). Slides were then incubated with peroxidase-conjugated anti-mouse immunoglobulin (Envision+; Dako Co., Carpinteria, CA) for 30 min. The reaction products were visualized with 0.05% Ni-diaminobenzidine solution containing 0.01% hydrogen peroxide for 5 min at room temperature.

Table 1. PCR oligonucleotide primer sequences, PCR products and annealing temperatures

Gene	Primer (5′-3′) forward/ reverse	Product (bp)	Annealing temperature (°C)
NRSF	CCCTTTGGCACTTCCTGACT GCATCCTACTTTGTCCTAAT	451	56
Synapsin I	CCTCCATTCTGTTCCCATCA CACCACCCCATCCGCATCTC	256	58
NF-M	AGTGAGGAGGAAGGGAGTGA AGTGACGGTTACAGATTTAG	294	55
CGA	TGTCCTGGCTCTTCTGCTCT TGTTTCTTCTGCTGATGTGC	276	56
GAPDH	GCCAAAAGGGTCAT-CATCTCTG CATGCCAGTGAGCTTCCCGT	348	52

PCR, polymerase chain reaction; NRSF, neuron-restrictive silencer factor; NF-M, neurofilament medium-size; CGA, chromogranin A; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Results

Morphological study (light microscopic findings). Under observation with a light microscope, the STM91-01, TTC549 and YAM-RTK1 MRT cell lines showed no significant morphological changes before or after treatment with TPA (data not shown). For the TM87-16 and TTC642 cell lines, the MRT cells exhibited a round appearance before induction (Fig. 1A), and actively produced elongated neuritic cytoplasmic processes with multiple varicosities after differentiation-induction with TPA (Fig. 1B). As expected, all five neuroblastoma cell lines exhibited elongated cytoplasmic processes with multiple varicosities after induction with TPA (Fig. 1, C and D).

RT-PCR study of the neural genes. NF-M mRNA was expressed only in the TM87-16 and TTC642 MRT cell lines before treatment with TPA, and the expression level of NF-M mRNA did not change throughout differentiation-induction with TPA. After induction with TPA, *de novo* expression of CGA mRNA was detected only in the TM87-16 cell line. In contrast, NF-M and CGA mRNAs were detected in all the neuroblastoma cell lines. NF-M and CGA mRNAs were stably expressed at a high level during TPA-induced differentiation (Table 2).

Competitive RT-PCR study of synapsin I and NRSF. Before treatment with TPA, synapsin I mRNA expression was found only in the TM87-16 and TTC642 MRT cells. In these two MRT cell lines, the cells co-expressed a low level of NRSF (Fig. 2A). In contrast, in the neuroblastoma cell lines, NRSF and synapsin I mRNAs expressions were varied. High concentrations of NRSF were found in NB-1 and TGW cell lines, while low expression of NRSF was seen in IMR-32 and NH-12 cell lines. IMR-32 and NH-12 cells co-expressed NRSF and synapsin I mRNAs (Fig. 2B).

Following differentiation-induction with TPA, there was no new expression of synapsin I mRNA in the STM91-01,

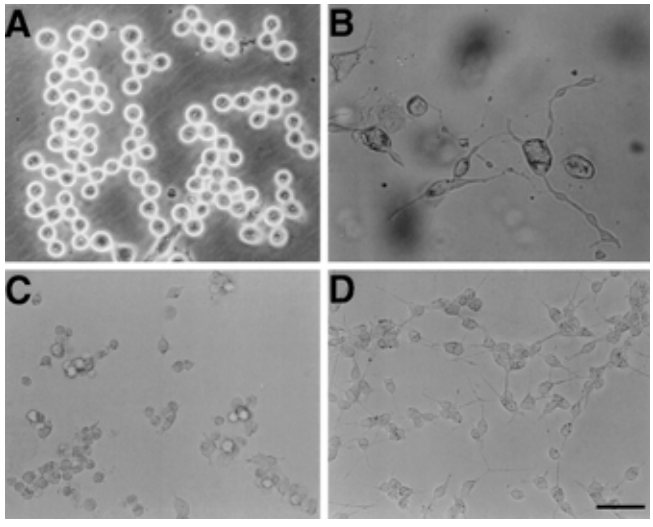

Fig. 1. Light microscopic findings on malignant rhabdoid tumor (MRT, TM87-16) cells and neuroblastoma (NB-1) cells (original magnification ×200 for A–D, bar=30 μm). (A) TM87-16 cells before treatment with TPA. (B) TM87-16 cells, demonstrating *de novo* propagation of elongated cytoplasmic processes, after differentiation-induction with TPA. (C) NB-1 cells, exhibiting small round cell appearance, before treatment with TPA. (D) NB-1 cells after differentiation-induction with TPA.

TTC549 and YAM-RTK1 cell lines. However, in TM87-16 and TTC642 MRT cells, we observed an increase in NRSF mRNA level concurrently with a marked decrease in synapsin I mRNA (Fig. 3A). Interestingly, these two MRT cell lines were differentiated in a very short time in the presence of TPA. Synapsin I mRNA was promptly increased after TPA differentiation, with the peak level at 6 h. Thereafter, synapsin I mRNA was rapidly decreased in a time-dependent manner (Fig. 3B). The NRSF mRNA level became stable in a short time after TPA-differentiation (data not shown). In contrast, expression of NRSF mRNA was significantly down-regulated in four out of five neuroblastoma cell lines (IMR-32, NB-1, NH-12 and TGW) after differentiation-induction with TPA. Synapsin I mRNA was significantly up-regulated in IMR-32 and NH-12, and *de novo* expression of synapsin I mRNA was detected in NB-1 and TGW (Fig. 4, A and B).

The results of the RT-PCR gene expression studies are summarized in Table 2.

***In situ* hybridization.** The hybridization signals for synapsin I mRNA were detected in the cytoplasm and on the cell membrane in TM87-16 and TTC642 MRT cells before treatment with TPA; however, these signals were rapidly down-regulated after induction of differentiation (Fig. 5, A and B). In contrast, in the NB-1 and TGW neuroblastoma cell lines, no signal for synapsin I mRNA was found before treatment of TPA, though an increased level of synapsin I mRNA was detected in the cytoplasm after differentiation-induction (Fig. 5, C and D).

Immunocytochemistry. TM87-16 and TTC642 MRT cells showed positive staining for the synapsin I protein in the cytoplasm and on the cell membrane before treatment with TPA, but the staining decreased and disappeared after induction with TPA (Fig. 6, A and B). In contrast, two neuroblastoma cell lines, NB-1 and TGW, showed negative staining for synapsin I protein before treatment with TPA, then positive staining for synapsin I protein appeared and increased after induction with TPA (Fig. 6, C and D).

Results for other markers analyzed by *in situ* hybridization and immunocytochemical studies corresponded to those of the mRNA expression detected by RT-PCR studies in all cell lines (see Table 2).

Discussion

The expression pattern of NRSF, also known as repressor ele-

Table 2. Summary of expression of the neural genes in the five MRT cell lines and five neuroblastoma cell lines before and after treatment with TPA

Cell lines	Before induction with TPA				After induction with TPA			
MRT	*NRSF*	*Synapsin I*	*NF-M*	*CGA*	*NRSF*	*Synapsin I*	*NF-M*	*CGA*
TM87-16	+	+	+	–	↑	↓	→	↑*
STM91-01	++	–	–	–	↑	–	–	–
TTC549	++	–	–	–	↑	–	–	–
TTC642	+	+	+	–	↑	↓	→	–
YAM-RTK1	++	–	–	–	↑	–	–	–
Neuroblastoma	*NRSF*	*Synapsin I*	*NF-M*	*CGA*	*NRSF*	*Synapsin I*	*NF-M*	*CGA*
IMR-32	+	+	+	+	↓	↑	↑	↑
NB-1	++	–	+	+	↓	↑*	↑	↑
NH-12	+	+	+	+	↓	↑	↑	↑
SCCH26	–	++	+	+	–	→	↑	↑
TGW	++	–	+	+	↓	↑*	↑	↑

MRT, malignant rhabdoid tumor; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; NRSF, neuron-restrictive silencer factor; NF-M, neurofilament medium-size; CGA, chromogranin A; +, expressed; ++, over-expressed; –, not expressed; →, no change; ↑, up-regulated; ↓, down-regulated; *, expressed only after TPA induction.

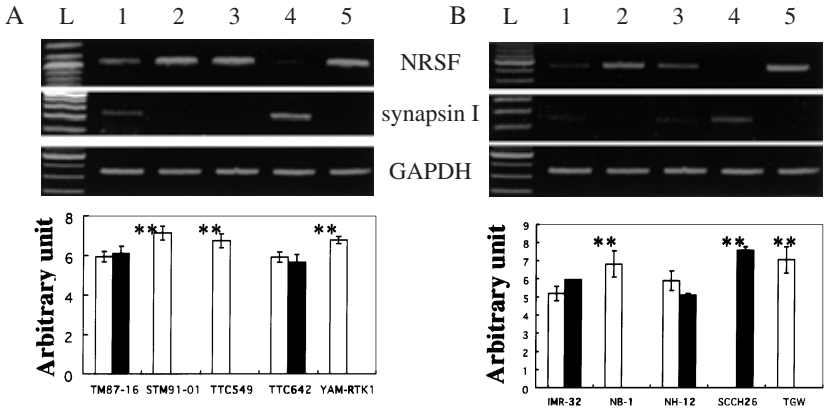

Fig. 2. Competitive RT-PCR analyses on NRSF and synapsin I mRNA expression before treatment with TPA in MRT cell lines (A) and neuroblastoma cell lines (B). (A) TM87-16 and TTC642 cell lines co-expressed a low level of NRSF and synapsin I mRNAs. ** $P<0.005$, relative expression of NRSF mRNA in STM91-01, TTC549 or YAM-RTK1 cells versus TM87-16. Lane L, 100 bp DNA ladder; lane 1, TM87-16; lane 2, STM91-01; lane 3, TTC549; lane 4, TTC642; lane 5, YAM-RTK1. □ NRSF, ■ synapsin I. (B) The expression level of NRSF in IMR-32 and NH-12, co-expressed NRSF and synapsin I, was lower than that of NB-1 and TGW. ** $P<0.005$, relative expression of NRSF or synapsin I mRNAs in NB-1 or TGW cells versus IMR-32. Lane L, 100 bp DNA ladder; lane 1, IMR-32; lane 2, NH-12; lane 3, NB-1; lane 4, SCCH26; lane 5, TGW. □ NRSF, ■ synapsin I.

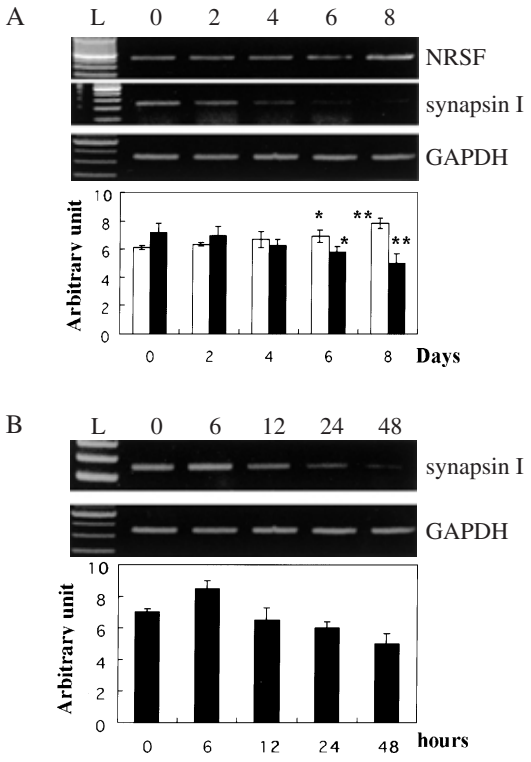

Fig. 3. Competitive RT-PCR analyses of NRSF and synapsin I mRNA expression in MRT cells during differentiation-induction with TPA. (A) In TM87-16 cells, expression of synapsin I mRNA was down-regulated while NRSF mRNA was significantly up-regulated after TPA treatment. * $P<0.05$, ** $P<0.005$, relative expression of NRSF or synapsin I mRNA on day 0 versus days 6 or 8 treated with TPA. □ NRSF, ■ synapsin I. Lane L, 100 bp DNA ladder; lane 0, day 0; lane 2, day 2; lane 4, day 4; lane 6, day 6; lane 8, day 8. (B) After differentiation-induction with TPA, TM87-16 cells expressed synapsin I with the peak level at 6 h. Lane L, 100 bp DNA ladder; lane 0, 0 h; lane 6, 6 h; lane 12, 12 h; lane 24, 24 h; lane 48, 48 h. ■ synapsin I.

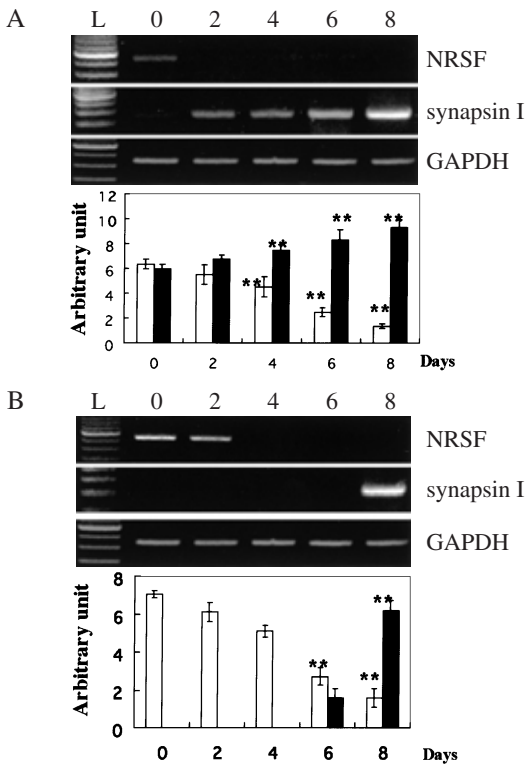

Fig. 4. Competitive RT-PCR analyses of NRSF and synapsin I mRNA expression in neuroblastoma cells during differentiation-induction with TPA. (A) In IMR-32 cells, expression of NRSF mRNA was down-regulated, but synapsin I mRNA was up-regulated. ** $P<0.005$, relative expression of NRSF or synapsin I mRNA on day 0 versus days 4, 6 or 8 treated with TPA. □ NRSF, ■ synapsin I. (B) In NB-1 cells, while significant down-regulation of NRSF mRNA was detected, *de novo* expression of synapsin I mRNA appeared on day 6. ** $P<0.005$, relative expression of NRSF mRNA on day 0 versus days 6 or 8 treated with TPA. ** $P<0.005$, relative expression of synapsin I mRNA on day 6 versus day 8 treated with TPA. □ NRSF, ■ synapsin I.

ment-1 silencing transcription factor (REST), in non-neural tissues suggests the role of NRSF as a negative regulator of neuron-specific genes, such as *synapsin I*.[19–24, 36] To our knowledge, studies on the expression of synapsin I and NRSF for MRT cell lines have not been reported so far. Moreover, studies on NRSF expression even in neuroblastoma have yielded conflicting results.[31, 32, 37] Therefore, we examined the expression of synapsin I, NRSF and other neural markers in MRT cell lines to investigate the neural characteristics of MRT possessing a neural phenotype.

As found by other investigators,[32, 37] neuroblastoma cells showed significant down-regulation of NRSF mRNA concomitantly with remarkable up-regulation of synapsin I mRNA during differentiation induced with TPA.

As for MRT cell lines, two MRT (TM87-16 and TTC642) cells exhibited neural differentiation, showing NF-M and synapsin I, and morphologically produced neuritic cytoplasmic process upon differentiation-induction. TM87-16 cells expressed CGA after treatment with TPA. The other three cells (STM91-01, TTC549 and YAM-RTK1) did not have neural phenotype. TM87-16 and TTC642 MRT cells showed a significantly lower expression of NRSF than the other three MRT cell lines. These two MRT cell lines co-expressed a low level of synapsin I. Lietz *et al.* reported an inverse expression pattern of NRSF and synapsin I in neuroblastoma cells.[37] Also in MRT cell lines, our results showed that the expression level of NRSF determined the expression level of synapsin I. NRSF subsequently repressed the transcription of synapsin I in MRT cells in the same manner as in neuroblastoma.[32, 37]

In these two MRT (TM87-16 and TTC642) cell lines, although synapsin I mRNA expression sharply increased with the peak level at 6 h, thereafter, significant down-regulation of synapsin I mRNA expression was observed concomitantly with an up-regulation of NRSF mRNA following TPA treatment. The changes in expression of NRSF and synapsin I following TPA-induced differentiation in these MRT cell lines were exactly opposite to those observed in the neuroblastoma cell lines.

It was reported that synapsin I might be involved in pre-release synaptic vesicle pooling through the binding of actin filaments at the synapse.[22, 26, 28, 30] Chin *et al.*[25] and Torri *et al.*[30] found that synapsin I expression was highest in the early progenitor stage of neural development and decreased in late progenitor stage. Additionally, Madison *et al.* reported that the expression of synapsin I was down-regulated in the course of neural differentiation.[38]

Our results showed that MRT cells with neural phenotype produced neuritic cytoplasmic process with varicosities upon TPA induction, but very interestingly, synapsin I was expressed highly only in an early phase of neural differentiation and was rapidly down-regulated. It was suggested that synapsin I expression is related to an early and undifferentiating stage of neuronal development in these MRT cells. Co-expression of NRSF and synapsin I would provide evidence that these MRT

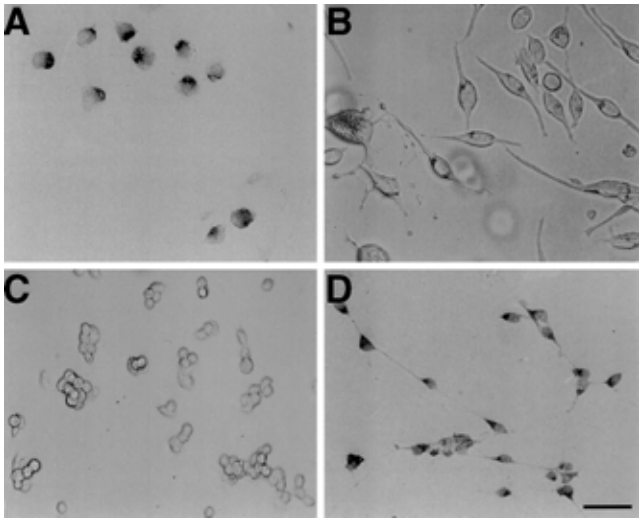

Fig. 5. *In situ* hybridization findings on MRT (TM87-16) cells and neuroblastoma (NB-1) cells (original magnification ×200 for A–D, bar=30 μm). TM87-16 cells before (A) and after (B) treatment with TPA. The hybridization signals for synapsin I were observed in TM87-16 cells, but disappeared after treatment with TPA. NB-1 cells before (C) and after (D) treatment with TPA. After differentiation-induction, the high level of hybridization signal for synapsin I was detected at the edge of polygonal cytoplasm.

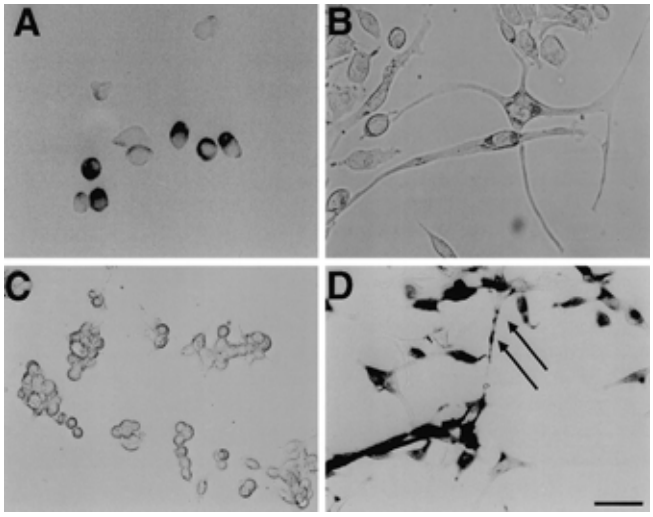

Fig. 6. Immunocytochemical findings on MRT (TM87-16) cells and neuroblastoma (NB-1) cells (original magnification ×200 for A–D, bar=30 μm). TM87-16 cells before (A) and after (B) treatment with TPA. Positive staining for synapsin I protein in TM87-16 cells was down-regulated after treatment with TPA. NB-1 cells before (C) and after (D) treatment with TPA. After induction with TPA, positive staining for synapsin I protein was detected in the elongated cytoplasm and on the multiple varicosities (arrows).

cells still remained in an early phase of neuronal development even after differentiation-induction. Some reports indicated that MRT cell lines with neural phenotype, including TM87-16 and TTC642, did not show the nerve growth factor (NGF)[13] and TrkA (high affinity nerve growth factor receptor).[16] Moreover, recently our laboratory reported that the expression of SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein recepter) complex was incomplete in these MRT cells even after differentiation-induction.[17] Totally different from neuroblastoma, neural differentiation of MRT cells was suspended very quickly. In neuroblastoma cells, the differentiation of the cells continued from the early progenitor stage to mature neuronal development. These MRT cell lines appeared to have only limited capability for neural differentiation, and showed a unique and incomplete neural differentiation. The characteristics of neural differentiation of these MRT cells are fairly distinct from those of neuroblastoma cells.

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