

Research report

Suppression of an actin-binding protein, drebrin, by antisense transfection attenuates neurite outgrowth in neuroblastoma B104 cells

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Abstract

Drebrins, actin-binding proteins, are dominantly expressed during embryogenesis and accumulated in neurite processes of postmigratory neurons. While the cytoskeletal proteins are the important factors for regulating neurite outgrowth, the cellular mechanism in neurons is still unclear. To address the role of drebrins in the neurite outgrowth, we have studied the effect of suppression of drebrin on a rat neuroblastoma B104 cell line, which constitutively expresses drebrin. Deprivation of serum or addition of gangliosides in the culture medium induced remarkable neurite outgrowth of B104 cells. We transfected B104 cells with an antisense construct of human drebrin E cDNA and found that the drebrin expression was significantly reduced in the stable antisense cell lines. In response to serum deprivation and gangliosides treatment, their ability to extend neurite processes was significantly attenuated. In contrast, the cell proliferation of the antisense transfectants was arrested by serum deprivation similar to control B104 cells. These data suggest that the drebrins are required for neurite outgrowth in neuronal cells. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The cytoskeletal proteins are the important endogenous factor for regulating neurite outgrowth. Actin-binding proteins have been also suggested to play important roles in the regulation of neurite motility through reorganization of actin filaments during neurite outgrowth [4,21]. Although a number of actin-binding proteins were identified in the brain [7,17,20], the regulatory mechanism for the actin cytoskeleton in neurite outgrowth is still unclear.

Drebrins are actin-binding proteins first detected in the embryonic chick brain and their expression was closely related to neuronal development [27,28]. Three isoforms are generated by alternative RNA splicing from a single gene of drebrin in the chicken brain [15,16]. The expression pattern of each isoform is regulated spatially and temporally in the developing brain. In the embryo, drebrins

are accumulated in the somata of migrating neurons and in neurite processes of postmigratory neurons. Drebrins overexpressed in cultured fibroblasts exclude tropomyosin from actin filaments, resulting in thick, curving bundles of actin filaments, and the formation of dendrite-like cell processes [11]. The data suggest that drebrins play an important role in regulating neurite outgrowth [24].

A neuroblastoma B104 cell lines is derived from a chemically induced brain tumor in rat [23]. The cells have a number of advantages for studying the *in vitro* regulation of neuronal development. While the cells proliferate actively in medium containing 10% serum, deprivation of serum induces arrest of proliferation and a morphological change from fibroblast-like to bipolar cell shape with processes. Gangliosides are differentiation factors to the cell line, as the natural differentiation factor, NGF, to PC12 cells [19].

In this study, we examined the effect of a forced reduction in drebrin levels by stable transfection with an antisense construct of the drebrin cDNA on a neuronal B104 cell line. By selective suppression of drebrin expression, the cells did not extend significant neurite outgrowth in response to serum deprivation or gangliosides treatment.

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2. Materials and methods

2.1. Plasmid construction and transfection

The antisense orientation plasmid pMIW/as-drebrin was constructed by inserting the coding region of the human drebrin E cDNA, blunt-ended *DraI*–*EcoRI* fragment from the gDbh13 [30] into the *EcoRV* restriction site under the β -actin promoter of pMIW-HEP plasmid [26]. Rat neuroblastoma B104 cells [23] were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and 50 μ g/ml kanamycin according to standard techniques. Twenty μ g of pMIW/as-drebrin and 2 μ g of pST-neoB, a neomycin resistance expression plasmid [13], were coprecipitated with calcium phosphate according to standard procedures [34]. Stable transfectants were selected in medium containing 600 μ g/ml of G418 (Sigma, St. Louis, MO), beginning 3 days subsequent to transfection. After limiting dilution of G418-resistant clones, colonies that were virtually nonreactive with the anti-drebrin monoclonal antibody (M2F6) [9] were cloned to the single cell stage and reevaluated by Western blot and immunofluorescence analysis. In all cases, B104 cells transfected only with the neomycin resistance gene were included as controls.

2.2. Western blot analysis

Cells were weighed, and homogenized with a micro tissue grinder in 10 vols. of sodium dodecyl sulfate (SDS)-sample buffer composed of 2% SDS, 5% 2-Mercaptoethanol, 10% glycerol, 1 mM EDTA, 40 mM Tris, and 240 mM glycine at pH 8.5. These homogenates were electrophoresed on SDS-polyacrylamide gel (7.5%) and the gels were stained with Coomassie Brilliant Blue or transferred to a nitrocellulose sheet (Toyo Roshi, Japan) by electroblotting for 12 h at 150 mA. After blocking with 5% skin milk for 60 min, the sheets were incubated with the anti-drebrin antibody or an anti-actin monoclonal antibody (Cederlane Laboratories, Canada) for 60 min at room temperature. After washing, the blots were incubated with peroxidase-conjugated anti-mouse second antibody (Organon Teknika Durham, NC) for 60 min at room temperature. The reaction products were visualized with 0.3% chloronaphthol, 0.3% hydrogen peroxide, and 27 mg/ml imidazole.

2.3. Cell culture

The antisense drebrin transfectants were grown in DMEM supplemented with 600 μ g/ml of G418. Subsequently, the medium was replaced by either fresh DMEM with or without 10% FCS, or serum-free DMEM with addition of 50 μ g/ml of gangliosides type II (Sigma), as described previously [19]. After 8 h incubation, the cultures were fixed, random fields were imaged by phase-con-

trast microscopy and recorded on photographs. The length of neurite processes of cells was taken as the distance from the tip of a process to the middle of the cell soma. The results show the mean length of the longest neurite process per cell for 50 individual cells.

2.4. Immunocytochemistry

After cells were grown on poly-L-lysine (100 μ g/ml) coated glass coverslips for 24 h, the cultures were fixed for 15 min in 3.7% formaldehyde in phosphate-buffered saline (PBS). The cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min and washed with PBS. Nonspecific binding was blocked by incubation in PBS with 3% bovine serum albumin for 30 min at room temperature. After removing the blocking solution, the anti-drebrin antibody was added for 60 min at room temperature. The slides were washed with PBS, then incubated for 60 min at room temperature in fluorescein-conjugated anti-mouse IgG (Organon Teknika) diluted to a final concentration of 1:300. The slides were mounted and viewed under a Nikon Optiphot micro-scope fitted for fluorescence microscopy.

For double staining with rhodamine-phalloidin (Sigma), which has affinity with filamentous (F)-actin [5], the cells were first incubated with rhodamine-phalloidin diluted 1:50 for 30 min at room temperature, washed PBS, and incubated with the anti-drebrin antibody.

2.5. Evaluation of proliferation

Replicating cells were identified immunologically using bromo-deoxyuridine (BrdU) as described previously [29]. In brief, the cultures in each condition were incubated with BrdU (Sigma) at a final concentration of 10^{-4} M for 4 h.

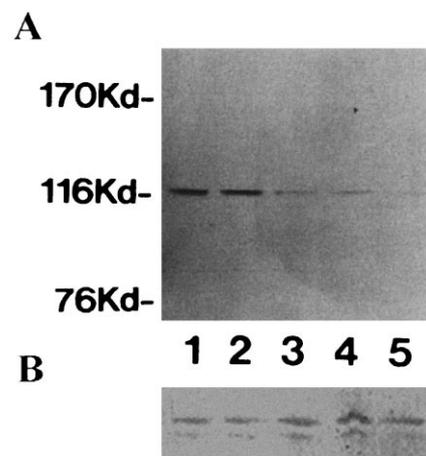


Fig. 1. Immunoblot analysis of drebrin expression. Each sample of the native B104 cells (lane 1), NeoB (lane 2), ADI-1 (lane 3), ADI-2 (lane 4), and ADII-4 (lane 5) was separated by SDS-PAGE, blotted to the nitrocellulose sheet, and reacted with an anti-drebrin antibody M2F6 (A) and an anti-actin monoclonal antibody (B). NeoB; B104 cells transfected only with the neomycin resistance gene. ADI-1, ADI-2, and ADII-4; stable antisense drebrin clones.

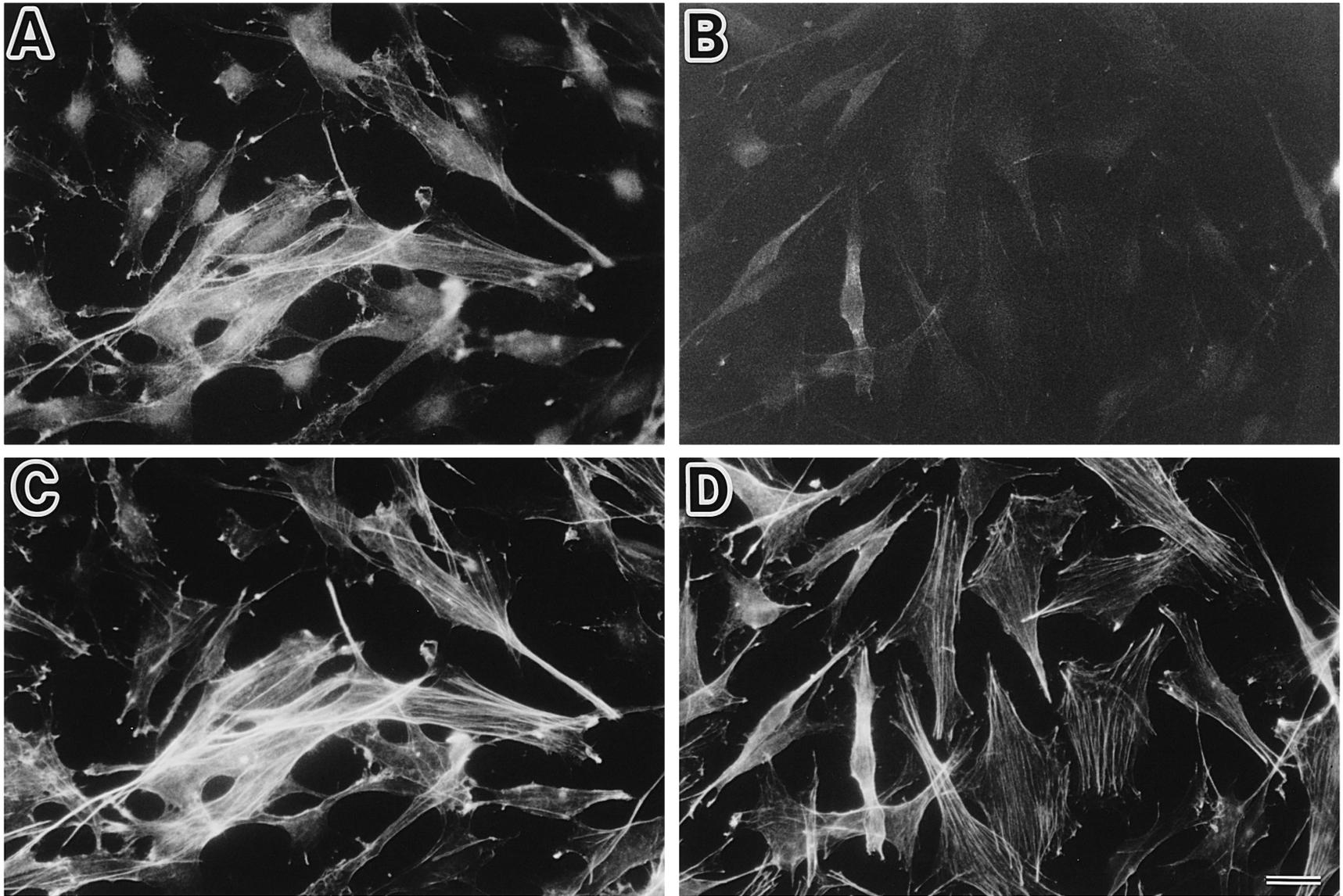
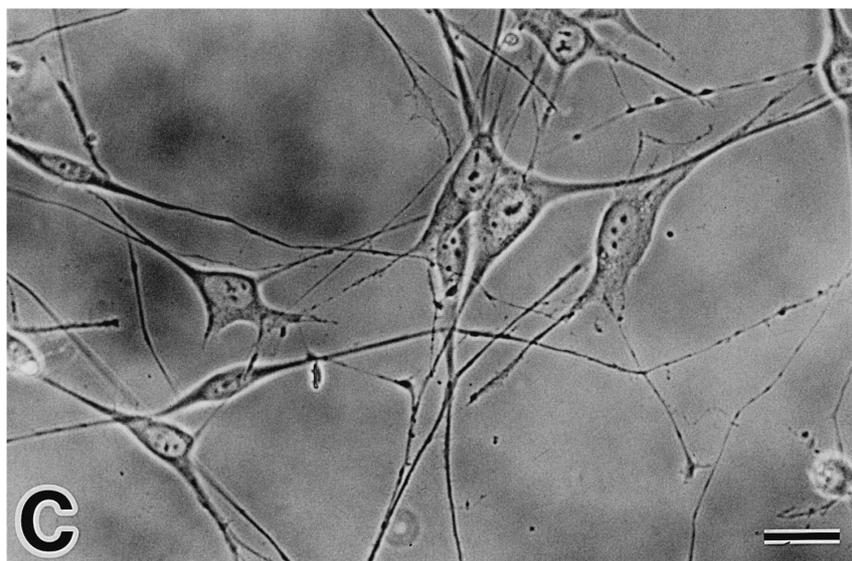
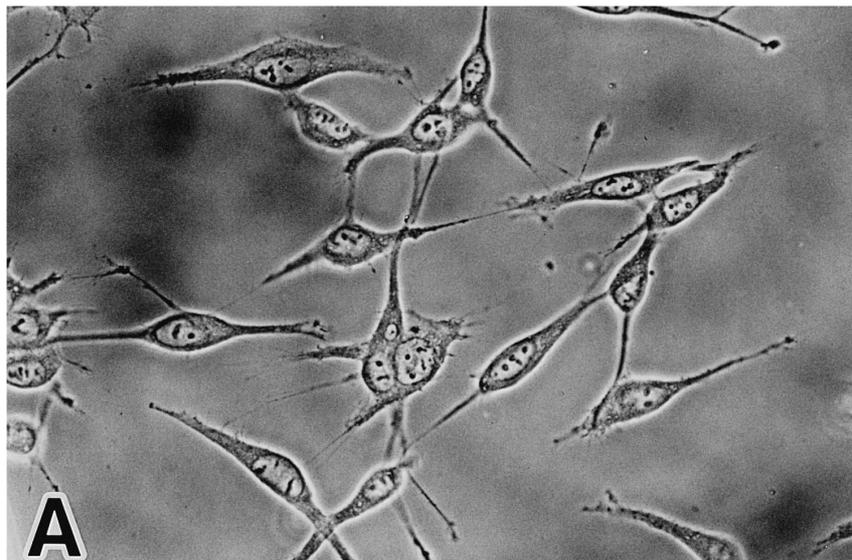


Fig. 2. Double-immunofluorescence labeling of drebrin and filamentous-actin. The control NeoB (A and C) and the antisense drebrin transfectant ADII-4 cells (B and D). The cells were stained with the anti-drebrin monoclonal antibody M2F6 (A and B) and rhodamine-phalloidin which has affinity with filamentous-actin (C and D). The same fields are shown in each pair of micrographs. Calibration bar = 28.6 μm .



After removal of the BrdU solution, the cells were fixed in methanol at -20°C . The DNA was denatured by addition of 2 N hydrochloric acid. After the acid was neutralized with 0.1 M sodium tetraborate (pH 9), the incorporation of BrdU was detected using a monoclonal mouse anti-BrdU antibody (Becton Dickinson, San Jose, CA) and the fluorescein-conjugated anti-mouse IgG.

3. Results

3.1. Reduction of drebrin expression in B104 cells by transfection with an antisense drebrin cDNA construct

Western blot analysis of B104 cells by the anti-drebrin antibody M2F6 revealed a single band, corresponding to a molecular weight of approximately 125 kDa (Fig. 1A, lane 1). In every two transfection experiments with the antisense drebrin cDNA construct in B104 cells, twelve clones resistant to G418 were isolated. The levels of drebrin expression in the various antisense drebrin (AD) clones were determined by Western blotting with the anti-drebrin antibody. Three antisense clones, ADI-1, ADI-2, and ADII-4, showed significantly low levels of drebrin expression (Fig. 1A, lanes 3–5). In contrast, drebrin expression in NeoB cells transfected only with the neomycin resistance gene was similar to the untransfected B104 cells (Fig. 1A, lane 2). The each sample from all the lines reacted similarly with a monoclonal antibody against actin (Fig. 1B).

Drebrin is localized at the similar sites as actin filaments in neuroblastoma of SH-SY5Y cells [1] and PC12 cells [22], and in fibroblasts transfected with drebrin cDNA [11,25]. The relationship between drebrin and actin filaments in B104 cells transfected with antisense drebrin cDNA was analyzed by double immunofluorescence staining using the anti-drebrin antibody and rhodamine-phalloidin which has affinity with F-actin. In control NeoB cells, drebrin was colocalized with well developed actin stress fibers (Fig. 2A,C) similar to that in native B104 cells (data not shown). Although most drebrin was distributed diffusely within the cell, some of the stress fibers were immunostained with the anti-drebrin antibody. In AD cells, well developed actin stress fibers were still present, while drebrin expression was suppressed (Fig. 2B,D).

3.2. Effect of low drebrin expression on neurite outgrowth in response to serum deprivation or gangliosides treatment

By deprivation of serum, native B104 cells become bipolar in cell shape with processes (Fig. 3B). After addi-

tional gangliosides treatment to serum-free medium, these cells attained a multipolar morphology with several long radical neurites (Fig. 3C). By further addition of fresh medium with 10% FCS to these cells, the cell shape changed reversibly to the native (Fig. 3A). To test the effect of the suppression of drebrin on neurite process formation, we have studied AD cells in response to serum deprivation and gangliosides treatment. In the case of AD cells, they responded only slightly to both serum deprivation and gangliosides treatment (Fig. 4B,D). In contrast, NeoB cells extended neurite processes in response to these treatments as nontransfected B104 cells (Fig. 4A,C). The results of quantitative analysis of neurite length in response to serum deprivation and gangliosides treatment are summarized in Table 1. There was no significant difference in means of neurite lengths among B104, control NeoB, and AD cells in medium with 10% FCS. However, in serum deprivation and gangliosides treatment, all of three AD cells clearly showed low responses, with increases in average process length of only 11–16% in serum deprivation and 20–39% in gangliosides treatment. The control NeoB cells responded like the untransfected B104 cells, with increases in average process length of 55% in deprivation of serum and 116% in response to gangliosides treatment.

3.3. Effect of low drebrin expression on cell proliferation

To evaluate the proliferative ability of cells, the active mitotic cells were identified immunohistochemically by mouse anti-BrdU antibodies after exposure to BrdU for 4 h. As shown in Table 2, there was no apparent difference in the uptakes of BrdU between control B104 and AD cells in medium with 10% FCS. Both cell lines similarly became quiescent in response to either deprivation of serum or gangliosides treatment.

4. Discussion

In the cell cycle of B104 cells, there exist two distinct states: a proliferating (P) state and a state of relative quiescence (G0 or resting (R) state) [19]. The transition from the P to the R state is accompanied by marked morphological alterations of the B104 cell such as neurite outgrowth promotion, which suggests cell differentiation. The transition is reversible and controlled in part by growth factors present in serum. In present study, reduction of drebrin expression induced little effects on the cell

Fig. 3. Phase contrast images of B104 cells. The cells in DMEM with 10% serum (A), in serum-free DMEM (B), and in serum-free DMEM with gangliosides (C). The cells growing in DMEM with 10% serum showed a fibroblast-like morphology (A). In serum-free medium, the cells became bipolar and promoted neurite outgrowth (B). By the treatment with gangliosides (50 $\mu\text{g}/\text{ml}$), the cells in serum-free medium (B) became a multipolar morphology with prolonged processes (C). Calibration bar = 28.6 μm .

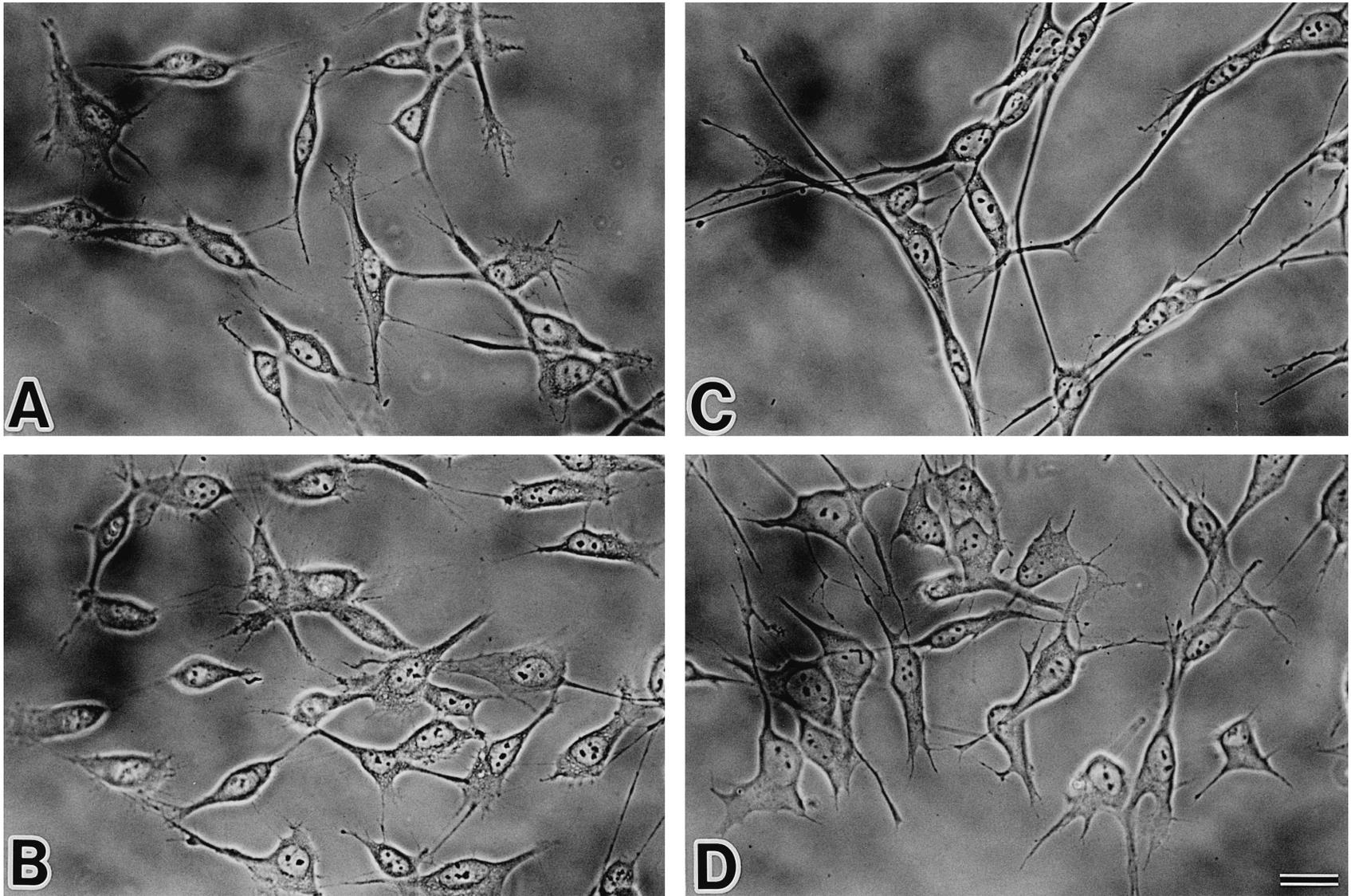


Fig. 4. Effect of gangliosides treatment on antisense drebrin transfected cells. Phase contrast micrographs after 8 h treatment with DMEM with 10% serum (A and B) and serum-free DMEM with gangliosides (50 $\mu\text{g}/\text{ml}$) (C and D). NeoB cells, transfected only with the neomycin resistance gene, extended neurite processes in response to gangliosides treatment as nontransfected B104 cells (A and C). In contrast, antisense drebrin transfected cells (ADII-4) did not extend significant processes by gangliosides treatments (B and D). Calibration bar = 28.6 μm .

cycle of B104 cells. Although AD cells became quiescent in response to deprivation of serum or gangliosides treatment as native B104 cells, they did not show significant neurite outgrowth by these treatments.

The strategy to reduce the gene expression by the antisense mRNA in target cells is widely used including cytoskeletal proteins [32,33]. This method is particularly useful for the study of vital genes, when the gene eliminated by targeted homologous recombination might be lethal. By transfection with a 2.5-kb antisense drebrin cDNA expression vector to neuroblastoma B104 cells, we obtained the AD cells whose ability were significantly reduced to extend neurite processes upon selective treatments. It appears unlikely that this result was due to transfection alone, because the neomycin resistance gene transfected cells responded like the untransfected cells. Furthermore, the three stable cell lines of ADI-1, ADI-2, and ADII-4, obtained by two transfection experiments, yielded similar results in terms of suppression of drebrin expression and morphological changes under selective conditions. However, the characteristic changes in AD cells were unstable. The AD cells became to reexpress drebrin in culture for a long time. Similar phenomena have been reported previously in the studies with cytoskeletal protein genes [14,18]. Although the mechanisms for reversion of antisense suppression are still unclear, there are several possibilities such as a change in the compartmentalization of the target RNA, making it inaccessible to the antisense RNA [10].

Table 1
Quantitative analysis of neurite length in response to serum deprivation and gangliosides treatment

	FCS (+)	FCS (–)	Gangliosides (+)
B104	63.3 ± 36.6 ^{ns}	96.3 ± 44.7 ^{ns}	128.6 ± 50.6 ^{ns}
(% increase in length)		(52%)	(103%)
NeoB	64.9 ± 25.0	100.8 ± 31.9	140.4 ± 49.5
(% increase in length)		(55%)	(116%)
ADI-1	61.5 ± 22.9 ^{ns}	71.5 ± 18.4*	85.2 ± 24.7*
(% increase in length)		(16%)	(39%)
ADI-2	64.4 ± 18.0 ^{ns}	71.2 ± 18.4*	77.3 ± 20.6*
(% increase in length)		(11%)	(20%)
ADII-4	62.7 ± 17.2 ^{ns}	72.0 ± 17.2*	75.4 ± 23.8*
(% increase in length)		(15%)	(20%)

B104 cells, NeoB cells transfected only with the neomycin resistance gene, and antisense drebrin transfectants, ADI-1, ADI-2, and ADII-4, were cultured in DMEM with 10% fetal calf serum, FCS (+); serum-free DMEM, FCS (–); or serum-free DMEM with gangliosides, gangliosides (+).

The length of neurite process was taken as the distance from the tip of a process to the middle of the cell soma ($n = 50$).

There was no significant difference in means of neurite lengths among B104, control NeoB, and AD cells in medium with 10% FCS (ns, not significant; unpaired *t*-test).

In serum deprivation and gangliosides treatment, all of three AD cells clearly showed low responses compared with the control NeoB cells (*, $p < 0.0001$; unpaired *t*-test). There was no significant difference in means of neurite lengths between B104 and control NeoB in these treatments.

Each value represents mean ± S.D.

Table 2

Percent of BrdU incorporation in control B104 cells and antisense drebrin transfectants

	FCS (+)	FCS (–)	Gangliosides (+)
Control B104	57 ± 8	19 ± 7	25 ± 7
AD	56 ± 7 ^{ns}	30 ± 6 ^{ns}	30 ± 2 ^{ns}

Cultured cells in DMEM with 10% fetal calf serum, FCS (+); serum-free DMEM, FCS (–); or serum-free DMEM with gangliosides, gangliosides (+), were reacted with BrdU in 4 h and visualized with a monoclonal antibody against BrdU.

There was no significant difference in the uptakes of BrdU between control B104 and AD cells in medium with 10% FCS, serum-free medium, or gangliosides treatment (ns, not significant; unpaired *t*-test).

Each value represents mean ± S.D.

It has been reported that the plasticity of neurites correlates inversely with the stability of its cytoskeletal components: microtubules in growth cones are very dynamic, whereas in mature neurites, microtubules are highly stabilized [8]. The binding of microtubule-associated proteins to the microtubules contributes to this stability, regulating neurite outgrowth and differentiation [2,6,31]. The actin cytoskeleton also regulates the motility of neurites [4,21]. The organization of actin is regulated by various kinds of actin-binding proteins. Drebrin binds to actin filaments competitively against tropomyosin on the similar actin-binding sites [24]. Tropomyosin stabilizes actin filaments by protecting them from actin-destabilizing proteins, such as gelsolin [12] and actin depolymerizing factors [3] in the brain. In contrast, drebrin-bound actin filaments are easily attacked by gelsolin [11]. Drebrins may destabilize actin filaments by freeing actin filaments from tropomyosin, thereby maintaining the actin filaments in a dynamic state suitable for the neurite outgrowth. Phalloidin stainings for F-actin in AD cells were similar to those in native B104 cells (Fig. 2), suggesting that drebrin does not affect on actin localization. Nevertheless, the AD cells did not extend significant neurite processes in response to serum deprivation and gangliosides treatment. In AD cells, actin filaments might become too rigid to form significant neurite processes due to the low drebrin expression. Further studies such as the drebrin gene transgenic experiments in animals are desired to understand better the molecular details of neurite formation.

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