Purification of human primary neuroblastomas by magnetic beads and their *in vitro* culture

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Abstract. In the present study we have successfully isolated neuroblastoma cells from primary human neuroblastoma tissues by using a magnetic bead-mediated purification system. Since primary neuroblastoma tissues contained CD3- and CD19-positive lymphocytes, total cell suspensions were prepared and incubated with magnetic beads coated with anti-CD3 or with anti-CD19 antibody. After magnetic separation, unbound materials were recovered and analyzed by immunohistochemical staining for NB84, one of the neuroblastoma markers. Immunohistochemical and FACS analyses demonstrated that NB84-positive cells were enriched in the unbound fraction. Subsequently, unbound materials were seeded on cell culture plates and maintained at 37˚C overnight. After incubation, non-adherent cells were collected and stained with anti-NB84 antibody. Under our experimental conditions, a significant increase in the number of NB84-positive cells was observed. Furthermore, our purified NB84-positive cells responded to all-*trans* retinoic acid and nerve growth factor better than the initial primary cells. Collectively, our present results suggest that magnetic bead-mediated purification enriches neuroblastoma cells which retain their biological properties.

Introduction

Neuroblastoma is one of the most common early childhood solid tumors of the peripheral nervous system arising from an as yet unidentified population of neural crest cells (1), and is clinically and cytogenetically divided into two major subgroups with distinct biological properties (2). One subgroup of tumors in the early stages displays favorable prognosis and usually occurs in patients <1 year of age. They have no *MYCN* gene amplification and often differentiate and/or regress spontaneously. In sharp contrast, tumors that occur in patients >1 year of age usually possess *MYCN* gene amplification and allelic loss in the distal part of the short arm of chromosome 1, and are often aggressive with an unfavorable prognosis in spite of an intensive multimodal therapy (3). Additionally, it has been shown that high expression levels of neurotrophin receptor genes *TrkA* and *TrkB* are favorable and unfavorable prognostic indicators, respectively (4-6). However, the precise molecular mechanisms behind disease progression and tumor regrowth are still unclear. From a clinical point of view, aggressive neuroblastomas are the most problematic, and it is quite difficult to decide which therapeutic strategy is suitable.

As described previously (7), neuroblastomas exhibit heterogenous morphologies with tumors composed of a mixture of neuroblasts, ganglion cells, non-neuronal Schwann-like cells and stromal cells. Indeed, human neuroblastoma-derived cell lines consist of heterogenous subpopulations of cells which show distinct morphological and biochemical features (7-10). The established neuroblastoma cell lines spontaneously give rise to diverse populations of neuroblastic (N-type) and Schwann-like (S-type) cells (9,10). In addition to N- and S-type cells, cells with intermediate morphology (I-type) have also been cloned (11), and these three subtypes have an ability to interconvert or transdifferentiate spontaneously (12), suggesting that N-type to S-type differentiation might reflect *in vivo* differentiation of the tumor. To better understand the biological behavior as well as molecular events *in vivo*, it seems to be important to remove the additional components such as non-neuronal Schwann-like and stromal cells from neuroblastomas in culture.

In the present study, we have obtained successful results isolating neuroblastoma cells from primary neuroblastoma tissues by using magnetic bead-mediated purification.

Materials and methods

Patient population. Tumors were staged following the International Neuroblastoma Staging System criteria (13).

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The patients were treated according to the protocols proposed by the Japanese Infantile Neuroblastoma Cooperative Study (14) and the Study Group of Japan for Treatment of Advanced Neuroblastoma (15). All patients agreed to participate and provided written informed consent. Our present study was approved by the institutional ethics review committee.

Primary culture. Fresh human neuroblastoma tissues were minced and incubated with RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and 100 μg/ml of OPI (Sigma, St. Louis, MO, USA) in the presence of 500 units/ml of collagenase (Sigma) at 37˚C for 1 h. After incubation, tumor tissues were dispersed by using a plastic syringe with an 18-gauge needle, seeded on tissue culture plates precoated with collagen, and cultured at 37˚C with 5% CO_2 in a humidified incubator. Where indicated, cells were treated with the indicated combinations of all-*trans* retinoic acid (ATRA, Sigma) and nerve growth factor (NGF, Sigma), and their effects on neurite outgrowth were examined with a phase-contrast microscope.

Purification of neuroblastoma tumors. The magnetic beads were conjugated with anti-mouse IgG. After incubation, the magnetic beads (Dynal, Oslo, Norway) were washed in PBS and then incubated for 4-8 h with monoclonal anti-CD3 (DHCT1, Ancell, Bayport, MN) or with anti-CD19 (BU12, Ancell) antibody at 4˚C. The indicated beads were mixed and incubated for 1 h at 4˚C with cell suspensions before magnetic separation to isolate the two fractions: cells bound to the beads (lymphocytes) versus cells not bound to the beads (neuroblastomas, Schwann cells and fibroblasts). The latter cell fraction was subsequently cultured in a standard medium overnight at 37˚C, and then separated into adherent cells (Schwann cells and fibroblasts) and non-adherent cells (neuroblastomas).

Measurement of cell viability. The trypan blue dye exclusion experiments were carried out by incubating the cell suspension with an equal amount of 0.3% trypan blue solution (Sigma) for 5 min at room temperature. After the incubation, the number of cells excluding trypan blue was measured by using a hemocytometer to estimate the number of intact viable cells.

Immunohistochemistry. Neuroblastoma tissues were fixed in 10% formaldehyde, embedded in paraffin, and 3-μm sections were subjected to immunostaining. Before incubating with anti-NB84 antibody (NB84a, Novocastra Laboratories, Newcastle, UK), the sections were treated with 0.05% Pronase in 50 mM Tris-HCl (pH 7.5) for 5 min. The sections were stained with anti-NB84 antibody at 4˚C overnight, processed for the biotin-streptavidin method (Nichirei, Tokyo, Japan), and visualized with diaminobenzidine solution. Cell nuclei were stained with hematoxylin and eosin (H&E).

Flow cytometry. Cells (3-5x10^5) were washed in ice-cold PBS, and fixed in 0.25% paraformaldehyde for 15 min at room temperature. After washing in PBS, cells were incubated with anti-CD3 or with anti-CD19 antibody followed by incubation with FITC-conjugated isotype-specific secondary antibody (Invitrogen, Carlsbad, CA) at 4˚C for 30 min. After washing in PBS, analysis was performed by FACS flow cytometer (Becton Dickinson, San Diego, CA). Cells were properly gated and histograms [FITC-fluorescence (x-axis) versus counts (y-axis)] were plotted to illustrate the logarithmic fluorescence intensity.

Results

Immunohistochemical analysis of human primary neuroblastomas. The 52 examined fresh human primary neuroblastoma samples were divided into 38 non-disseminated tumors with excellent outcome (stage 1, 2 and 4s) and 14 disseminated tumors with unfavorable outcome (stage 3 and 4). These primary samples were subjected to primary cultures according to a standard protocol, and their viability was examined by trypan blue dye exclusion assay. Under our experimental conditions, 91 and 94% of non-disseminated and disseminated cells were viable, respectively (data not shown). In accordance with recent observations (16), the represented immunohistochemical analysis revealed that surgically resected tumor tissues (case 625) contained NB84-positive neuroblastoma cells as well as lymphocytes, fibroblasts and Schwann cells (Fig. 1A). Similar results were also obtained in immunohistochemical analysis of total cell suspensions prepared from case 625 (Fig. 1B). NB84 has been considered to be one of the neuroblastoma markers (17).

Purification of neuroblastoma cells by using magnetic beads. To examine the amounts of lymphocytes included in primary neuroblastoma samples, we performed FACS analysis. For this purpose, total cell suspensions prepared from 24 cases including 14 non-disseminated tumors and 10 disseminated tumors were incubated with magnetic beads conjugated with anti-CD3 or with anti-CD19 antibody, and the unbound materials were again subjected to the magnetic bead treatment. Then unbound materials were processed for FACS analysis to determine the number of CD3- or CD19-positive cells. The representative data (case 625) are shown in Fig. 2A. Total cell suspensions contained 41% CD3-positive cells (T cells) but no CD19-positive cells (B cells). As expected, magnetic bead treatment resulted in a complete removal of CD3-positive cells. The cell viability of the bound materials (lymphocyte fractions) derived from non-disseminated and disseminated tumors was 81 and 79%, respectively (data not shown). On the other hand, the cell viability of the unbound materials prepared from non-disseminated and disseminated tumors was 94 and 92%, respectively (data not shown). We then examined the unbound materials by immunohistochemical analysis. As shown in Fig. 2B, NB84-positive cells were relatively enriched after magnetic bead-mediated separation. The unbound materials still contained NB84-positive neuroblastoma cells as well as fibroblasts and Schwann cells. According to our preliminary observations, neuroblastoma cells were less adherent than Schwann cells as well as fibroblasts to culture plates (data not shown). To separate NB84-positive neuroblastoma cells from fibroblasts and Schwann cells, the unbound materials were seeded on cell culture plates and incubated at 37˚C overnight. After incubation, non-adherent cells were recovered and subjected

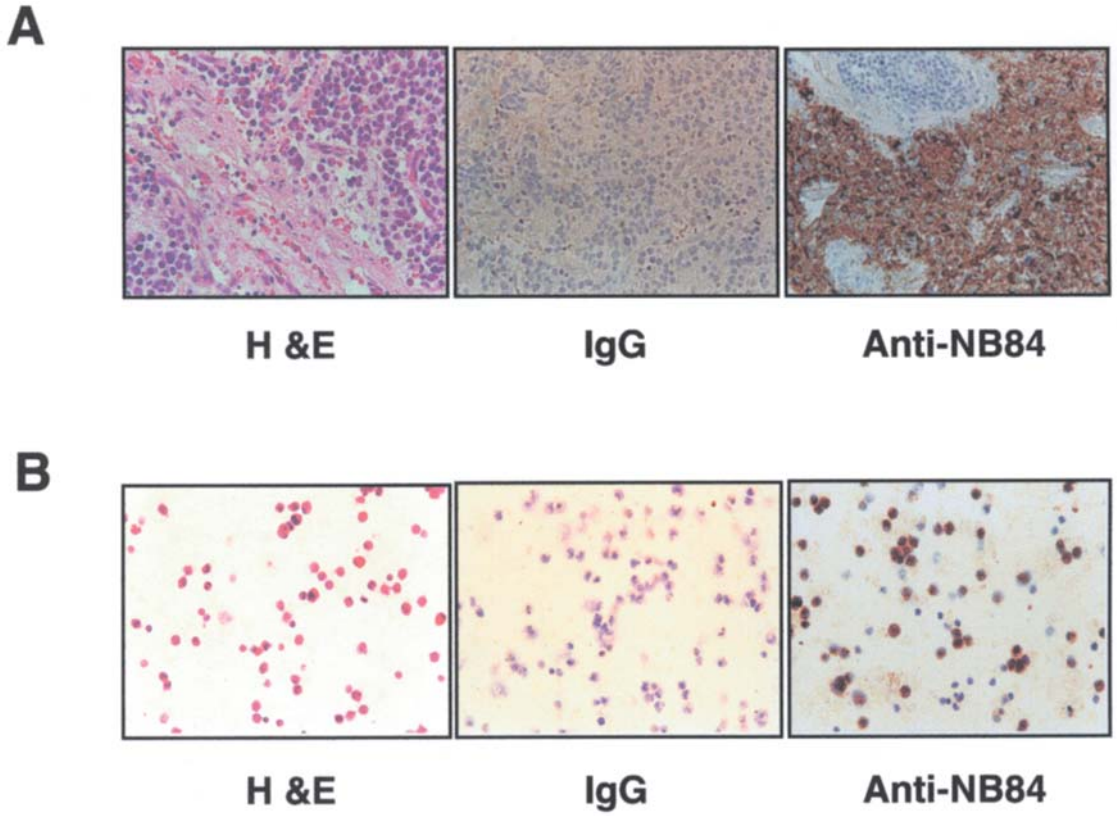

Figure 1. Immunohistochemical staining for NB84 in human primary neuroblastoma tissues. (A) Clinical samples of the patient with neuroblastoma (case 625) were subjected to H&E staining. Alternatively, these primary samples were processed for immunohistochemical staining with control IgG or with anti-NB84 antibody. (B) H&E and immunohistochemical staining of cell suspensions prepared from primary neuroblastoma (case 625).

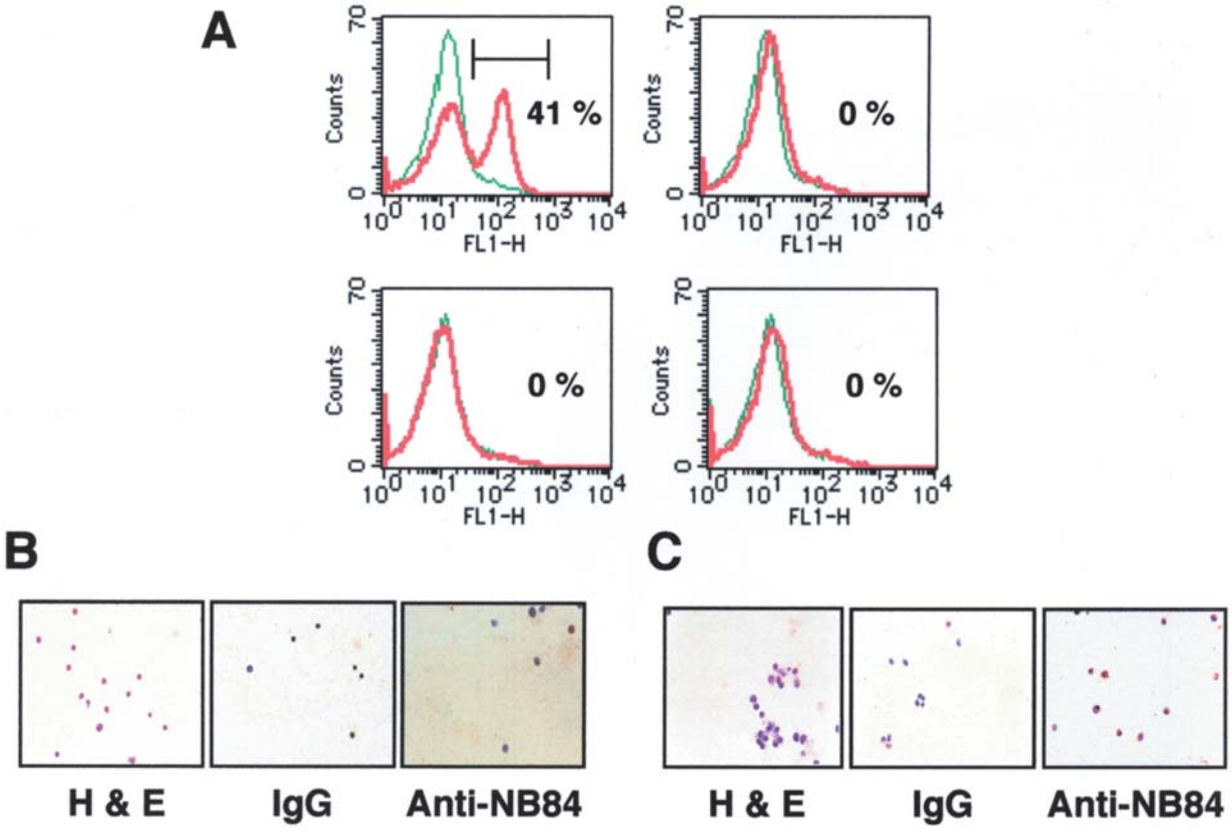

Figure 2. Flow cytometric analysis of primary neuroblastoma. (A) Total cell suspensions prepared from primary neuroblastoma (case 625) were analyzed by flow cytometry for the expression of CD3 and CD19 before and after magnetic separation. (B) Immunohistochemical analysis of neuroblastoma cell suspensions (case 625) after magnetic separation (CD3- and CD19-negative cells). (C) After magnetic separation of neuroblastoma cell suspensions (case 625), CD3- and CD19-negative cells were maintained overnight, and non-adherent cells were collected followed by immunohistochemical staining.

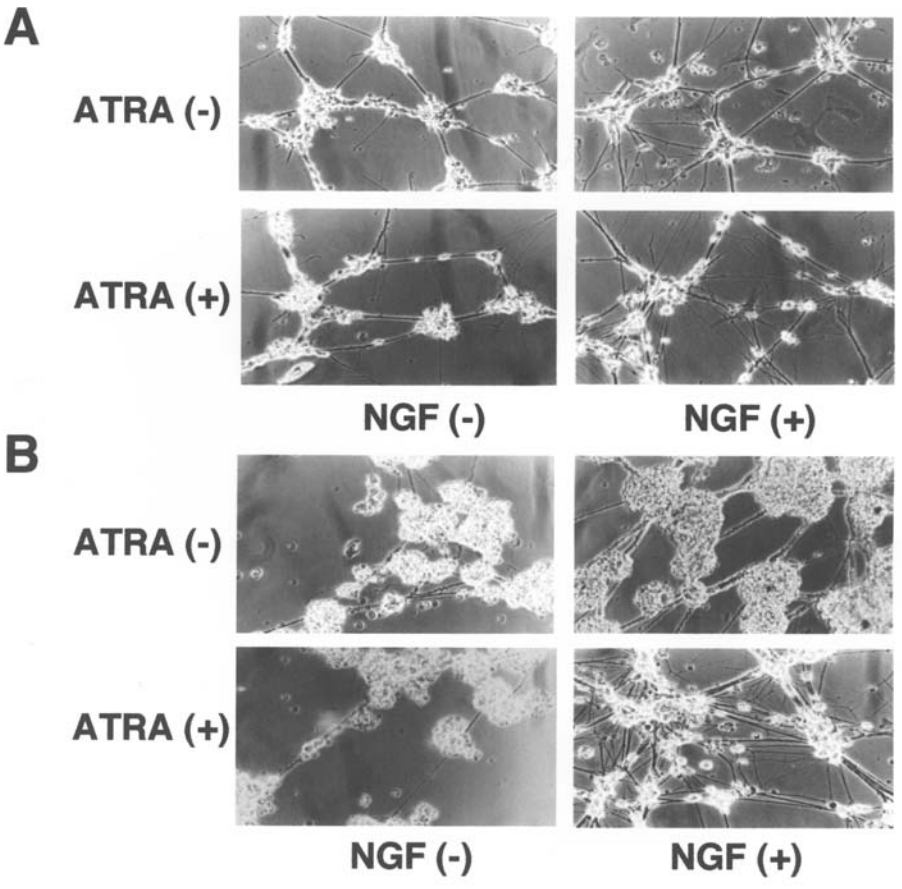

Figure 3. Neurite extension of primary neuroblastomas in response to NGF and/or ATRA. Total cell suspensions (A) and CD3- and CD19-negative non-adherent cells (B) prepared from case 625 were treated with or without the indicated combinations of NGF (100 ng/ml) and ATRA (5 μM) for seven days, and their neurite outgrowth was examined.

to immunohistochemical staining with anti-NB84 antibody. As shown in Fig. 2C, NB84-positive neuroblastoma cells were efficiently enriched by our present procedure.

Neurite extension in response to ATRA and/or NGF. To address whether magnetic bead-mediated enrichment of neuroblastoma cells could affect their biological behavior, we compared the degree of neurite outgrowth between cells before and after the selection. To this end, total cell suspensions and CD3- and CD19-negative non-adherent cells prepared from case 625 were cultured in the presence or absence of ATRA and/or NGF. Seven days after treatment, cells were observed with a phase-contrast microscope. Representative data are shown in Fig. 3A and B. ATRA alone had undetectable effects on both cells, whereas NGF treatment led to a formation of neurites in both cells. To note, treatment of CD3- and CD19-negative non-adherent cells with the combination of ATRA and NGF significantly enhanced neurite outgrowth as compared with total cell suspensions, suggesting that NB84-positive neuroblastoma cells purified by magnetic bead-mediated separation retain the biological properties of primary neuroblastomas.

Discussion

To understand the biological properties of neuroblastomas *in vivo*, it is necessary to isolate neuroblastoma cells from fresh neuroblastoma tissues in culture. As described previously (18), growth factors have an ability to promote proliferation or differentiation of neuroblastoma cells through interactions with their specific receptors. Among them, NGF, which induces normal adrenal cells to differentiate into cells identical to sympathetic neurons, plays a key role in the development of neuroblastomas (19). It has been well established that the high-affinity NGF receptor *TrkA* gene is highly expressed in low-stage neuroblastomas but not in advanced neuroblastomas (4). Several lines of evidence suggest that NGF responsiveness of neuroblastoma-derived cell lines is closely associated with the expression levels of *TrkA* and the low-affinity NGF receptor gene $p75^{NTR}$ (20,21). Since $p75^{NTR}$ alone had undetectable effects on NGF responsiveness of neuroblastoma cell lines (22), we sought to isolate neuroblastoma cells from primary neuroblastoma tissues by using magnetic beads coated with anti-$p75^{NTR}$ antibody. Under our experimental conditions, we failed to enrich neuroblastoma cells due to diverse expression levels of $p75^{NTR}$ in primary neuroblastoma samples (data not shown).

During the preparation of primary neuroblastoma samples, we noticed that primary neuroblastoma tissues contain substantial amounts of lymphocytes. We then employed magnetic beads coated with anti-CD3 or anti-CD19 antibody to remove lymphocytes from primary neuroblastoma samples. After magnetic separation, the unbound materials were cultured overnight and the non-adherent cells were collected.

Table I. FACS analysis of advanced neuroblastomas.

			Total cell suspension				Cells after treatment with beads		
			Lymphocytes			NB cells	Lymphocytes		NB cells
Case	Age	Stage	Total (%)	CD3 (%)	CD19 (%)	NB84 (%)	CD3 (%)	CD19 (%)	NB84 (%)
548	2 years	4	4	4	0	ND	ND	ND	ND
559	1 year	3	20	20	0	ND	0	0	ND
564	2 years	4	20	7	13	ND	0	0	ND
580	9 months	3	21	9	12	ND	ND	ND	ND
581	1 year	4	31	31	0	ND	ND	ND	ND
602	1 year	4	13	13	0	17	0	0	50
603	2 years	4	15	4	11	ND	ND	ND	ND
613	4 years	4	56	28	28	16	0	0	51
615	7 months	3	15	11	4	ND	0	0	ND
649	6 years	4	0	0	0	22	0	0	56

ND, not determined.

Table II. FACS analysis of neuroblastomas in stages 1, 2 and 4s.

			Total cell suspension				Cells after treatment with beads		
			Lymphocytes			NB cells	Lymphocytes		NB cells
Case	Age	Stage	Total (%)	CD3 (%)	CD19 (%)	NB84 (%)	CD3 (%)	CD19 (%)	NB84 (%)
601	9 months	2	28	20	8	ND	ND	ND	ND
609	7 months	1	58	44	14	ND	0	0	ND
611	7 months	4s	57	57	0	ND	0	0	ND
619	9 months	2	83	72	11	50	0	0	ND
624	8 months	1	96	48	48	ND	40	34	ND
625	7 months	1	41	41	0	66	0	0	72
627	7 months	1	15	15	0	0	0	0	ND
641	7 months	1	0	0	0	64	0	0	65
643	1 year	1	0	0	0	78	0	0	86
678	7 months	1	15	14	1	86	2	0	95
684	8 months	1	4	4	0	95	0	0	97
687	8 months	1	11	10	1	86	2	1	75
711	7 months	1	20	19	1	79	0	0	95
716	8 months	1	20	18	2	22	2	1	60

ND, not determined.

FACS analysis revealed that our procedure successfully enriches NB84-positive viable neuroblastoma cells (Tables I and II), indicating that NB84-positive neuroblastoma cells can be separated from Schwann cells as well as from fibroblasts by taking advantage of their differential adhesion. Moreover, NB84-positive neuroblastoma cells responded to ATRA and NGF, suggesting that our enriched materials retained the biological properties of primary neuroblastomas. Collectively, our magnetic bead-mediated isolation system provides fresh and enriched neuroblastoma cells in culture.

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