Pergamon

European Journal of Cancer, Vol. 33, No. 12, pp. 1937–1941, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(97)00207-4

Original Paper

Expression of a 260 kDa Neuroblastoma Surface Antigen, the Target of Cytotoxic Natural Human IgM: Correlation to *MYCN* Amplification and Effects of Retinoic Acid

K. David,[1] A. Ehrhardt,[1†] M.W. Ollert,[1¶] R. Erttmann,[2] R. Bredehorst[1] and C.-W. Vogel[1⋆]

[1]Department of Biochemistry and Molecular Biology, University of Hamburg, Martin-Luther-King-Pl. 6, 20146 Hamburg; and [2]Department of Paediatric Haematology and Oncology, University Hospital Eppendorf, 20246 Hamburg, Germany

Human neuroblastoma cells contain a 260 kDa surface-associated antigen (NB-p260) that is recognised by natural cytotoxic IgM antibodies. In this study we demonstrate that NB-p260 is expressed *in vivo* in a neuroblastoma tumour specimen but not in normal human tissues of neuronal origin. Since *MYCN* amplification is a clinical marker of neuroblastoma disease progression, we analysed the expression of NB-p260 in human neuroblastoma cell lines with different *MYCN* amplification status. However, both amplified and non-amplified neuroblastoma cell lines exhibited comparable NB-p260 expression. Treatment of neuroblastoma cells with the differentiation-inducing agent retinoic acid (RA) also had no effect on the expression of NB-p260. Collectively, the data suggest that expression of NB-p260 on human neuroblastoma cells is independent of malignancy and differentiation status of neuroblastoma. © **1997 Elsevier Science Ltd.**

Key words: neuroblastoma, tumour antigen, natural antibodies, *MYCN*, retinoid acid
Eur J Cancer, Vol. 33, No. 12, pp. 1937–1941, 1997

INTRODUCTION

HUMAN NEUROBLASTOMA cell lines express a 260 kDa surface antigen (NB-p260) that is recognised by cytotoxic natural human anti-neuroblastoma IgM antibodies [1]. *In vitro*, the cytoxicity of these antibodies is mediated both by activation of the classical pathway of complement and induction of apoptosis [2]. *In vivo*, the therapeutic application of the natural anti-neuroblastoma IgM induces growth suppression of established human neuroblastoma xenografts in nude rats [3]. The results of these animal studies and the observation that neuroblastoma patients lack significant levels of natural anti-neuroblastoma IgM antibodies suggest a potential of these antibodies as a novel therapeutic modality in the treatment of human neuroblastoma. However, more information on the expression of the NB-p260 antigen *in vivo* and its regulation is necessary before therapeutic studies can be realised. One important aspect is the effect of the status of differentiation and malignancy on the expression of NB-p260. To address the issue of malignancy, the oncogene *MYCN* was used as a surrogate marker. In a clinical setting, *MYCN* amplification of neuroblastoma tumours is an unfavourable prognostic marker which correlates with poor outcome [4, 5]. Human neuroblastoma cell lines that differ in their *MYCN* amplification status were investigated for NB-p260 expression. Furthermore, neuroblastoma cells were treated with retinoic acid (RA) to evaluate the effect of RA-induced differentiation on the expression of NB-p260 [6, 7]. Our data show that expression of NB-p260, the target of natural cytotoxic IgM antibodies on neuroblastoma cell lines, is independent of *MYCN* amplification and RA-induced differentiation.

MATERIAL AND METHODS

Chemicals

All-*trans*-retinoic acid (RA) (Sigma, Deisenhofen, Germany) was dissolved in absolute ethanol at a concentration of 10^{-2} M and stored at –20°C for a maximum of 2 weeks.

⋆Correspondence to C.-W. Vogel.
†Current address: Fraunhofer Institute for Toxicology und Aerosol Studies, Nikolai Fuchs-Str. 1, 30625 Hannover, Germany.
¶Current address: Department of Dermatology, Technical University of Munich, Biedersteiner Str. 29, 80802 Munich, Germany.

Cell cultures

Human neuroblastoma cell lines were cultivated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 IU/ml), and streptomycin (100 µg/ml) [1]. Cells were harvested with a trypsin–EDTA solution. All cell culture reagents were obtained from Gibco BRL (Berlin, Germany). For induction of differentiation, neuroblastoma cells were seeded at a density of 5×10^3 cells/cm^2. RA was added at a final concentration of 10 µM the day after cell seeding [6]. The culture medium was replaced with fresh medium containing RA every other day.

Southern blot analysis

Genomic DNA was isolated from the tumour cell lines by phenol–chloroform extraction, digested with restriction endonuclease Eco-RI and fractionated on 0.8% agarose gels [8]. After transfer to Nytran filter (Schleicher and Schuell, Dassel, Germany), it was hybridised with the ^{32}P labelled *MYCN* probe Nb-1 (Oncor, Heidelberg, Germany) and washed under stringent conditions.

Northern blot analysis

Total RNA from each cell line was isolated using the guanidinium–phenol–chloroform extraction method [9]. mRNA was purified with the Oligo-dT-Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. Purified mRNA was separated on 1% formaldehyde–agarose gel, Northern blotted and subsequently hybridised with ^{32}P labelled Nb-1 [10]. Equal loading of gels was confirmed by stripping the filter and rehybridisation to a probe for β-actin [8].

Western blot analysis

Cellular extracts were fractionated on 10% SDS–polyacrylamide gels and transferred (120 min at 50 V) on to polyvinylidene difluoride membrane (Millipore, Eschborn, Germany) in 3-(cyclohexylamino)propyl sulphonic acid transfer buffer according to established procedures [11]. The blots were immunostained with a *MYCN* specific monoclonal antibody (Dianova, Hamburg, Germany) followed by incubation with an alkaline-phosphatase labelled secondary antibody against mouse IgG (Sigma).

Immunofluorescence staining

Staining for MYCN. Neuroblastoma cells cultivated in Permanox chamber slides (Nunc, Wiesbaden, Germany) were fixed with 3.7% formaldehyde followed by permeation with 2.5% Triton X-100. Immunostaining was performed with a primary antibody against *MYCN* (Dianova) followed by incubation with a fluorescent Cy3-conjugate against mouse IgG (Sigma).

Staining for microtubule-associated proteins (MAPs). Cultivated neuroblastoma cells were methanol-fixed and incubated with a polyclonal antibody against MAPs (Sigma). A second incubation was performed with a dichlorotriacinylamino-fluorescein conjugated goat anti-rabbit immunoglobulin (Dianova). The preparations were examined with a Zeiss microscope (Oberkochen, Germany) equipped for epifluorescence.

Histological techniques

Serial cryostat sections (4–6 µm) of a human neuroblastoma tumour and normal human adrenal gland were chloroform–acetone fixed and incubated with human anti-neuroblastoma IgM. Detection of bound IgM was performed with an alkaline phosphatase conjugated goat anti-human IgM (Sigma) as described [3]. Colour development of alkaline phosphatase activity was performed with Fast Red/naphtol (Sigma). Nuclei were counterstained with haematoxylin (Merck, Darmstadt, Germany) [3].

In vitro *binding and cytotoxicity assays*

Natural IgM binding to neuroblastoma cells was determined by indirect immunofluorescence. Aliquots of 1×10^6 neuroblastoma cells were incubated in 100 µl normal human serum (NHS) from anti-neuroblastoma-positive individuals as described [1]. A dichlorotriacinylamino–fluorescein conjugated goat anti-human IgM (Dianova) was used as a secondary antibody. Cells were analysed in a flow cytometer (FACScan, Becton Dickinson, Heidelberg, Germany). For cytotoxicity assays, aliquots of 1×10^6 neuroblastoma cells were incubated with 100 µl normal human serum from anti-neuroblastoma-positive individuals, followed by incubation with non-cytotoxic NHS as a source of complement as described [1]. Complement-mediated cytotoxicity was determined by flow cytometry using a propidium iodide assay [1].

RESULTS

In vivo *expression of NB-p260 in human tissue*

The expression of NB-p260 was investigated in cryostat sections of normal human tissues of neuronal origin and neuroblastoma tumour tissue. Binding of human anti-neuro-

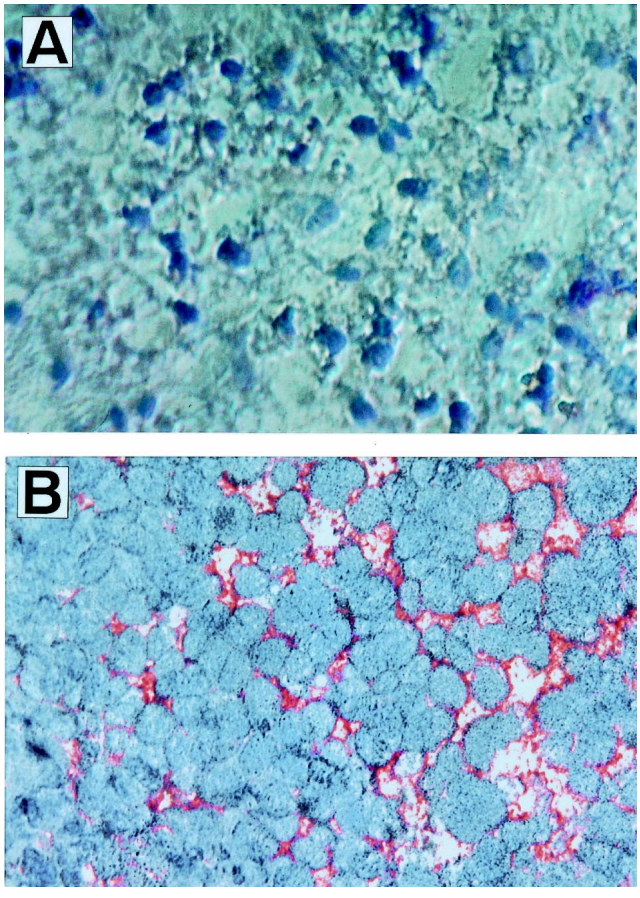

Figure 1. Binding of natural human anti-neuroblastoma IgM (red staining) in cryostat sections of normal human adrenal medulla (a) and human neuroblastoma tumour (b). Bound anti-neuroblastoma IgM was detected by an alkaline phosphatase labelled goat antibody against human IgM (magnification: ×400).

blastoma IgM was detectable neither in normal human adrenal medulla (Figure 1a) nor in brain tissue (not shown), but extensive binding to tumour tissue from a neuroblastoma patient was observed (Figure 1b). Comparison of serial neuroblastoma tumour sections revealed a relatively homogeneous expression pattern of NB-p260. Based on these data, NB-p260 appears to be expressed *in vivo* at least by the vast majority of neuroblastoma cells.

Effect of MYC*N amplification on NB-p*260 *expression*

Two *MYCN* amplified neuroblastoma cell lines (LA-N-1, LA-N-5) [12] and one non-amplified neuroblastoma cell line (SK-N-SH) [13] were used to investigate the effect of *MYCN* amplification on the expression of NB-p260. The *MYCN* status of these neuroblastoma cell lines was verified by genomic, m-RNA and protein analyses. The amplified copy status of LA-N-1 and LA-N-5 cells and the single copy status of SK-N-SH cells was confirmed by Southern blot analysis (not shown). Hybridisation of Eco RI digested genomic DNA from LA-N-1 and LA-N-5 cells with a *MYCN* specific probe yielded a strong signal of a 2.0 kb fragment, while under identical conditions only a weak signal was obtained with DNA from SK-N-SH cells. Analysis of the 3.2 kb *MYCN* RNA transcript demonstrated that the expression of *MYCN* in LA-N-1, LA-N-5 and SK-N-SH neuroblastoma cells correlates with the gene amplification status of these cell lines. The amplified neuroblastoma cell lines showed a strong staining of the *MYCN* transcript, whereas no mRNA to *MYCN* was detectable in SK-N-SH cells (not shown). In accordance with these data the 65 kDa *MYCN* protein was detectable via immunoblot analysis in LA-N-1 and LA-N-5 cell extracts, but not in SK-N-SH cell extracts (not shown). The relative amount of *MYCN* protein paralleled those of *MYCN* RNA. A corresponding expression pattern was observed by immunofluorescence analysis of the *MYCN* protein in LA-N-1, LA-N-5 and SK-N-SH neuroblastoma cells. LA-N-1 and LA-N-5 cells (Figures 2a and b) showed intense fluorescent staining signal, while the staining intensity was much weaker in SK-N-SH cells. They showed a heterogeneous pattern with a spectrum of lightly stained cells and cells with no detectable stain (Figure 2c). In the absence of the primary anti-*MYCN* antibody, no fluorescence was observed (Figure 2d).

The *in vitro* expression of NB-p260 in these cell lines was analysed by the extent of binding of anti-neuroblastoma IgM and anti-neuroblastoma IgM-mediated cytotoxicity. FACS analysis revealed comparable IgM binding to the 3 neuroblastoma cell lines (Table 1). These data correlate with the degree of cytotoxicity, which was almost identical for all 3 neuroblastoma-cell lines (Table 1). Collectively, the results demonstrate that the expression of NB-p260 is not affected by the *MYCN* amplification status.

*Effect of RA-induced differentiation on NB-p*260 *expression*

In order to evaluate the effect of a varying differentiation status on the expression of NB-p260, SK-N-SH neuroblastoma cells were exposed to the differentiation-inducing agent RA. After 2 days of RA treatment, SK-N-SH cells showed a

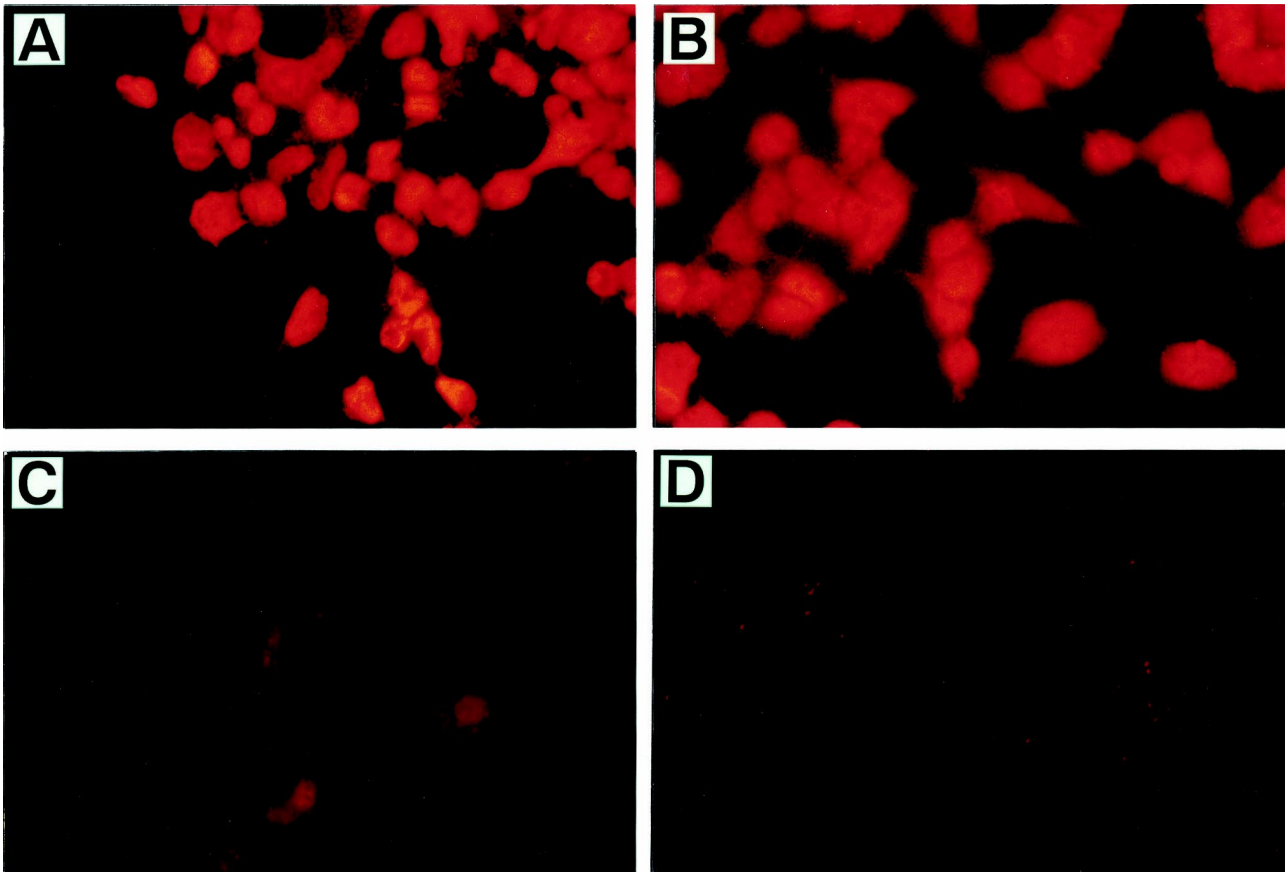

Figure 2. Immunofluorescence staining of adherent LA-N-1 (a), LA-N-5 (b) and SK-N-SH (c) cells for *MYCN* protein after formaldehyde fixation, with a Cy3 conjugate as secondary antibody (magnification: ×1000). In a control experiment, the primary anti-*MYCN* antibody was omitted (d).

Table 1. *Cytofluorometric analysis of anti-neuroblastoma IgM binding and anti-neuroblastoma IgM-mediated cytotoxicity*

Neuroblastoma cell line	Binding of anti-neuroblastoma IgM (mean channel)	Anti-neuroblastoma IgM-mediated cytotoxicity (%)
LA-N-1	519	87
LA-N-5	631	91
SK-N-SH	490	81

IgM binding and IgM-mediated cytotoxicity were analysed by flow cytometry as described in Material and Methods.

Table 2. *Effect of retinoic acid treatment on binding and cytoxicity of anti-neuroblastoma IgM to SK-N-SH neuroblastoma cells*

Treatment with retinoic acid	Binding of anti-neuroblastoma IgM (mean channel)	Anti-neuroblastoma IgM-mediated cytotoxicity (%)
Control	546	63
1×10^{-5} M	693	59

IgM binding and IgM-mediated cytotoxicity were analysed by flow cytometry as described in Material and Methods.

significantly reduced cell proliferation (not shown) and developed morphologically defined neurites (Figure 3b). The outgrowth of neurites was accompanied by a significantly increased synthesis of MAPs as confirmed by an immunofluorescence assay (Figure 3d). Table 2 shows that the extent of anti-neuroblastoma IgM binding to SK-N-SH cells after treatment with RA was slightly higher as compared to untreated SK-N-SH cells. The degree of anti-neuroblastoma IgM-mediated cytotoxicity, however, was similar for control and RA-treated SK-N-SH cells (Table 2). Based on these data, RA-induced differentiation had no notable effect on the expression of NB-p260.

DISCUSSION

The results of this study indicate a lack of expression of NB-p260, the target of cytotoxic natural human anti-neuroblastoma IgM, in normal human tissues of neuronal origin including adrenal medulla and brain tissue. Since previous analyses have demonstrated that NB-p260 is also not expressed on the surface of cultured normal human melanocytes, fibroblasts and epidermal keratinocytes [1], normal human tissues appear to be devoid of surface-associated NB-p260. This assumption is in accordance with the observation that cytotoxic anti-neuroblastoma IgM antibodies are present in the sera of healthy individuals [1]. Our results revealed abundant *in vivo* expression of NB-p260 in a tumour specimen from a human neuroblastoma patient. The expression patterns were relatively homogeneous in serial tumour sections suggesting that NB-p260 is expressed at least by the vast majority of neuroblastoma cells in this tumour.

Based on these expression data, NB-p260 could be a promising target for an antibody-based therapy. However,

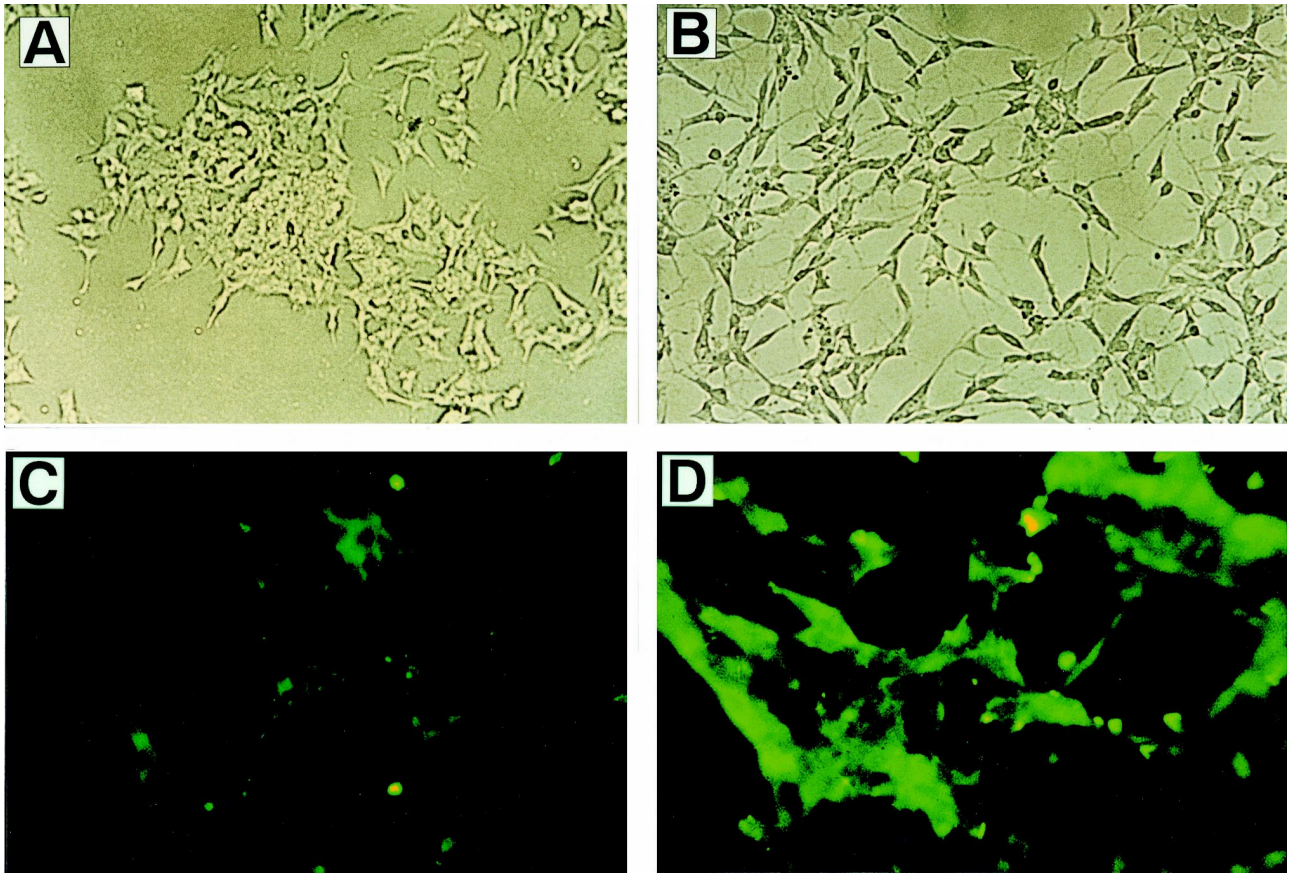

Figure 3. Effect of RA on neurite outgrowth (a) and (b) and the expression of MAPs (c) and (d) in SK-N-SH neuroblastoma cells. Shown are SK-N-SH cells before (a and c) and after treatment with RA for 5 days (b) and (d). MAPS were detected by an immunofluorescence assay using a fluorophore (DTAF)-conjugated secondary antibody.

neuroblastoma tumours are known to be heterogeneous with regard to their degree of malignancy [14]. Therefore, the expression of NB-p260 was evaluated in neuroblastoma cell lines with different *MYC*N amplification status. Amplification of *MYC*N of neuroblastoma tumours correlates with a poor outcome of the disease [4, 5] and has been shown to affect the expression of several important proteins such as the multidrug-resistance-associated protein [15]. The *MYC*N amplification status of the different neuroblastoma cell lines was investigated by protein, mRNA and genomic analyses, since in recent publications evidence has been presented that overexpression of the *MYC*N protein is not always correlated with gene copies or RNA transcripts [8, 16]. Comparison of amplified and non-amplified neuroblastoma cell lines, revealed no significant differences with regard to anti-neuroblastoma IgM binding and anti-neuroblastoma IgM-mediated cytotoxicity, indicating that the expression of NB-p260 is independent of the *MYC*N amplification status.

The impact of varying differentiation status on the expression of NB-p260 was evaluated in another set of experiments using RA-treated SK-N-SH neuroblastoma cells. RA is known to exert various important biological effects in the control of normal growth and differentiation. Treatment of neuroblastoma cells induces several morphological changes including the outgrowth of neurites [6, 7]. Since microtubule-associated proteins (MAPs) are key regulators of neurite outgrowth [17], the enhanced expression of these cytoskeletal proteins by RA treatment was used in this study as marker of RA-induced differentiation. Subsequent analyses indicated that the expression of NB-p260 was not affected by RA treatment despite the induction of significant cellular morphology changes and an enhanced synthesis of MAPs.

Collectively, our data suggest that expression of NB-p260 on human cells is independent of the malignancy and differentiation status of neuroblastoma. As a consequence, NB-p260 represents an excellent target for an antibody-based anti-neuroblastoma therapeutic approach since (i) NB-p260 was not expressed in normal human tissue, at least not in the corresponding normal human tissue, (ii) NB-p260 was expressed in all neuroblastoma cell lines investigated so far, regardless of neuroblastoma differentiation and malignancy status and (iii) NB-p260 was expressed *in vivo* by the vast majority if not all neuroblastoma cells in 1 neuroblastoma tumour specimen.

1. Ollert MW, David K, Schmitt C, *et al.* Normal human serum contains a natural IgM antibody cytotoxic for human neuroblastoma cells. *Proc Natl Acad Sci USA* 1996, **93**, 4498–4503.
2. Ollert MW, David K, Vollmert C, Bredehorst R, Vogel C-W. Human natural IgM antibodies induce apoptosis of human neuroblastoma cells both *in vivo* and *in vitro*. Annual Meeting of the German Society for Cell Biology, Hamburg, March 1996. *Eur J Cell Biol* 1996, **69**, A 100.
3. David K, Ollert MW, Juhl H, *et al.* Growth arrest of solid human neuroblastoma xenografts in nude rats by natural IgM from healthy humans. *Nature Med* 1996, **2**, 686–689.
4. Seeger RC, Brodeur GM, Sather H, *et al.* Association of multiple copies of the N-*myc* oncogene with rapid progression of neuroblastomas. *N Engl J Med* 1985, **313**, 1111–1116.
5. Duffy MJ. Cellular oncogenes and suppressor genes as prognostic markers in cancer. *Clin Biochem* 1993, **26**, 439–447.
6. Thiele CJ, Reynolds CP, Israel MA. Decreased expression of N-*myc* precedes retinoic acid-induced morphological differentiation of human neuroblastoma. *Nature* 1985, **313**, 404–406.
7. Ponzoni M, Bocca P, Chiesa V, *et al.* Differential effects of *N*-(4-hydroxyphenyl) retinamide and retinoic acid on neuroblastoma cells: apoptosis versus differentiation. *Cancer Res* 1995, **55**, 853–861.
8. Wada RK, Seeger RC, Brodeur GM, *et al.* Human neuroblastoma cell lines that express N-myc without gene amplification. *Cancer* 1993, **72**, 3346–3353.
9. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 1987, **162**, 156–159.
10. Matsunaga T, Shirasawa H, Tanabe M, *et al.* Expression of alternatively spliced *src* messenger RNAs related to neuronal differentiation in human neuroblastomas. *Cancer Res* 1993, **53**, 3179–3185.
11. Ollert MW, Calderone RA. A monoclonal antibody that defines a surface antigen on *Candida albicans* hyphae cross-reacts with yeast cell protoplasts. *Infect Immunol* 1990, **58**, 625–631.
12. Kohl NE, Gee CE, Alt FW. Activated expression of the N-*myc* gene in human neuroblastomas and related tumors. *Science* 1984, **226**, 1335–1336.
13. Gazitt Y, He YJ, Chang L, *et al.* Expression of N-myc, c-myc and MDR-1 proteins in newly established neuroblastoma cell lines: A study by immunofluorescence staining and flow cytometrie. *Cancer Res* 1992, **52**, 2957–2965.
14. Siegel ES, Sato JK. Neuroblastoma. In Moossa AR, Robson MC, Schimpfl SC, eds. *Oncology*, Baltimore, Maryland, Williams & Wilkins, 1986, 1211–1231.
15. Bordow SB, Haber M, Madafiglio J, Cheung B, Marshall GM, Norris MD. Expression of the multidrug resistance-associated protein (MRP) gene correlates with the amplification and overexpression of the N-myc oncogene in childhood neuroblastoma. *Cancer Res* 1994, **54**, 5036–5040.
16. Slavc I, Ellenbogen R, Jung W-H, *et al. myc* gene amplification and expression in primary human neuroblastoma. *Cancer Res* 1990, **50**, 1459–1463.
17. Ponzoni M, Casalaro A, Lanciotti M, Montaldo PG, Cornaglia-Ferraris P. The combination of γ-interferon and tumor necrosis factor causes a rapid and extensive differentiation of human neuroblastoma cells. *Cancer Res* 1992, **52**, 931–939.

Acknowledgements—This work was supported in part by the Deutsche Krebshilfe e.V. and the Fördergemeinschaft Kinderkrebszentrum Hamburg e.V.