Integrin up-regulation as marker of neuroblastoma cell differentiation: correlation with neurite extension

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Abstract

We have characterized the adhesion properties, integrin expression, and morphological changes due to extracellular matrix (ECM)-integrin interactions in a neuronal model. We showed that a modulation of some integrin heterodimers occurs during interferon- γ (IFN- γ) induced neuroblastoma (NB) cell differentiation. To better elucidate the possible implication and function of integrin receptors during neuronal maturation, we analyzed the changes in integrin expression in two human NB cell lines, LAN-5 and GI-LI-N, which represent different stages of neuronal differentiation. These models show opposite morphological maturation after interferon- γ and tumor necrosis factor- α (IFN- γ +TNF) treatment. While LAN-5 cells acquired the ability to extend long and branched neurites, GI-LI-N cells did not. Both cell lines showed enhanced expression of phenotypical and biochemical markers of neural maturation. Moreover, retinoic acid (RA) had different effects on the two NB cell lines: on LAN-5 cells it acts as a differentiation-promoting agent, while on GI-LI-N cells it has an antiproliferative effect, driving them to apoptosis. RT-PCR experiments and immunoprecipitation assays showed a late but marked increase in the expression of α_1 , α_2 , α_3 , and β_1 chains after IFN- γ +TNF treatment of LAN-5 cells, and only α_1 and β_1 chains upon RA induction. Treatment with IFN- γ +TNF induced GI-LI-N cells to show only a late and remarkable increase of α_1/β_1 heterodimer; on the contrary, RA treatment caused a decrease in all integrin chains. These changes are accompanied in differentiated cells by substantial increases in cell attachment to all purified ECM components tested and an increase of neurite-bearing cells and of average neurite length. In conclusion, these findings indicate a close correlation between up-regulation of integrins and neuronal morphogenesis.

Keywords: neuroblastoma; retinoic acid; interferon- γ ; differentiation; integrins; neuritogenesis

Abbreviations: ECM, extracellular matrix; IFN- γ , interferon- γ ; NB, neuroblastoma; TNF, tumor necrosis factor- α ; RA, retinoic acid; TNF-Rs, TNF receptors; NFs, neurofilaments, MAP₂, MAP₅, MAP τ , microtubule-associated proteins 2,5, (τ); GAPDH, glyceraldehyde-3-phosphate-dehydrogenase

Introduction

Integrins are the major family of cell surface receptors that mediate cell-to-cell and cell-to-extracellular matrix (ECM) interactions (Hynes, 1992). These integrin-mediated adhesive interactions are intimately involved in the regulation of many cellular functions, including embryonic development, homeostasis, leukocyte homing and activation, angiogenesis, tumor cell growth and metastasis (Juliano and Haskill, 1993; Stoker *et al*, 1968; Adams and Watt, 1993; Brooks *et al*, 1994; Blau *et al*, 1991). Indeed, intracellular contacts and cell-matrix interactions profoundly effect phenotypic features, such as gene regulation, cytoskeletal structure, cell growth control, and differentiation (Blau and Baltimore, 1991; Frisch and Francis, 1994; Ingber, 1993). Moreover, integrins are the major class of adhesion receptors in the nervous system, mediating the differentiating effects of ECM components on neural cells during development (Reichardt and Tomaselli, 1991).

Neuroblastoma (NB) is the most common extracranial malignant solid tumor of childhood arising from the sympathetic nervous system (De Bernardi *et al*, 1987). Although NB cells are presumably derived from a common transformed progenitor (Tsokos *et al*, 1987), they usually express multiple phenotypes, including the neuronal, neurilemmal, epithelioid, and melanocytic ones (Tsokos *et al*, 1987; Biedler *et al*, 1973). All phenotypes may interconvert *in vitro* either spontaneously or in a tissue culture condition-dependent manner (Ross *et al*, 1983; Ciccarone *et al*, 1989). A variety of drugs and biological agents have been reported to induce differentiation of NB cells *in vitro* (Abemayor and Sidell, 1989; Lanciotti *et al*, 1992; Ponzoni *et al*, 1992). Therefore, NB cell lines are suitable models for investigating the mechanisms of neuronal death and their relation to differentiation (Piacentini *et al*, 1992; Ponzoni *et al*, 1995; Hanada *et al*, 1993). Moreover, treatment-induced maturation has been well documented in some patients, similarly resulting in differentiation primarily along neural crest, neuronal and Schwannian cell lineages. Thus, this neural crest-derived tumor retains its ability to differentiate along physiological lineages for an adrenal medullary precursor cell, including the chromaffin and non-chromaffin lineages present in the adult adrenal medulla (Tsokos *et al*, 1987; Ciccarone *et al*, 1989; Abemayor and Sidell, 1989). Although the two human NB cell lines, GI-LI-N and LAN-5, used in this study as a well-defined model system (Abemayor and Sidell, 1989; Ponzoni *et al*, 1992, 1995; Montaldo *et al*, 1994), represent

different stages of chromaffin maturation of the adrenal medulla (Cooper *et al*, 1990a,b), they are both inducible along neuronal cell lineage using interferon- γ (IFN- γ) plus tumor necrosis factor- α (TNF) (Ponzoni *et al*, 1992; Montaldo *et al*, 1994).

The expression of integrins can be regulated by growth and differentiation factors (Dedhar *et al*, 1991; Heino *et al*, 1989). Indeed, the expression of certain integrins may be crucial for a differentiating phenotype (Menko and Boettiger, 1987) and consequently the expression of different integrins may be up- or down-regulated according to the differentiation state of a given cell (Dedhar *et al*, 1989).

Recently, we have also shown that integrin heterodimers are modulated during human NB cell differentiation (Rozzo *et al*, 1993). To further investigate the role of integrins in cell-type specific differentiation, and since NB cell adhesion to matrix is an absolute requirement for survival, proliferation or differentiation, we reasoned that modulation of their adhesion capability may represent a signal to activate a differentiation process in these cells, or alternatively may be a marker of ongoing differentiation, correlating with the process of neuritogenesis. Our results demonstrate that ECM, acting through integrin receptors, directs already committed NB cells to establish and maintain the differentiated state, regulating their neurite outgrowth capability.

Results

Effects of various biological agents on NB cell maturation

The hallmarks of NB cell differentiation *in vitro* following treatment with a variety of compounds or biological agents have been described in detail (Abemayor and Sidell, 1989; Ponzoni *et al*, 1992; Wuarin *et al*, 1991). Although the changes observed vary broadly among the different cell lines and inducing treatments, the general features of NB cell differentiation include slowing or complete arrest of proliferation, loosening of cell cluster and emission of neuritic processes. Moreover, a series of biochemical changes involving cytoskeletal proteins, membrane antigens and neurotransmitters biosynthetic pathways takes place. Morphological changes and expression of mature cytoskeletal proteins have been checked in the present study to assess cell differentiation.

Morphological evaluation The phenotypic characteristics of NB cell lines were evaluated by microscopic analysis of their morphology after 5 days of treatment. Treatment of LAN-5 cells with IFN- γ alone or in combination with TNF or with the addition of RA resulted in marked differentiation toward the neuronal phenotype. Cells appeared to be more spread out and polar, more adherent, with longer and interconnected neurites (Figure 1C, E, and I, respectively). TNF alone did not induce any morphological change (Figure 1G) in LAN-5 cells because the latter cells constitutively do not express TNF-Rs, while the p80 form of TNF-R is induced by IFN- γ treatment (Montaldo *et al*,

Figure 1 Morphological changes induced by differentiating treatments in LAN-5 (left side of the panel) and GI-LI-N (right side of the panel) NB cells. Phase contrast micrographs, bar 75 μ m. (A, B) control cells; (C, D) cells cultured in the presence of 1000 IU/ml IFN- γ ; (E, F) 1000 IU/ml IFN- γ plus 200 IU/ml TNF; (G, H) 200 IU/ml TNF; (I, L) 3×10^{-6} M RA.

1994). A less pronounced morphological differentiation was noted in GI-LI-N cells. Although also this cell line expresses IFN- γ -R (Montaldo *et al*, 1994), it seems almost insensitive to IFN- γ in terms of morphological maturation (Figure 1D). However, the synergistic effect of IFN- γ and TNF (Figure 1F) caused the appearance of a minor population of large flattened epithelial-like cells (also observed in TNF-treated cells, Figure 1H) among a majority of small spindle-shaped cells with elongated short neuritic processes, similar to those observed in cells treated with IFN- γ alone (Figure 1D).

By contrast, GI-LI-N cells treated with RA rounded up, reduced their diameter and eventually detached from the dish (Figure 1L). The same morphological features were observed by microscopy of May Grünwald-Giemsa-stained

Figure 2 Time-dependent induction of DNA fragmentation by RA-treatment of GI-LI-N cells. NB cells (1×10^6 cells/time point) either untreated (–) or treated with 3×10^{-6} M RA (+) were lysed and the DNA was isolated and electrophoresed as described in the Materials and Methods section. (P) pellets of intact DNA; (S) supernatants containing fragmented DNA; (M) *HaeIII*-digested ϕ X DNA as molecular size marker.

cells (data not shown). Thus, the morphology of GI-LI-N cells following RA treatment may be due to programmed cell death rather than to necrosis and/or terminal differentiation (Piacentini *et al*, 1992; Ponzone *et al*, 1995; Thompson, 1995; Rozzo *et al*, 1997). This hypothesis has been confirmed by electrophoretic analysis. Indeed, as shown in Figure 2 the characteristic pattern of DNA fragmentation in multiples of 180–200 bp was observed in GI-LI-N cells starting from 36 h of treatment with 3×10^{-6} M RA.

Cytoskeletal protein modulation Expression of cytoskeletal proteins by treated and control LAN-5 and GI-LI-N cells was investigated by immunofluorescence analysis of permeabilized cells. A representative panel of photomicrographs of NB cells stained with some mAbs to cytoskeletal proteins is shown in Figure 3, while the overall results are summarized in Table 1. The high molecular weight NFs and, in particular, their phosphorylated forms were up-modulated in both NB cell lines by treatment with the combination of IFN- γ and TNF, with a shift in fluorescence localization from a diffuse somatic to an intense perinuclear and neuritic pattern. By contrast, RA treatment induced a marked increase of NFs in LAN-5 cells, while it induced a faint decrease of these cytoskeletal proteins in GI-LI-N cells. As for NFs, treatment of NB cells with IFN- γ +TNF induced a drastic enhancement in the expression of MAP₂, MAP₅, and MAP (τ) with a sharp immunolocalization in the perinuclear and neuritic compartments. As expected, treatment with RA increased the expression of MAPs in LAN-5 cells, while it induced a modest down-modulation in GI-LI-N cells, further indicating the inability of RA to trigger neuronal differentiation in this cell line.

Modulation of integrin expression during NB cell differentiation

Recently, we (Rozzo *et al*, 1993) and others (Rossino *et al*, 1991) have shown that modulation of some integrin heterodimers occurs during NB cell differentiation. To better elucidate the possible implication and function of these integrins during neuronal maturation, we analyzed the changes in integrin expression in LAN-5 and GI-LI-N cell lines, which represent different stages of neuronal differentiation (Cooper *et al*, 1990a,b). To this end, both NB cell lines were treated with IFN- γ , IFN- γ +TNF, or RA, total RNA was extracted at different time intervals and utilized for the evaluation of integrin mRNA expression by RT-PCR. Oligonucleotide primers specific for α_1 , α_2 , α_3 , and β_1 integrin chains were used and the results are shown in Figure 4A, B and C. Treatment with IFN- γ induced a late but consistent up-regulation of all integrin chains in LAN-5 cells, responsive to IFN- γ in terms of differentiation capability, while no significant changes were observed in IFN- γ -resistant GI-LI-N cells. Treatment of both cell lines with the combination of IFN- γ +TNF, that appears to promote biochemical and functional changes associated with differentiation of both NB cell lines, drastically enhanced the mRNA expression for all integrin chains with time. As expected from the biological studies, we did not observe any changes in TNF-treated cells. By contrast, opposite effects were observed in the two cell lines after treatment with RA. Indeed, a clear up-regulation of the α_1 and β_1 mRNAs expression was detected after 3 days of treatment in LAN-5 cells, that are also induced into terminal differentiation, while an early and drastic down-modulation of all integrin chains was obtained in GI-LI-N cells, that are driven to apoptotic cell death by RA (Rozzo *et al*, 1997).

The pattern of expression of the corresponding protein products after 5 days of treatment was then analyzed by immunoprecipitation of lysates from treated and untreated surface radioiodinated NB cells (Figure 5). Using an antiserum to the β_1 chain, we co-immunoprecipitated both β_1 chain and the associated α chains with the following molecular weights: β_1 110 Kda, α_2 and α_3 150 Kda, α_1 200 Kda (Rozzo *et al*, 1993; Rossino *et al*, 1991; and Figure 5). These results have been confirmed using specific monoclonal antibodies to the various α chains (data not shown and Rozzo *et al*, 1993; Rossino *et al*, 1991). A significant enhancement of the $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ heterodimers was seen in both cell lines treated with the combination of IFN- γ +TNF. A similar increase was observed in IFN- γ -treated LAN-5 cells, while no integrin increase was visible when treated with TNF alone. Moreover, in cells in which the proliferation rate was arrested by starvation with 2% FCS-containing medium without affecting cell maturation (Lancioti *et al*, 1992; Rozzo *et al*, 1993), the amounts of such integrins were comparable to those seen in untreated cells. Finally, following treatment with RA, a very marked increase of the $\alpha_1\beta_1$ heterodimer was seen in LAN-5 cells, while a drastic down-modulation of integrin expression was observed in GI-LI-N cells. We

Figure 3 Immunofluorescence analysis of cytoskeletal proteins in LAN-5 cells. NB cells were untreated (A,a,B,b,C,c) or treated for 5 days (D,d,E,e,F,f) with IFN- γ +TNF and analyzed by fluorescence microscopy after staining with mAbs to MAP-5 (a,d), NF-200 Kda (b,e) and NF-200 Kda-P (c,f). Phase contrast pictures of the same fields (A,B,C,D,E,F). bar=20 μ m.

then investigated the time-dependence of these modulations by performing immunoprecipitation experiments at earlier times. In agreement with RT-PCR data, we did not observe any protein modulation before 2–3 days of treatment with the various differentiation protocols (data not shown).

Modulation of NB cell adhesion during differentiation

To assess the biological significance of these integrin modulations, we performed cell adhesion assays after 5 days of treatment. As shown in Figure 6, differentiation protocols induced a drastic enhancement in the attachment of LAN-5 cells to the most important ECM proteins. As expected, treatment with IFN- γ +TNF increased the ability of GI-LI-N cells to attach to ECM proteins, while RA treatment decreased the percentage of adhesion of these cells.

When testing the precocity of the effects of the differentiating agents, we did not observe early up-modulation of NB cell adhesion (Figure 7). Indeed, although the changes detected vary between the two NB cell lines, the inducing treatments and, overall, the ECM proteins tested, we generally observed a time-dependent up-regulation of cell attachment starting from 48–72 h after RA- and IFN- γ +TNF-treatments, thus confirming the data obtained by RT-PCR and immunoprecipitation.

Since biochemical features of NB cell differentiation are already detectable after 2 days of treatment with the differentiation promoting agents (Lanciotti *et al*, 1992; Ponzoni *et al*, 1992; Montaldo *et al*, 1994), these findings clearly indicate a role for integrin receptors in neuronal morphogenesis thus ruling out their possible involvement in the triggering of NB cell maturation.

This conclusion was confirmed by the fact that when differentiated LAN-5 cells were plated on ECM proteins,

Table 1 Effects of various differentiation-promoting agents on cytoskeletal proteins of LAN-5 and GI-LI-N human NB cells assessed by immunofluorescence analysis after 5 days

Treatment	NF-200 Kda	NF-200 KDa-P	MAP2	MAP5	τ
LAN-5 cells	78 $\pm$ 8 ^a	45 $\pm$ 4	58 $\pm$ 8	55 $\pm$ 4	19 $\pm$ 6
Control	++ ^b S ^c	+ S,P	+ S,P	+ S,P	+ S
IFN- γ (1000U/ml)	92 $\pm$ 7 ++ S,P,N	80 $\pm$ 10 ++ P,N	90 $\pm$ 6 ++ S,P	82 $\pm$ 8 ++ S,P,N	69 $\pm$ 5 ++ S,P,N
TNF (200U/ml)	70 $\pm$ 3 ++ S	44 $\pm$ 7 + S,P	59 $\pm$ 6 + S,P	58 $\pm$ 6 + S,P	20 $\pm$ 5 + S
INF- γ (1000U/ml)+ TNF (200U/ml)	94 $\pm$ 6 +++ S,P,N	90 $\pm$ 5 +++ P,N	93 $\pm$ 7 +++ S,P	91 $\pm$ 7 ++ P,N	82 $\pm$ 5 +++ S,P,N
RA (3 $\times$ 10 ⁻⁶ M)	92 $\pm$ 5 ++ S,P,N	83 $\pm$ 8 +++ P,N	79 $\pm$ 6 ++ S,P,N	93 $\pm$ 7 ++ P,N	60 $\pm$ 8 ++ P,N
GI-LI-N cells	80 $\pm$ 9	35 $\pm$ 7	60 $\pm$ 8	43 $\pm$ 5	32 $\pm$ 6
Control	+ S	+ S,P	+ S	+ S,P	+ S
IFN- γ (1000U/ml)	88 $\pm$ 6 + S,P	48 $\pm$ 5 + S,P	68 $\pm$ 6 + S,P	49 $\pm$ 7 + S,P	39 $\pm$ 4 + S,P
TNF (200U/ml)	90 $\pm$ 8 + S,P	44 $\pm$ 6 + S,P	73 $\pm$ 4 + S,P	50 $\pm$ 4 + S,P	42 $\pm$ 7 + S,P
IFN- γ (1000U/ml)+ TNF (200U/ml)	93 $\pm$ 7 ++ S,P	57 $\pm$ 5 ++ S,P,N	94 $\pm$ 6 ++ S,P	56 $\pm$ 5 ++ S,P,N	72 $\pm$ 6 ++ S,P
RA (3 $\times$ 10 ⁻⁶ M)	70 $\pm$ 7 + S	21 $\pm$ 4 + S	50 $\pm$ 9 + S	28 $\pm$ 5 + S	22 $\pm$ 5 + S

^aPercent of positive cells. Data are the means $\pm$ SD of three independent experiments. ^bFluorescence intensity: +, weakly positive; ++, moderately positive; +++, strongly positive. ^cFluorescence localization: S, somatic; P, perinuclear; N, neuritic

there was a great increase in their capacity to extend neurites with respect to untreated cells (Figure 8). In keeping with the working hypothesis, the fraction of neurite-bearing cells and mean neurite length increased with 2 days of IFN- γ +TNF treatment of both NB cell lines, when cells were plated for 12 h over culture dishes coated with collagen I (Table 2). Similar results were obtained when differentiated NB cells were plated on collagen IV and laminin (data not shown).

Discussion

The results presented in this paper are consistent with the interpretation that ECM, acting through integrin receptors, directs committed NB cells to establish and maintain the differentiated state.

NB is a peculiar tumour in its behavior inasmuch as it can occasionally undergo *in vivo* spontaneous or chemically-induced maturation or regression (Hass, 1988). One mechanism of tumour regression probably involves maturation of neoplastic cells into terminally differentiated, nonproliferating, ganglion-like cells (Cushing and Wolbach,

1927). A second mechanism, closely associated with differentiation and reported even for non-neuronal models (Hanada *et al*, 1993; Jensen *et al*, 1992) may involve the induction of programmed cell death.

Several reports have shown that retinoids have antitumor properties. Besides the well known differentiation-inducing ability of RA (Abemayor and Sidell, 1989; Sidell, 1982), it has recently been reported that RA can induce programmed cell death in neuronal models, depending on the phenotype of NB cells used (Piacentini *et al*, 1992), or can induce terminal differentiation followed by apoptosis independently of the cellular phenotype (Ponzoni *et al*, 1995). Thus, we investigated the effects of RA in two human, stage unrelated, neuroblastic-like NB cell lines in comparison with those of the combination of IFN- γ +TNF, which induce terminal differentiation of NB cells (Ponzoni *et al*, 1992; Montaldo *et al*, 1994). We found that RA completely differentiated LAN-5 cells, while it did not induce any morphological change or cytoskeletal protein modulation, typical of neuronal maturation in GI-LI-N cells. On the contrary, it drove GI-LI-N cells to programmed cell death (Rozzo *et al*, 1997). Moreover, the combination of

Figure 4 Expression of $\alpha 1$, $\alpha 2$, $\alpha 3$ and $\beta 1$ mRNAs during differentiation of LAN-5 and GI-LI-N cells. The data are obtained by densitometric scanning (A and B) of RT-PCR products analyzed by agarose gel electrophoresis using the AppleScan program. Different integrins and the housekeeping gene GAPDH were coamplified by RT-PCR of total RNA from control and treated LAN-5 (A) or GI-LI-N (B) cells. The data are calculated as the ratio between the optical density of the integrin chain band and that of GAPDH. The results are expressed as mean $\pm$ S.D. of the percentage of control level obtained in three different experiments. (C) Representative up-regulation of $\alpha 1$ mRNA expression by IFN- γ +TNF treatment. M, *Hae*III-digested ϕ X DNA as molecular size marker; lanes 1 to 3, LAN-5 cells; lanes 4 to 6, GI-LI-N cells. Lanes 1 and 4, untreated NB cells; lanes 2 and 5, NB cells treated with TNF (200 IU/ml); lanes 3 and 6, NB cells treated with IFN- γ +TNF (1000 IU/ml+200 IU/ml).

Figure 5 Integrin expression in LAN-3 and GI-LI-N cells treated for 5 days with differentiation promoting agents. Surface radioiodinated cell lysates of control and treated cells were immunoprecipitated using an anti β_1 antiserum as described in the Materials and Methods section. Molecular mass markers are in kilodaltons.

Figure 6 Adhesion properties of LAN-5 and GI-LI-N cells on ECM proteins after treatment with differentiating agents. Cell adhesion assays were carried out on control and treated NB cells as described in the Materials and methods section. The data are expressed as mean $\pm$ S.D. of the percentage of treated and untreated attached cells in three independent experiments each done in triplicate.

Figure 7 Time-dependent up-regulation of NB cell adhesion by differentiating agents. Cell adhesion assays were carried out on control and treated NB cells, at different times (Materials and Methods section). Data have been calculated as in Figure 6, but in this case, cell adhesion of untreated cells at each time-point has been taken as 100%. (A) LAN-5 cells treated with IFN- γ +TNF (1000 IU/ml+200 IU/ml); (B) LAN-5 cells treated with 3×10^{-6} MRA; (C) GI-LI-N cells treated with IFN- γ +TNF (1000 IU/ml+200 IU/ml) – (■) Coll I; (□) Coll IV; (●) LN; (△) FN; (○) VN.

Figure 8 Induction of neurite extension by differentiating agents in LAN-5 cells. Untreated NB cells (A, B, C) or treated for 2 days with 1000 IU/ml+200 IU/ml of IFN- γ +TNF (D, E, F) or with 3×10^{-6} M of RA (G, H, I) were removed from the culture plates and seeded on coated wells in serum-free RPMI medium for 2 h at 37°C. Phase contrast micrographs; bar corresponds to 100 μ m. Substrates were coated with 10 μ g/ml of (A, D, G) laminin, (B, E, H) collagen I, and (C, F, I) collagen IV.

Table 2 IFN- γ +TNF regulation of neurite outgrowth in NB cell lines

Conditions ^a	Neurite-bearing cells ^b (%)		Mean neurite length per cell ^c (μ m)	
IFN- γ +TNF pretreatment	LAN-5	GI-LI-N	LAN-5	GI-LI-N
no	15 $\pm$ 3	3 $\pm$ 1	55 $\pm$ 9	47 $\pm$ 10
yes	75 $\pm$ 15	30 $\pm$ 7	165 $\pm$ 25	88 $\pm$ 15

^aCulture dishes were coated with collagen I (10 μ g/ml). ^bNeurites, defined as process > 1 cell diameter in length, were scored for single cells in 20 randomly chosen microscopic fields on each dish. The value presented are the means of triplicate dishes $\pm$ standard deviations. ^cMean total length of neurite processes (including branches) measured for neurite-bearing cells

IFN- γ +TNF induced the morphological and biochemical differentiation of both NB cell lines. Indeed, changes in the type and amount of intermediate filaments have been shown to accompany neuronal cell differentiation (Shaw and Weber, 1982). We observed that in LAN-5 cells RA induces high molecular weight neurofilament proteins, particularly in the phosphorylated form, typical of mature neurons (Shaw and Weber, 1982; Carden *et al*, 1987), as does the treatment with IFN- γ +TNF. Moreover, data in chromaffin (Drubin *et al*, 1985) and NB cells (Ponzoni *et al*, 1992, 1995) induced to differentiate indicated that MAPs are key regulators of neurite outgrowth. The enhanced expression in both cell lines of these cytoskeletal proteins by treatment with IFN- γ +TNF and in LAN-5 cells by RA treatment confirms the ability of these biological agents to

induce true differentiation of NB cells. On the other hand, GI-LI-N cells treated with RA showed a slight but consistent down-modulation of almost all cytoskeletal proteins, thus confirming that cytoskeletal reorganization/disruption may occur during programmed cell death (Ponzoni *et al*, 1995; Thompson, 1995; Rozzo *et al*, 1997).

Adhesion of cells to matrix components is mostly mediated by integrin receptors (Hynes, 1992). These molecules, after binding to their specific ligands, cluster and promote the assembly of cytoskeletal proteins and, in turn, promote cell flattening on the substratum (Hynes, 1992; Burridge *et al*, 1988). Cells often display multiple integrins capable of interaction with a particular ECM protein ligand (Defilippi *et al*, 1991; Hemler, 1990). However, the precise mechanism(s) whereby signals from ECM proteins are transduced via integrins to the intracellular machinery controlling cell growth, behavior, differentiation, and apoptosis remains poorly defined (Juliano and Haskill, 1993; Clark and Brugge, 1995). There are many compelling examples of control of cell differentiation and gene expression through adhesive interactions with ECM (Defilippi *et al*, 1991; Dhawan *et al*, 1991; Kubota *et al*, 1992). Specifically, the extracellular matrix can influence various aspects of neuronal differentiation. Purified glycoproteins of the ECM such as laminin, collagens, and fibronectin have been shown to promote neurite outgrowth and survival *in vitro* (Davis *et al*, 1987; Tomaselli *et al*, 1987). The interactions of the

glycoproteins with the cells are mediated by integrins, cell surface receptors, shown to be involved in attachment and neuronal process outgrowth (Tomaselli *et al*, 1987). Moreover, neurite outgrowth, a hallmark of neuronal cells, requires the interaction of the cell with the ECM (Tomaselli *et al*, 1986), and integrins have been shown to mediate this interaction (Tomaselli *et al*, 1987). In this paper, we provide evidence that real differentiation-promoting protocols induced specific up-regulation of integrin heterodimers in NB cells. Since biochemical features of NB cell differentiation are already detectable after 2 days of treatment with differentiation agents (Lanciotti *et al*, 1992; Ponzoni *et al*, 1992), our findings clearly indicate a role for integrin receptors in neuronal morphogenesis, thus ruling out their possible involvement in the triggering of NB maturation. Indeed, the increased expression of integrin receptors occurs within 48–72 h of IFN- γ +TNF treatment, before neurite outgrowth can be morphologically appreciated (usually 72–96 h); thus the kinetics of induction is compatible with the role of these receptors in neurite extension. These results confirm and extend our previous ones (Rozzo *et al*, 1993) and the data of Rossino *et al* (1991), demonstrating that the increased expression of integrin complex leads to increased neurite extension on specific matrix proteins. Furthermore, the fact that RA did not increase, as did the IFN- γ +TNF treatment, the expression of α_2/β_1 and α_3/β_1 on LAN-5 cells, although it enhanced their capability to attach to ECM proteins, seems to confirm the overlapping and apparently redundant expression of integrin receptors on various cell types (Defilippi *et al*, 1991; Hemler, 1990).

It has also been suggested that growth cone-to substrate adhesion could be the rate-limiting step in neurite outgrowth (Turner *et al*, 1989; Zhang *et al*, 1993). This hypothesis is further supported by the findings of Rivas *et al* (1992), who observed that the addition of laminin to neuronal cultures grown on polylysine rapidly increases the rate of neurite extrusion by decreasing filopodial retraction, accelerating the outflow of organelles and microtubules into the adherent protrusions.

Mechanisms of extension are likely to be fairly similar in all cases of axon-like outgrowth. We have shown here that in NB cells, β_1 heterodimers play an essential role in differentiation-induced neurite outgrowth on ECM-coated substrata. We suggest that the role of integrins is to provide adequate traction at the growth cone and to allow for the cytoskeletal rearrangements necessary for the consolidation of the neuritic shaft by linking their cytoplasmic domains to the actin cytoskeleton.

Materials and Methods

Chemicals

Human recombinant interferon- γ (IFN- γ) and human recombinant tumour necrosis factor- α (TNF) were generous gifts of Boehringer Ingelheim (Milano, Italy); they were stored in aliquots (30 000 IU/ μ l) at -80°C . All-trans-retinoic acid (RA) (Sigma Chemical Co., St. Louis, MO) was dissolved in absolute ethanol at the concentration of 10^{-2} M and stored in aliquots at -20°C (up to 1 week). All the other reagents of biochemical and molecular biology grade were obtained from Sigma.

Cell cultures and treatments

The human NB cell lines, LAN-5 (Seeger *et al*, 1982) and GI-LI-N (Longo *et al*, 1988) were maintained in the logarithmic growth phase in 75 cm² plastic culture flasks (Corning, Bibby Sterlin, Ltd., Staffordshire, United Kingdom) in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 15% heat-inactivated FCS (Biochrom), sodium penicillin G (50 IU/ml), and streptomycin sulfate (50 μ g/ml) (complete medium) at 37°C in a 5% CO₂-95% air humidified incubator. Cells were split following treatment with 1 mM EDTA in Hanks' salts solution (Flow Laboratories, Milan, Italy), washed, counted, and replated in fresh complete medium. For induction of differentiation, cells were plated 2 days prior to the addition of inducing agents; the culture medium was replaced with fresh medium containing the inducing agents or their solvent control every other day. Cells were treated with 1000 IU/ml IFN- γ , and/or 200 IU/ml TNF, or 3×10^{-6} M RA, or starved in 2% FCS-complete medium for different times, as previously reported (Ponzoni *et al*, 1992, 1995; Rozzo *et al*, 1993).

Cell morphology

LAN-5 cells (5×10^5) and GI-LI-N cells (1×10^5) were seeded into T-25 flasks with 5 ml of culture medium and treated with the inducers as described above. From day 1 of treatment, 200 cells/culture from at least three different random areas of the flasks were examined daily with a phase contrast microscope (Olympus IMT-2).

Immunofluorescence analysis

Cytoskeletal proteins were detected as previously reported (Ponzoni *et al*, 1992), using mAbs with the following specificities: M_r 200 000 neurofilaments (NF-200 Kda) and their phosphorylated forms (NF-200 Kda-P), and microtubule associated proteins (MAP2, MAP5, and MAP(τ)) (Sigma-Aldrich, Milan, Italy). Briefly, cells were seeded at 2.5×10^4 /ml into multiwell slides and incubated as described. At defined times the slides were fixed in methanol/acetone (1/4, v/v) for 10 min at -20°C and incubated for 1 h at 37°C in a humid chamber with 200 μ l of the appropriately diluted mAbs. After three washings with PBS for 3 min at room temperature, they were incubated again with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Sigma-Aldrich). Cells were washed twice, observed under a microscope (Leitz Orthoplan; Leitz GmbH, Wetzlar, Germany) equipped with a UV 100-W mercury bulb, and evaluated as described elsewhere (Ponzoni *et al*, 1992, 1995).

The exposure time of an automatic camera has been quantified at a magnitude of $\times 250$ on fields of comparable numbers of cells labeled with different antibodies and used as the indirect index of fluorescence intensity. The overall staining intensity was uniform in control and treated cultures. The following score was used (exposure time): infinite (–); 60 s ($\pm$); 40 s (+); 20 s (++); less than 10 s (+++) (Ponzoni *et al*, 1992, 1995).

DNA fragmentation assay

After the RA treatment GI-LI-N cells were washed with ice-cold PBS, centrifuged at 1200 rpm for 10 min, and resuspended in lysis buffer consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2% Triton X-100. Cell lysis was carried out for 5 min at $+4^\circ\text{C}$ after brief vortexing. Cell lysates were then centrifuged at 13 000 rpm for 30', at $+4^\circ\text{C}$. Supernatants (S) were treated with 100 μ g/ml Proteinase K and incubated o.n. at 37°C . Pellets (P) were resuspended in 10 mM Tris-HCl (pH 8), 1 mM EDTA, 0.5% SDS, 100 μ g/ml Proteinase K, and incubated o.n. at 37°C . Both S and P were phenol and chloroform

extracted, then precipitated o.n. at -20°C by adding 1 : 100 (v/v) 1 M MgCl_2 , 1 : 10 (v/v) 3 M Na Acetate (pH 5.2) and 1 volume of isopropyl alcohol. DNA was then pelleted by centrifugation at 13 000 rpm, for 20 min, washed with 1 volume of 70% ethanol; and dried under vacuum. DNA was resuspended with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA buffer supplemented with 50 $\mu\text{g}/\text{ml}$ RNase A and incubated for 2 h at 37°C . Samples were then analyzed on 1.2% agarose gel electrophoresis, in 40 mM Tris-acetate, 1 mM EDTA (TAE) buffer (pH 8.0). One μg of *Hae*III-digested $\phi\chi$ DNA (Pharmacia Biotech, Uppsala, Sweden) or of 100 bp ladder (Boehringer Mannheim GmbH, Mannheim, Germany) was applied to each gel to provide molecular size markers.

RNA extraction, and semiquantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from control or treated cells ($\sim 1 \times 10^6$) using the guanidine-thiocyanate-chloroform method (Chomczynski and Sacchi, 1982). cDNA synthesis was made by priming different amounts of total RNA (ranging from 200 to 1 ng, the interval of linear signal generation by the two coamplified cDNAs, see below) with random hexamers by means of a commercial kit (Gene Amp RNA-PCR kit, Perking Elmer Corporation, Norwalk, CT) according to the suggested procedure. At the end of the synthesis, an integrin specific pair of primers together with two primers synthesized on the basis of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sequence were added in the same tube at the final concentration of 1 μM each. PCR reactions were carried out according to the kit suggestions for 30 cycles, each comprised of the following three steps: 1 min at 94°C , 2 min at 58° or 60°C , 3 min at 72°C . These were followed by a final extension step of 10 min at 72°C . The integrin specific primers, synthesized by standard procedure with an Applied Biosystem 391 DNA synthesizer (Foster City, CA), on the basis of the various human integrin cDNA published sequences were as follows: β_1 primers (forward 5'-GCGAAGGCATCCCTGAAAGT-3'; reverse 5'-GGACACAGGATCAGGTTGGA); α_1 primers (forward 5'-GAGCCTG-GAACCTATTAAGC-3'; reverse 5'-CGAACTGTGATGTTCTTTG-3'); α_2 primers (forward 5'-GTGTGGACATCAGTCTGGAA-3'; reverse 5'-CTGTGCACGATTGAAGGAAC-3'); α_3 primers (forward 5'-GCTGTATCCACGAGATCA-3'; reverse 5'-GTCAGCCTCTGTCTCTGA-3'). The GAPDH specific primers were: forward 5'-TGAAGGTCGGAGTCAACGGAT-3'; reverse 5'-CATGTGGCCATGAGGTCC-3'.

PCR products were run on 2% agarose gel electrophoresis in TBE buffer (90 mM Tris-HCl (pH 8.3), 90 mM Boric Acid, and 2 mM EDTA) and stained with ethidium bromide. The gels were then photographed under UV transillumination. Two bands with the expected size of 982 base pairs (bp) for GAPDH and 663 bp, or 635 bp, or 618 bp, or 653 bp for β_1 , α_1 , α_2 , or α_3 integrin chains, respectively, were detected. Quantitation was made by scanning the photographs with the aid of the AppleScan program. For each time point, the ratio between the densitometric value of integrin and GAPDH bands was calculated in both treated and control cells and variations were expressed as percent of the corresponding control value.

Cell surface radioiodination and immunoprecipitation

Cells were grown for different times in the presence or absence of the various biological agents listed above, then harvested and surface labeled with Na^{125}I , according to the procedure previously described (Rozzo *et al*, 1993). The anti β_1 rabbit polyclonal antiserum was generously provided by Dr. G. Tarone, University of Turin, Italy.

Immunoprecipitations were carried out as described (Rozzo *et al*, 1993). Briefly, the antibody was adsorbed onto protein G-agarose (Pierce, Rockford, IL, USA) by incubation for 1 h at 4°C with rotation in 10 mM Tris-HCl (pH 8.5) 150 mM NaCl, 0.5% Tween 20, 0.1% Renex, 0.01% ovalbumin, 2.5 mM NaN_3 (PORT). These immunoadsorbents were incubated overnight at 4°C with aliquots of radioiodinated cell lysates diluted in PORT, washed several times and eluted by boiling in Laemmli sample buffer (Laemmli, 1970). Samples were analyzed by SDS-PAGE on 7.5% polyacrylamide gels, followed by autoradiography on Hyperfilm MP (Amersham, Buckinghamshire, UK).

Cell adhesion and neurite outgrowth assay

Control and treated LAN-5 and GI-LI-N cells were metabolically labeled o.n. using 0.5 $\mu\text{Ci}/\text{ml}$ of Trans [^{35}S] Label (ICN) (spec. at: 1125 Ci/mmol) in Met-Cys-free RPMI 1640 (ICN) supplemented with 10% dialyzed FCS, sodium penicillin G (50 IU/ml) and streptomycin sulfate (50 $\mu\text{g}/\text{ml}$). Floating and adherent cells were then harvested with ice cold PBS without Ca^{++} and Mg^{++} , washed twice, resuspended in RPMI 1640 without FCS, and seeded on 96 well-plates (1×10^5 cells/well) previously coated with the following ECM purified components, at the concentration of 10 $\mu\text{g}/\text{ml}$ each, as previously described (Rozzo *et al*, 1993): Collagen type I (Coll I); collagen type IV (Coll IV); laminin (LN); fibronectin (FN); vitronectin (VN) (Sigma). Cells were allowed to adhere for 1 h at 37°C and then non-attached cells were washed out with PBS. Adherent cells were lysed with 200 μl of 1% Triton X-100 in PBS for 30' at 4°C , the lysates transferred to scintillation vials and the radioactivity associated to the single samples was measured with a β -counter (Packard). Percentage of adherent cells was determined as the ratio bound of total radioactivity added. To check whether differences between control and treated cells were not due to difference in metabolic labeling, some adhesion experiments were performed using Cresyl violet as described (Rozzo *et al*, 1993) with comparable results.

Methods for coating dishes and for preparing cells for the neurite outgrowth assay were the same as described for cell attachment assay except that cells were not radioactively labeled. Neurite outgrowth was assayed in triplicate in 35 mm dishes coated as for the attachment assay, using serum-free RPMI medium with or without $\text{IFN-}\gamma$ +TNF (1000 IU/ml+200 IU/ml) as indicated. After incubation at 37°C for 12 h, cells were fixed with 2.5% glutaraldehyde (Sigma) for 30 min and rinsed twice with PBS. Twenty microscopic fields were chosen randomly from each dish and single cells were scored. Cells with at least one process greater than one cell diameter were counted as neurite-bearing cells.

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