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Vitronectin Expression in Differentiating Neuroblastic Tumors

Integrin αvβ5 Mediates Vitronectin-Dependent Adhesion of Retinoic-Acid-Differentiated Neuroblastoma Cells

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The metastatic potential of undifferentiated neuroblastomas is typically lost when differentiation into ganglioneuroblastomas occurs spontaneously or is induced. Cell adhesion may play a role in metastasis, and we have shown recently that expression of integrin αvβ5 protein and mRNA is up-regulated in ganglioneuroblastomas* in vivo. *To investigate whether interactions of αvβ5 with matrix components play a role in the loss of metastatic potential, we used immunohistochemical and* in situ *hybridization to analyze neuroblastic tumors at various stages of differentiation for expression of the αvβ5 ligands, vitronectin and osteopontin, and determined the ability of vitronectin to promote attachment and neurite outgrowth* in vitro *in a retinoic-acid-differentiated neuroblastoma cell model. We found that vitronectin, but not osteopontin, was expressed in 5 of 5 ganglioneuroblastomas but was absent or weakly expressed in 6 of 6 undifferentiated neuroblastomas. Neuronal cell vitronectin was detected in 7 of 9 ganglioneuromas, 5 of 8 peripheral ganglia, and 14 of 21 adrenal gland medullae, confirming expression of vitronectin in mature peripheral neurons.* In vitro, *vitronectin promoted attachment of both undifferentiated and retinoic-acid-differentiated neuroblastoma cells, which was inhibited 20 and 60%, respectively, by monoclonal antibody anti-integrin αvβ5. Vitronectin-promoted neurite outgrowth of retinoic-acid-differentiated neuroblastoma cells was not inhibited by monoclonal antibody anti-αvβ5. These data suggest that the synthesis of vitronectin and the ability of integrin αvβ5 to mediate vitronectin adhesion on retinoic-acid-differentiated neuroblastoma cells may promote differentiation of neuroblastoma cells* in vivo. *(Am J Pathol 1997, 150:1631–1646)

Undifferentiated neuroblastoma tumors are rapidly invasive and metastatic.[1,2] These tumors, which generally arise in the peripheral nervous system, are capable of differentiating into neurons or ganglioneuroblastomas.[1,2] The mechanism underlying this differentiation, which can occur spontaneously or with treatment,[1,2] is unclear. However, comparative analysis of these two types of tumor may indicate the mechanism contributing to invasive and metastatic potential. *In vitro*, extracellular matrix proteins, including fibronectin, laminin, tenascin, and several proteoglycans, have been reported to induce differentiation, or neurite outgrowth, of undifferentiated neuroblastoma cells.[3–8] Interaction with matrix proteins, however, is not sufficient to induce complete differentiation, as differentiating agents such as retinoic acid or nerve growth factor are also necessary.[3–8] This suggests that interaction with the extracellular matrix protein is probably one of several cellular signals required to induce differentiation of undifferentiated neuroblastoma cells. A role for the extracellular matrix in the development of the periph-

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eral nervous system has been suggested.[9–13] Several extracellular matrix proteins, such as fibronectin, laminin, and tenascin, are expressed in a spatially and temporally specific manner during peripheral nerve development.[9–13] Certain extracellular matrix proteins, such as fibronectin, are also expressed in a specific manner during regeneration of peripheral nerve.[11]

Extracellular matrix proteins can signal or communicate with the cell through the integrin family of cell adhesion receptors.[14–16] Integrins are a large family of receptors that mediate a number of important cellular functions including cell-matrix adhesion, cell-cell adhesion, cell migration, neurite outgrowth, and matrix protein turnover.[14–16] The integrin receptors are heterodimers composed of an α- and β-subunit, both of which span the cell membrane lipid bilayer. The subunits are noncovalently associated but interact with high affinity.[14–16] Fifteen α- and eight β-subunits have been identified to date, and it has been shown that heterodimeric pairing is necessary for receptor function. Although pairing of α- and β-subunits determines, in part, ligand specificity, a number of other factors also can influence ligand specificity, such as cell type, differentiation state of the cell, divalent cations, and the affinity state of the receptor.[14–16] Previous studies regarding integrin expression of neuroblastoma cells have focused on the β1 subfamily.[17–25] Expression of the β1 integrin subunit in neuroblastomas appears to be affected by amplification and overexpression of the N-*myc* oncogene,[23–25] which is a marker of poor prognosis in these tumors.[1,2] Overexpression of transfected N-*myc* in a human neuroblastoma cell line can result in down-regulation of β1 integrin subunit expression.[23–25] These data, combined with animal studies, have led to the suggestion that human neuroblastoma cell lines that exhibit reduced integrin expression display more aggressive tumor behavior.[23–25] Functional studies have shown that at least four β1 integrins mediate neurite outgrowth *in vitro*, including $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$, and $\alpha8\beta1$.[17–19,26] *In vivo*, neuroblastic tumors have been reported to express the β1 subunit and the α1, α2, and α3 subunits, suggesting that integrins $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ are expressed on the cell surface.[20,21] To date, no studies of neuroblastic tumors *in vivo* have investigated the expression of the matrix proteins recognized by these three β1 integrins.

We recently have demonstrated that ganglioneuroblastoma cells express integrin $\alpha v\beta5$ mRNA and protein *in vivo*.[27] The ligands recognized by integrin $\alpha v\beta5$ (vitronectin and osteopontin) are distinct from those recognized by integrins $\alpha1\beta1$, $\alpha2\beta1$, and $\alpha3\beta1$ (eg, collagen, fibronectin, and laminin).[14–16,28] To determine whether the same ganglioneuroblastoma tumor cells express a ligand for integrin $\alpha v\beta5$ *in vivo*, we investigated the expression of matrix protein ligands of integrin $\alpha v\beta5$ in these tumors. *In vivo*, the expression of vitronectin protein was up-regulated in ganglioneuroblastoma cells as compared with undifferentiated neuroblastoma cells, and vitronectin was detected in normal peripheral nervous system neurons. To determine whether vitronectin promotes peripheral neuronal cell adhesion and neurite outgrowth, we performed adhesion and neurite outgrowth assays, utilizing undifferentiated and retinoic-acid-differentiated neuroblastoma cells. Our data identify an integrin $\alpha v\beta5$-mediated vitronectin-dependent cell adhesion mechanism in ganglioneuroblastoma tumors. These findings, taken together with the absence or minimal expression of vitronectin in undifferentiated neuroblastoma, lead us to speculate that integrin $\alpha v\beta5$ recognition of vitronectin contributes to the differentiation of undifferentiated neuroblastoma cells and to the nonmetastatic phenotype of ganglioneuroblastoma cells.

Materials and Methods

Tissue Collection

Formalin-fixed, paraffin-embedded tissues from biopsies of 16 peripheral neuroblastic tumors, 1 ganglioneuroma, and 1 adrenal gland, and 18 adrenal glands obtained at autopsy were provided by The Children's Hospital of Alabama. Eight of the eighteen adrenal glands contained peripheral ganglia in the capsule or medulla. In addition, formalin-fixed, paraffin-embedded tissues from biopsies of eight ganglioneuromas and two adrenal glands were obtained from The University of Alabama at Birmingham Hospital. Two differentiating neuroblastoma biopsies and one ganglioneuroblastoma biopsy were obtained from the Cooperative Tissue Procurement Network of the National Cancer Institute and were snap-frozen at −70°C.

These neuroblastic tumors were classified into undifferentiated neuroblastoma, differentiating neuroblastoma, and ganglioneuroblastoma using the Shimada classification as previously described.[1,2] The histological grading of neuroblastic tumors suggests the prognosis. Undifferentiated neuroblastomas, which are composed of undifferentiated neuroblasts, are typically associated with a poor prognosis and rapidly invade and metastasize.[1,2] In contrast, ganglioneuroblastomas, which are composed predominantly of mature ganglion or neuronal cells as well as

Schwann cells are associated with a better prognosis and metastasize less frequently.[1,2] An intermediate histological grade indicated by undifferentiated neuroblasts and immature ganglion cells, termed differentiating neuroblastoma, is associated with an intermediate prognosis. Formalin-fixed tumor tissues obtained from consecutive cases over a 4-year period in which formalin fixation was initiated in the operating room were utilized to optimize cellular mRNA preservation.

Antibodies, Immunohistochemistry, and Western Blot Analysis

The following antibodies were purchased: normal rabbit serum from Sigma Chemical Co. (St. Louis, MO), affinity-isolated rabbit anti-synaptophysin antiserum from Dako Laboratories (Glostrup, Denmark), rabbit anti-human fibronectin antiserum from Gibco BRL (Gaithersburg, MD), mouse monoclonal antibody (MAb) anti-collagen type IV IgG from ICN Biomedicals (Costa Mesa, CA), and goat anti-rabbit IgG and goat anti-mouse IgG horseradish peroxidase conjugates as well as goat anti-mouse IgG alkaline phosphatase conjugate from BioRad Laboratories (Richmond, CA). MAb anti-human laminin ascites (LAM-89) from Sigma and clones I to III from Gibco BRL were pooled in equal parts. MAb anti-osteopontin (MAb 53)[29] was a gift from Dr. John F. Harris (London Regional Cancer Center, London, Canada). Rabbit anti-integrin β5 IgG,[30] MAb anti-integrin αv (LM142),[31] and neutralizing MAbs anti-integrin $\alpha v\beta 3$ (LM609),[31] anti-integrin $\alpha v\beta 5$ (P3G2),[32] and anti-integrin $\beta 1$ (P4C10)[33] were gifts from Dr. David Cheresh (Scripps Research Institute, La Jolla, CA). Neutralizing MAb anti-αv (L230)[34] hybridoma (HB8448), which recognizes the αv subunit paired with any β-subunit, was purchased from the American Type Culture Collection (Rockville, MD), propagated in culture, and purified by protein A Sepharose chromatography. Antibody dilutions utilized for immunohistochemical analysis were as follows: rabbit anti-fibronectin antisera (1:500), rabbit anti-synaptophysin (1:50), normal rabbit serum IgG (10 μg/ml), rabbit anti-human vitronectin IgG (7.5 μg/ml), MAb anti-collagen type IV IgG (10 μg/ml), MAb anti-osteopontin IgG (10 μg/ml), and pooled MAb anti-human laminin ascites (1:200). The use of rabbit anti-human vitronectin IgG antibody and rabbit anti-human fibronectin antibody in immunohistochemical analysis was as described previously.[35] Rabbit anti-human vitronectin IgG antibody preadsorbed for 1 hour[35] with purified vitronectin (100 μg/ml) did not stain the tissue whereas preadsorbance with fibronectin (100 μg/ml) did not affect the staining pattern. Vitronectin was purified by the method of Yatohgo et al[36] and, after disulfide reduction, migrated on 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as a 65/75-kd doublet. Fibronectin was purchased from Gibco BRL.

Immunohistochemical analysis was performed as described previously.[27,35] Pepsin (Sigma) digestion (1 mg/ml in phosphate-buffered saline (PBS) for 1 hour at 22°C) was performed before reaction with anti-laminin, anti-collagen, anti-fibronectin, and anti-synaptophysin antibodies to enhance antibody reactivity. Antigen retrieval was performed before reaction with anti-osteopontin MAb to enhance antigen detection.[27] Positive control tissues for matrix protein antibody reactivities were as follows: liver for anti-vitronectin, decalcified bone for anti-osteopontin, endothelial cells of glioblastoma tumors for anti-tenascin, and blood vessel basement membrane for anti-fibronectin, anti-laminin, and anti-collagen type IV antibody reactivity.[27,29,35,37] Reticulin and trichrome stains were performed as described previously.[38] The grading scale for analysis of immunohistochemical positivity and intensity, and the method to determine the threshold of positivity as well as the percentage of positive cells, was as described previously.[27] Normal rabbit serum IgG was used as a negative control.[27,35,39] Gangliocytic cells and Schwann cells were identified as described previously,[27] and adrenal medulla gland pheochromocytes were identified by histological criteria as well as reactivity with rabbit anti-synaptophysin antibody on serial tissue sections.[1,2]

Western blot analysis was performed essentially as described previously.[40] The cell monolayer was lysed on ice in RIPA buffer,[35] centrifuged, subjected to 5% SDS-polyacrylamide gel electrophoresis, transferred to nylon membrane, blocked, reacted with MAb anti-human laminin (1:5000 dilution of ascites; clone I, Gibco BRL)[41,42] overnight at 4°C in 5% bovine serum albumin/PBS, washed, reacted with alkaline-phosphatase-conjugated goat anti-mouse IgG (1:3000 dilution) for 1 hour at 22°C, washed, and developed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) substrates.

cDNAs, Riboprobe Transcription, and in Situ *Hybridization Analysis*

The vitronectin and fibronectin constructs utilized for transcribing antisense riboprobes and the riboprobe transcription and *in situ* hybridization analysis were

performed as described previously.[27,39,43] The hybridized sections were graded as positive if ≥4 silver grains were observed over a nucleus or perinuclear area.[27,39] The fibroblasts in connective tissue and the endothelial cells in blood vessels of each tissue section served as a negative control for the vitronectin antisense riboprobe. The grading system to assess the intensity of hybridization and the method to determine the threshold of positivity as well as the percentage of positive cells was as described previously.[27]

Fluorescent Activated Cell Sorter (FACS) Analysis

SK-N-SH human undifferentiated neuroblastoma cells were purchased from the American Type Culture Collection and were propagated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Gibco BRL), as described.[44] Cells were maintained free of mycoplasma. SK-N-SH cells were induced to undergo differentiation with 1 μmol/L all-*trans* retinoic acid (Sigma) for 6 days, as described.[44] Consistent with a previous report,[44] retinoic acid treatment of SK-N-SH cells (1 μmol/L, 6 days) resulted in neurite outgrowth as well as decreased proliferation. FACS analysis was performed as described previously.[45]

Cell Adhesion and Neurite Outgrowth Assays

Retinoic-acid-differentiated SK-N-SH cells were metabolically labeled as a monolayer overnight (0.12 mCi of [^{35}S]methionine/ml of complete media), harvested with buffered EDTA, resuspended in adhesion assay buffer, and plated onto 96-well plates (20,000 cells/well) that had been previously coated with vitronectin, laminin, or ovalbumin (10 μg/ml) and allowed to attach (30 minutes at 37°C and 5% CO_2), as described previously.[35] Laminin was purchased from Sigma. For the adhesion assay, cells were incubated with neutralizing anti-integrin antibodies or mouse IgG (50 μg/ml) for 30 minutes before aliquots were placed into the wells. Adhesion was stopped at 30 minutes, followed by washing with PBS (2×), harvesting, and scintillation counting.[35] All experiments were performed in replicates of five, and background counts estimated from adhesion to ovalbumin-coated wells were subtracted. For the neurite outgrowth assay, cells were plated onto the coated wells in the presence of neutralizing anti-integrin antibody or mouse IgG (50 μg/ml), the plate was incubated at 37°C (5% CO_2) for 4 hours, and each well was photographed at 4 hours (×20 magnification).[17,18] All conditions were performed in replicates of five. The mean process outgrowth obtained on ovalbumin-coated control wells was subtracted. Neurite outgrowth, defined as process outgrowth greater than the mean process outgrowth on ovalbumin, was calculated for 20 cells for each antibody condition and averaged. Neurite outgrowth on laminin was used as the positive control and taken to be 100% after subtraction of process outgrowth on the control ovalbumin-coated plates.[17,18]

Results

Vitronectin Protein and mRNA Expression in Gangliocytic Cells of Ganglioneuroblastoma in Vivo

To determine the repertoire of matrix proteins expressed in neuroblastic tumors, neuroblastic tumor biopsies representing various stages of differentiation were subjected to immunohistochemical analysis with anti-vitronectin, osteopontin, fibronectin, laminin, tenascin, and collagen type IV antibodies.[27] In five of six undifferentiated neuroblastomas, tumor-cell-associated vitronectin was not detectable (Figure 1A and Table 1). In the one specimen that was weakly positive for protein (case 5, Table 1), only 3% of tumor cells expressed vitronectin. In contrast, in gangliocytic cells in five of five ganglioneuroblastomas, vitronectin protein was present in nearly all of the gangliocytic cells and strongly expressed in greater than 30% (Figure 1B, Table 2). Furthermore, vitronectin labeling was present in the adjacent extracellular matrix (Figure 1B). Normal rabbit serum IgG failed to react with the ganglioneuroblastoma (Figure 1C). Similarly, in >20% of gangliocytic cells in five of five neuroblastic tumors of intermediate stage, known as differentiating neuroblastoma, vitronectin protein was present. Although vitronectin protein was present in gangliocytic cells, it was not detected in neuroblasts in three of five ganglioneuroblastomas (Table 2). These results indicate that the expression of vitronectin protein is up-regulated with differentiation of peripheral neuroblasts *in vivo*. We further investigated vitronectin expression in a benign tumor of adults, termed ganglioneuroma, which is composed of ganglion cells (neurons) and Schwann cells. These tumors may be related to neuroblastoma, as it has been speculated that ganglioneuromas arise from neuroblastomas that have differentiated.[1,2] Immunohistochemistry revealed high expression of vitronectin protein in ~50% of ganglion cells in seven of nine ganglioneuromas (Table 3),

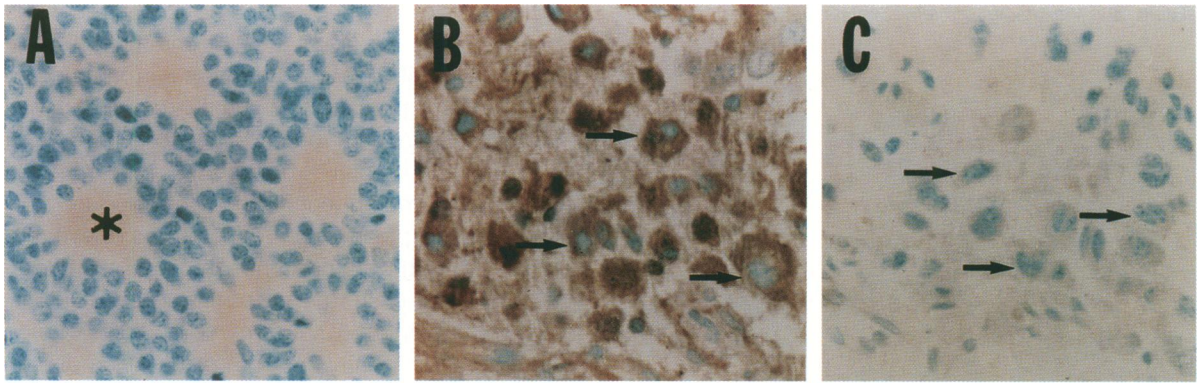

Figure 1. *Vitronectin protein expression in gangliocytic cells* in vivo. *Paraffin sections of undifferentiated neuroblastoma (case 2, Table 1) and ganglioneuroblastoma (case 4, Table 2) were subjected to immunohistochemical analysis with rabbit anti-vitronectin IgG antibody or normal rabbit serum IgG, as described in Materials and Methods.*[27,39] *Vitronectin reactivity was not detected in undifferentiated neuroblastoma* (A). *In contrast, vitronectin protein was detected in gangliocytic cells of ganglioneuroblastoma, within both the cytoplasm and the extracellular matrix* (B), *but not in the normal rabbit serum IgG control* (C). *The* asterisk *in* A *denotes the center of a Homer Wright rosette.* Arrows *in* B *and* C *denote gangliocytic cells. Magnification, ×100.*

consistent with the results obtained for gangliocytic cells of ganglioneuroblastoma.

Laminin and fibronectin were identified in a similar tumor distribution to that of vitronectin, in that fibronectin and laminin could not be detected in five of six undifferentiated neuroblastomas but were detected in gangliocytic cells or in the adjacent extracellular matrix in four of five ganglioneuroblastomas (Table 2). Tenascin and osteopontin could not be detected in any of the undifferentiated neuroblastomas or ganglioneuroblastomas. Tumor-cell-associated collagen IV was not detected by immunohistochemical analysis; however, collagen type IV was detected in the blood vessel basement membranes of all neuroblastic tumors, independent of the stage of differentiation, as were fibronectin and laminin.

Table 1. *Vitronectin mRNA and Protein Expression in Undifferentiated Neuroblastoma*

	VN				
Case	mRNA	Protein	FN	LM	COL
1	wk+	–	–	–	+
2	–	–	+	+	+
3	+	–	–	–	+
4	wk+	–	–	–	+
5	–	wk+	–	–	+
6	–	–	–	–	+

In situ hybridization and immunohistochemical analysis were performed as described in Materials and Methods.[27,39,43] Collagen was detected by trichrome and reticulin staining. The intensity of hybridization of the antisense vitronectin riboprobe was graded as follows: – (negative hybridization signal), ≤3 grains over the nucleus or perinuclear area; wk+ (weak hybridization signal), 4 to 5 grains over the nucleus or perinuclear area; + (positive hybridization signal), ≥6 grains over the nucleus or perinuclear area. The intensity of staining by immunohistochemical analysis was graded as follows: light brown staining, weakly positive (wk+); medium brown staining, positive (+); and dark brown staining, strongly positive (++). The age range for patients, denoted as cases 1 to 6 above, was 4 days to 2 years. VN, vitronectin, FN, fibronectin; LM, laminin; COL, collagen.

Table 2. *Vitronectin mRNA and Protein Expression in Ganglioneuroblastoma*

	Neuroblasts					Gangliocytic cells				
	VN					VN				
Case	mRNA	Protein	FN	LM	COL	mRNA	Protein	FN	LM	COL
1	wk+	–	–	–	+	+	++	wk+	wk+	+
2	–	wk+	–	–	+	+	++	++	wk+	+
3	–	–	–	–	+	+	++	–	–	+
4	–	+	–	–	+	+	++	–	+	+
5	–	–	–	–	+	–	++	wk+	+	+

In situ hybridization and immunohistochemical analysis were performed as described in Materials and Methods.[27,39,43] Ganglioneuroblastomas are composed of neuroblasts and gangliocytic cells as well as Schwann cells and neural stroma.[1,2] In all cases, ≥80% of tumor cells were gangliocytic cells. The age range for patients, denoted as cases 1 to 5 above, was 9 months to 3 years. See Table 1 for explanation of grading and abbreviations.

Table 3. *Vitronectin Protein Expression in Ganglioneuroma*

Case	VN protein	
	Ganglion cells	Schwann cells
1	+	wk+
2	+	−
3	++	++
4	++	++
5	++	++
6	+	wk+
7	−	+
8	−	−
9	+	++

Immunohistochemical analysis was performed as described in Materials and Methods.[27,39] The age range for patients, denoted as cases 1 to 9 above, was 7 to 78 years. See Table 1 for explanation of grading.

Both trichrome and reticulin stains, which nonspecifically stain collagen, demonstrated tumor-cell-associated positivity, which was most prominent in the five ganglioneuroblastomas.

To determine whether gangliocytic cells are capable of synthesizing vitronectin *in vivo*, *in situ* hybridization with an antisense vitronectin riboprobe was carried out on serial sections of the neuroblastic tumor biopsies described above.[27,39,43] In five of six undifferentiated neuroblastomas, vitronectin mRNA was undetectable or only weakly expressed in tumor cells (Figure 2A, Table 1), consistent with the absent or weak expression of vitronectin protein revealed by immunohistochemical analysis. In contrast, in four of five ganglioneuroblastomas, vitronectin mRNA was clearly demonstrated in gangliocytic cells (Figure 2C, Table 2), again consistent with the immunohistochemical analysis for vitronectin protein. Hematoxylin and eosin (H&E)-stained tissue sections of the undifferentiated neuroblastoma and of the ganglioneuroblastoma biopsies are shown in Figure 2, B and D, respectively. As a control, antisense fibronectin riboprobe was hybridized to a subset of these tumors, and a positive signal was detected in <5% of undifferentiated neuroblastoma cells and in 10 to 20% of gangliocytic cells in ganglioneuroblastoma, supporting the immunohistochemical findings. These results, together with the vitronectin immunohistochemical analyses, are the first to demonstrate that gangliocytic cells are capable of synthesizing vitronectin protein *in vivo*.

Vitronectin Expression in Adrenal Gland Pheochromocytes and Peripheral Ganglia

To determine whether pheochromocytes (specialized peripheral neurons) express vitronectin, 21 adrenal glands were subjected to immunohistochemistry using rabbit anti-vitronectin IgG antibody. In 14 of 21 adrenal glands, vitronectin protein was present in the cytoplasm of pheochromocytes (Figure 3A, Table 4). The negative control, normal rabbit serum IgG, failed to react with the pheochromocytes (Figure 3B), whereas the positive control, rabbit anti-synaptophysin, strongly stained pheochromocytes (Figure 3C). An H&E-stained slide of the adrenal medulla is shown in Figure 3D. Furthermore, in five of eight peripheral ganglia, vitronectin protein was present in the cytoplasm of ganglion cells (Table 4), indicating that vitronectin is expressed in mature peripheral neurons. The mean percentage of ganglion cells and pheochromocytes expressing vitronectin within individual sections was 34 and 41%, respectively, indicating that a significant portion of mature peripheral neurons express this protein.

Matrix Protein Expression in Schwann Cells

Vitronectin reactivity was associated with Schwann cells in four of five ganglioneuroblastomas (cases 1 to 3 and 5, Table 2), in the seven ganglioneuromas in which vitronectin was detected in ganglion cells (Table 3), and in all five peripheral ganglia in which vitronectin was detected in ganglion cells (Table 4). Antisense vitronectin riboprobe hybridized to a small percentage of Schwann cell nuclei in ganglioneuroblastomas, consistent with the immunohistochemical results and indicating that Schwann cells are capable of synthesizing vitronectin. Fibronectin, collagen, and laminin proteins were detected in a diffuse pattern that was associated with Schwann cells in all ganglioneuroblastomas, confirming and extending the results of studies of Schwann cell matrix protein expression in Schwannoma tumors.[3,4,9–12] Synthesis of fibronectin by Schwann cells was confirmed by *in situ* hybridization with an antisense fibronectin riboprobe, which resulted in detection of a strong fibronectin mRNA signal located over Schwann cell nuclei of ganglioneuroblastomas. Fibronectin mRNA was also detected over fibroblasts and smooth muscle cells in neuroblastic tumors of all stages of differentiation (data not shown).

Integrin αvβ5 Expression on Neuroblastoma Cells

We have recently reported the expression of a vitronectin receptor, integrin αvβ5, in ganglioneuroblas-

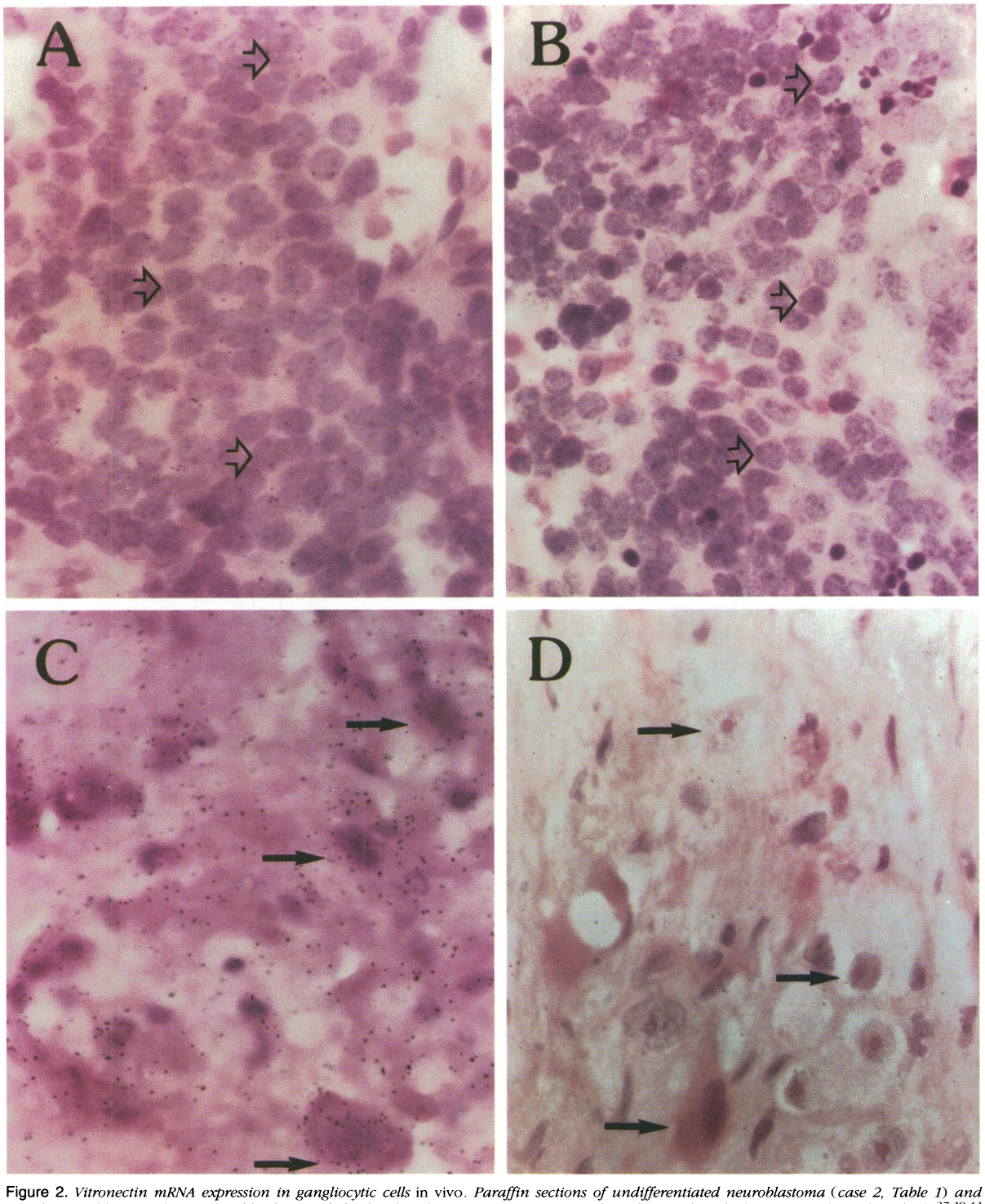

Figure 2. *Vitronectin mRNA expression in gangliocytic cells* in vivo. *Paraffin sections of undifferentiated neuroblastoma (case 2, Table 1) and ganglioneuroblastoma (case 1, Table 2) were hybridized with antisense vitronectin riboprobe, as described in Materials and Methods.*[27,39,43] *Vitronectin mRNA could not be detected in the undifferentiated neuroblastoma* (A). *In contrast, clusters of nuclear and perinuclear silver grains were detected in gangliocytic cells of ganglioneuroblastoma* (C), *indicating vitronectin mRNA expression. An H&E-stained section of the undifferentiated neuroblastoma and of the ganglioneuroblastoma is seen in* B *and* D, *respectively.* Open arrows *denote undifferentiated neuroblasts in the undifferentiated neuroblastoma* (A *and* B), *and* closed arrows *denote gangliocytes in the ganglioneuroblastoma* (C *and* D). *Magnification,* ×100.

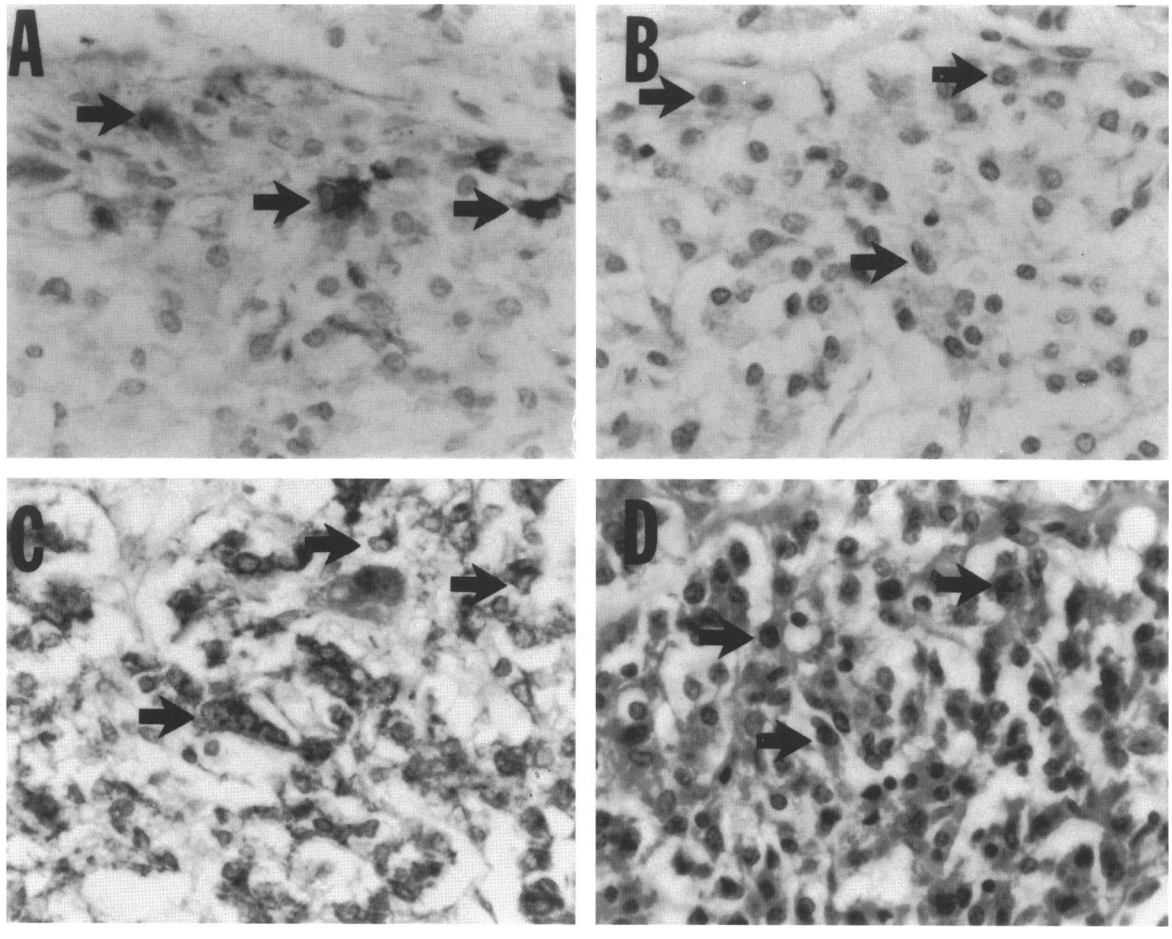

Figure 3. *Vitronectin expression in adrenal gland medulla pheochromocytes* in vivo. *Paraffin sections of normal adrenal medulla (case 7, Table 4) were reacted with rabbit anti-vitronectin IgG antibody, normal rabbit serum IgG, or rabbit anti-synaptophysin antibody, as described in Materials and Methods.[27,39] Strong vitronectin reactivity was demonstrated in medullae pheochromocytes* (**A**) *but not by the normal rabbit serum IgG control* (**B**). *Pheochromocytes were additionally identified by positive staining with anti-synaptophysin antibody* (**C**). *An H&E-stained section is shown in* **D**. **Arrows** *denote pheochromocytes. Magnification, ×100.*

toma cells *in vivo*.[27] In contrast to integrin αvβ5, integrin αvβ3 expression was found to be down-regulated with differentiation of neuroblastic tumors *in vivo*.[27] To determine whether integrin αvβ5 mediates vitronectin-dependent adhesion of ganglioneuroblastoma cells, we utilized an *in vitro* model based on retinoic-acid-induced differentiation of SK-N-SH human neuroblastoma cells.[44] Retinoic acid treatment (1 μmol/L, 6 days) induced neuronal differentiation of SK-N-SH cells identical to that previously reported (Figure 4B).[44] The morphology of untreated SK-N-SH neuroblastoma cells is shown in Figure 4A. By FACS analysis, we found that undifferentiated neuroblastoma cells (Figure 5A) and retinoic-acid-differentiated neuroblastoma cells (Figure 5B) expressed integrin αvβ5 similarly. The number of cells expressing integrin αvβ5, integrin αvβ3, and the β1 integrin subunit, as well as the mean fluorescent intensity were not significantly different between the undifferentiated and the retinoic-acid-differentiated neuroblastoma cells. Other investigators have previously reported expression of integrins αvβ3, αvβ1, α1β1, α2β1, α3β1, α4β1, and α5β1 on several neuroblastoma cell lines.[17–19,22]

Integrin αvβ5 Mediates Vitronectin-Dependent Adhesion of Retinoic-Acid-Differentiated Neuroblastoma Cells

To determine whether neuroblastoma cells adhered to vitronectin and whether integrin αvβ5 mediated this adhesion, adhesion assays were performed, as described.[35] The optimal concentration for vitronectin attachment was initially determined using a range of vitronectin concentrations (0.01 to 20 μg/ml). We found that 10 μg/ml vitronectin was optimal for attachment of both undifferentiated and retinoic-acid-

Table 4. *Vitronectin Protein Expression in Normal Peripheral Neurons*

Case	Age	Adrenal gland pheochromocytes	Ganglion cells
1	4 days	wk+	
2	1 months	wk+	
3	2 months	−	
4	2 months	+	
5	3 months	+	
6	4 months	−	
7	4.5 months	++	
8	5 months	−	
9	11 months	++	++
10	13 months	−	−
11	22 months	wk+	wk+
12	3 years	−	
13	3 years	+	+
14	4 years	wk+	−
15	5 years	−	
16	6 years	wk+	
17	9 years	+	+
18	9 years	−	−
19	13 years	+	+
20	59 years	+	
21	66 years	wk+	

Immunohistochemical analysis was performed as described in Materials and Methods.[27,39] The blank spaces denote absence of detectable ganglion in the adrenal gland or its capsule. See Table 1 for explanation of grading.

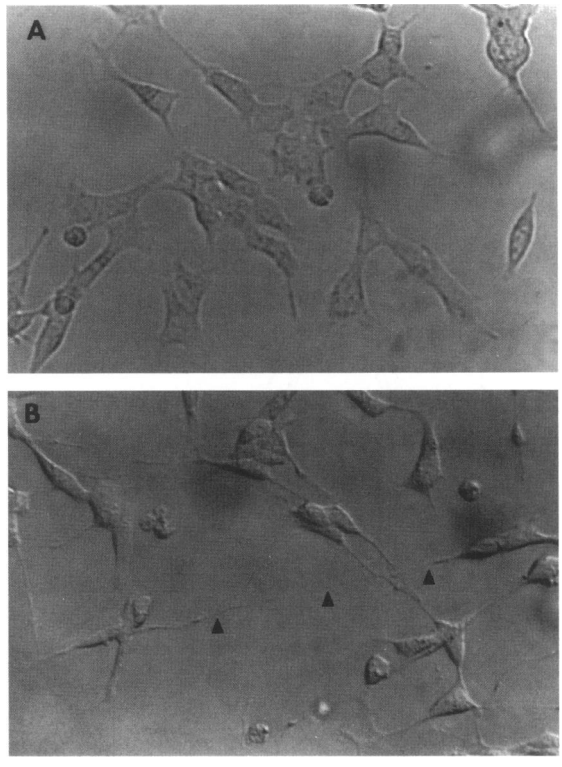

Figure 4. *Retinoic acid induces neurite outgrowth of human neuroblastoma cells. SK-N-SH human undifferentiated neuroblastoma cells were plated subconfluently in complete media and treated with 1 μmol/L all-trans retinoic acid for 6 days.[44] The medium was changed every 2 days with addition of fresh retinoic acid. Untreated neuroblastoma cells failed to demonstrate significant process outgrowth* (**A**) *as compared with the neurite outgrowth seen in retinoic-acid-treated cells* (**B**). **Arrowheads** *in* **B** *denote cell processes or neurite outgrowth. Magnification, ×100.*

differentiated neuroblastoma cells (Figure 6). In a typical 30-minute adhesion assay, the percent attachment of the undifferentiated and retinoic-acid-differentiated SK-N-SH cells was not statistically different, 30%, or 12,000, undifferentiated SK-N-SH cells and 25%, or 10,000, retinoic-acid-differentiated SK-N-SH cells attached to 10 μg/ml vitronectin (data not shown).

Neutralizing MAb anti-αvβ5 (P3G2) differentially inhibited attachment of undifferentiated neuroblastoma cells (20% inhibition; Figure 7A) and retinoic-acid-differentiated neuroblastoma cells (60% inhibition; Figure 7B), indicating that integrin αvβ5 on differentiated neuroblastoma cells mediates vitronectin attachment, in part. Neutralizing MAb anti-αv (L230) inhibited vitronectin attachment of both undifferentiated and retinoic-acid-differentiated SK-N-SH cells by 70% (Figure 7, A and B, respectively). Neutralizing MAb anti-integrin β1 (P4C10) inhibited attachment of undifferentiated and of retinoic-acid-differentiated neuroblastoma cells by 99 and 80%, respectively, indicating that, independent of the differentiation state, a β1 integrin(s) also promotes cell attachment to vitronectin. The similar inhibition of vitronectin attachment on retinoic-acid-differentiated neuroblastoma cells demonstrated with MAb anti-αv and MAb anti-αvβ5 is consistent with integrin αvβ5 being the major αv integrin mediating vitronectin attachment on these cells. These data indicate that integrin αvβ5 and a β1 integrin(s), such as α8β1, mediate vitronectin adhesion of the retinoic-acid-differentiated neuroblastoma cells, whereas integrin αvβ5, another αv integrin, and a β1 integrin mediate, at least in part, vitronectin adhesion of the undifferentiated neuroblastoma cells.

To determine whether vitronectin promotes neurite outgrowth of retinoic-acid-differentiated SK-N-SH cells and the role of integrin αvβ5, neurite outgrowth assays were performed in the presence or absence of neutralizing anti-integrin antibodies.[17,18] Retinoic-acid-differentiated SK-N-SH cells rapidly extended neurites on vitronectin (Figure 8B) as well as on the matrix protein laminin, which was used as a positive control (Figure 8A). At 4 hours in the presence of mouse IgG, the mean neurite outgrowth on vitronectin was 80% of that on laminin (Figure 9). Neutralizing MAbs anti-integrin αvβ5 (P3G2) and anti-integrin αvβ3 (LM609) failed to inhibit neurite outgrowth on vitronectin (Figure 9); however, neutralizing MAb anti-integrin β1 (P4C10) resulted in 50% inhibition, indicating that a β1 integrin(s) mediates neuroblas-

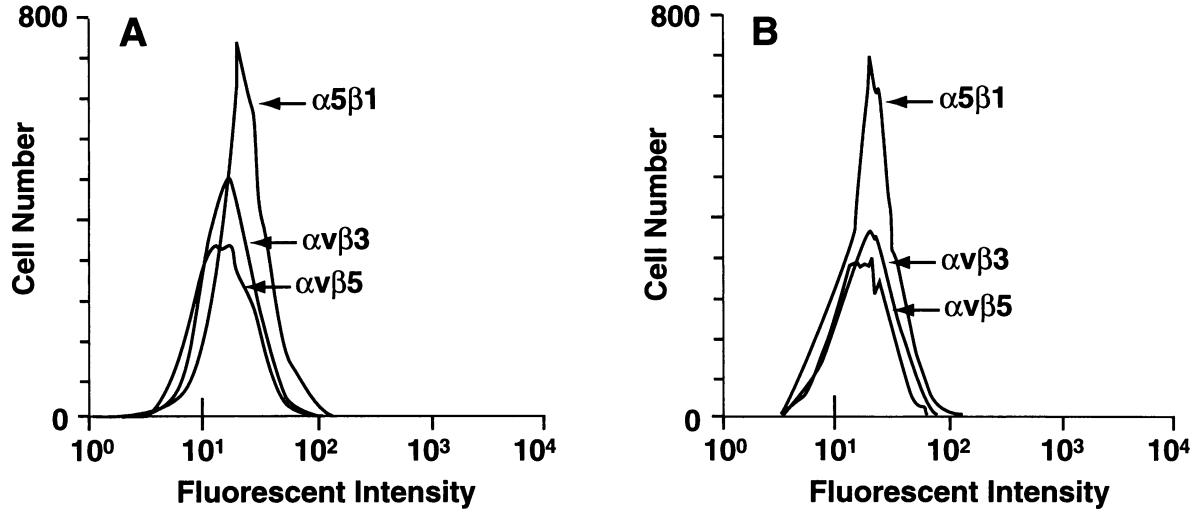

Figure 5. *Integrin αvβ5 expression on neuroblastoma cells. Undifferentiated and retinoic-acid-differentiated (1 μmol/L, 6 days) SK-N-SH neuroblastoma cells were harvested with buffered EDTA, reacted with primary anti-integrin antibodies, followed by goat anti-mouse or goat anti-rabbit IgG FITC-conjugated secondary antibody, and analyzed on a FACStar, as described in Materials and Methods.*[15] *MAb anti-integrin αvβ5 IgG was detected on 60% of the undifferentiated and 68% of the retinoic-acid-differentiated neuroblastoma cells; rabbit anti-integrin β1 IgG was detected on 95% of the undifferentiated and 92% of the retinoic-acid-differentiated neuroblastoma cells; and MAb anti-integrin αvβ3 IgG was detected on 75% of the undifferentiated and 80% of the retinoic-acid-differentiated neuroblastoma cells. The mean fluorescent intensity with mouse IgG as the primary antibody served as the negative control, and that fluorescence is denoted to the left of the vertical bar.*

toma cell neurite outgrowth on vitronectin. Neurite outgrowth on laminin was 55% inhibited by MAb P4C10 (Figure 9), consistent with previous reports of β1-integrin-mediated neurite outgrowth on laminin.[17,18] To confirm that laminin was not responsible for the neurite outgrowth of retinoic-acid-differentiated neuroblastoma cells adherent to vitronectin, Western blot analysis of the cell medium and monolayer with MAb anti-human laminin was performed at the end of the neurite outgrowth assay. Laminin was not detected in the cell media or cell monolayer of retinoic-acid-differentiated neuroblastoma cells adherent to vitronectin; however, the purified laminin positive control was readily detected (data not shown). These data suggest that vitronectin does indeed promote neurite outgrowth of retinoic-acid-differentiated neuroblastoma cells and that integrin αvβ5 mediates, in large part, vitronectin adhesion, whereas a β1 integrin(s) mediates vitronectin-dependent neurite outgrowth of retinoic-acid-differentiated neuroblastoma cells.

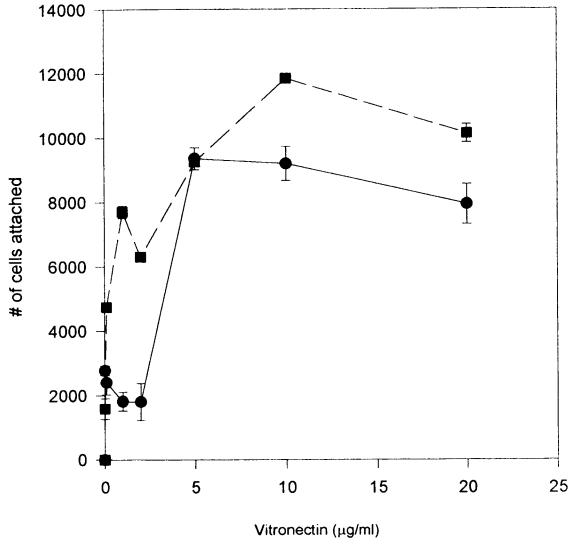

Figure 6. *Concentration dependence of vitronectin attachment by neuroblastoma cells. Undifferentiated and retinoic-acid-differentiated (1 μmol/L, 6 days) SK-N-SH neuroblastoma cells were metabolically labeled overnight with [*^{35}*S]methionine, harvested with buffered EDTA, resuspended in adhesion assay buffer, plated onto a 96-well plate previously coated with various concentrations of vitronectin (0.01 to 20 μg/ml), and allowed to attach (30 minutes at 37°C and 5% CO_2), as described in Materials and Methods.*[15] *Subsequently, the wells were washed with PBS (2×), and adherent cells were harvested with trypsin and scintillation counted. ■, undifferentiated SK-N-SH cells; ●, retinoic-acid-differentiated SK-N-SH cells. Adhesion to ovalbumin was subtracted out from each well. Results are the mean ± SEM of replicates of five for each condition.*

Discussion

The extracellular matrix plays a key role in regulating cell function,[3–16] but to date, no information has been available regarding extracellular matrix protein expression in neuroblastic tumors *in vivo*. Based on the well established role of the extracellular matrix in the development of the peripheral nervous system,[3–13] we postulated that the extracellular matrix of neuroblastic tumors would change with the stage of differentiation *in vivo*. Recently we have shown expression of a vitronectin receptor (integrin αvβ5) in the ganglioctic cells of ganglioneuroblastoma tumors.[27]

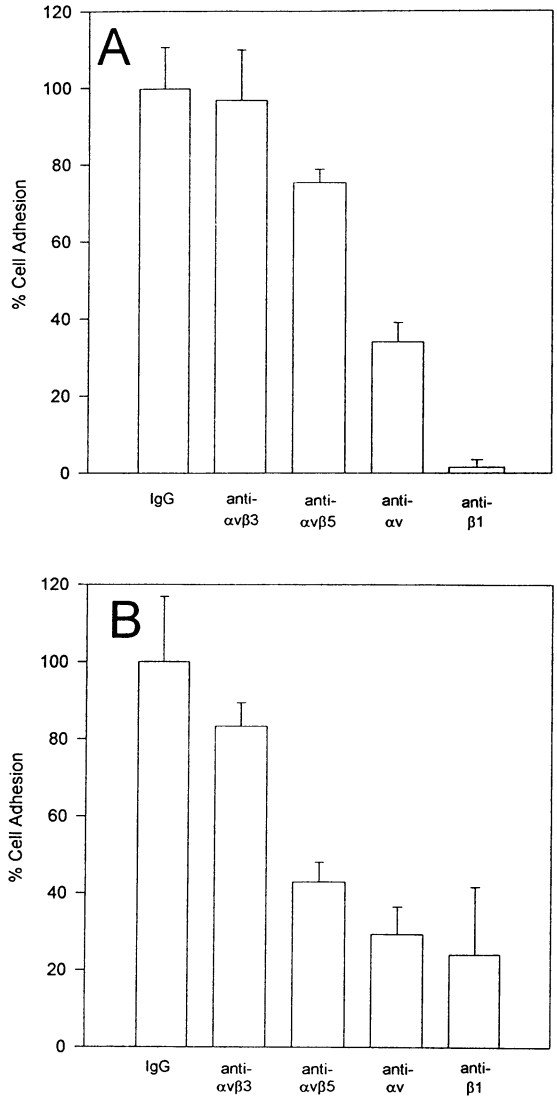

Figure 7. *Integrin αvβ5 on retinoic-acid-differentiated neuroblastoma cells mediates vitronectin adhesion. Undifferentiated and retinoic-acid-differentiated (1 μmol/L, 6 days) SK-N-SH neuroblastoma cells were metabolically labeled overnight with [^{35}S]methionine, harvested with buffered EDTA, resuspended in adhesion assay buffer, followed by incubation with 50 μg/ml neutralizing anti-integrin antibody or mouse IgG for 30 minutes, and then plated onto a 96-well plate previously coated with vitronectin (30 minutes at 37°C and 5% CO_2), as described in Materials and Methods.*[35] *Subsequently, wells were washed with PBS (2×), and adherent cells were harvested with trypsin and scintillation counted. MAb anti-integrin αvβ5 (P3G2) produced 20 and 60% inhibition of undifferentiated and retinoic-acid-differentiated neuroblastoma cell adhesion to vitronectin, as compared with the mouse IgG control, respectively. The latter indicates that integrin αvβ5 plays a role in mediating vitronectin adhesion of retinoic-acid-differentiated neuroblastoma cells. MAb anti-αv inhibited attachment of both cells by 70%. MAb anti-integrin β1 (P4C10) inhibited adhesion of both cells to vitronectin, indicating that αβ1 integrin(s) also participates in mediating vitronectin adhesion of these cells. MAb anti-integrin αvβ3 (LM609) failed to significantly inhibit adhesion of either cell to vitronectin. Adhesion to ovalbumin was subtracted out from each well. Results are the mean ± SEM of replicates of five for each condition.*

Therefore, we examined expression of those extracellular matrix proteins recognized by integrin αvβ5 (vitronectin and osteopontin) as well as several control proteins. We found that expression of vitronectin protein is up-regulated as neuroblastoma cells differentiate into neurons or gangliocytic cells *in vivo*, whereas osteopontin was not detectable. In addition, we found that vitronectin promotes attachment of retinoic-acid-differentiated neuroblastoma cells *in vitro*, suggesting that as neuroblastoma cells differentiate *in vivo* their synthesis of vitronectin may lead to a more stationary (adhesive) phenotype. Undifferentiated neuroblastoma cells also attach to vitronectin *in vitro*; however, the physiological significance of this is unclear as vitronectin is not detectable in most undifferentiated neuroblastomas *in vivo*.

Vitronectin also promoted neurite outgrowth of retinoic-acid-differentiated neuroblastoma cells, which at 4 hours was 80% of the neurite outgrowth observed on laminin. This neurite outgrowth on vitronectin does not appear to be secondary to laminin synthesis by the cells, as Western blot analysis of the cell medium and monolayer collected under the same conditions failed to detect laminin. The MAb anti-human laminin utilized for this Western blot analysis was directed toward an epitope in the β1 portion of intact laminin[41,42] and, therefore, detects a laminin isoform which contains the B1 chain. To date, seven isoforms of laminin have been identified, four of which do not contain the B1 chain.[46,47] It is not known which laminin isoform(s) is synthesized by undifferentiated or differentiated neuroblastoma cells *in vivo* or *in vitro*. The endoneurium (extracellular matrix) of human peripheral nerve *in situ* expresses the B1 chain.[48] Gangliocytic cells *in situ* are found within peripheral nerve, and neuroblastomas are thought to arise from undifferentiated cells within peripheral nerve[1,2]; thus, it is likely that laminin produced by differentiated neuroblastoma cells *in vitro* also includes a laminin isoform containing the B1 chain. We cannot rule out the possibility that a laminin isoform not containing the B1 chain may be synthesized by these cells and also contribute to neurite outgrowth on vitronectin. Vitronectin has been reported as promoting neurite outgrowth of PC12 pheochromocytoma cells,[49] and more recently, vitronectin has been shown to be developmentally expressed in avian retinal neurons[50] and in mouse tissue.[51,52] The role of vitronectin in neuronal development of neuroblastoma cells is unclear; however, it is unlikely that vitronectin alone promotes neuronal differentiation as vitronectin is a component of serum (approximately 250 μg/ml)[53] and neuroblastoma cells grown in medium containing 10% fetal bovine

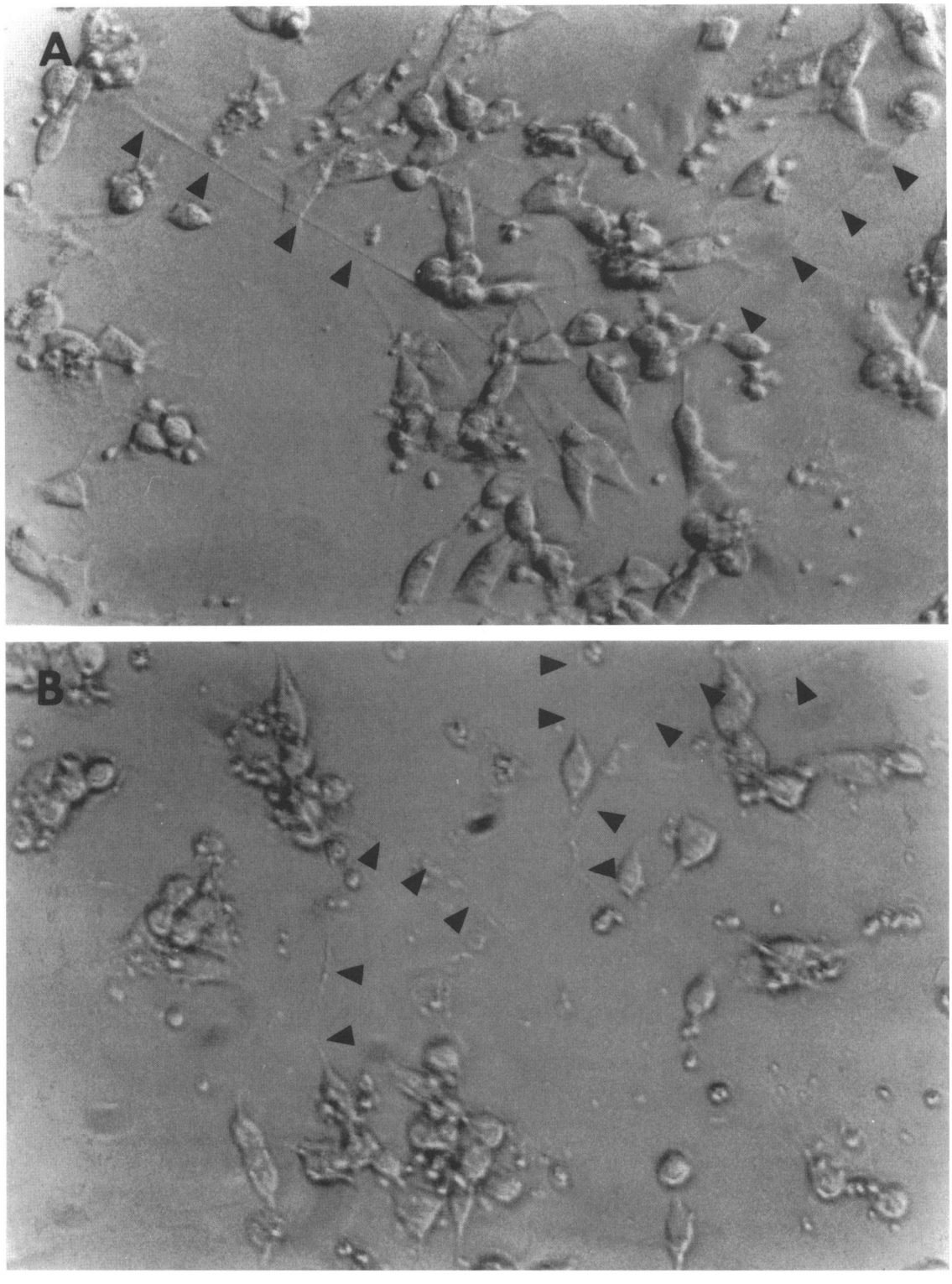

Figure 8. *Vitronectin promotes neurite outgrowth of retinoic-acid-differentiated neuroblastoma cells in serum-free conditions. Retinoic-acid-differentiated (1 μmol/L, 6 days) SK-N-SH cells were harvested with buffered EDTA, resuspended in serum-free adhesion assay buffer, and plated onto 96-well plates previously coated with vitronectin or laminin for 4 hours (37°C, 5% CO_2), as described in Materials and Methods.*[17,18] *In* **A**, *cells plated on laminin demonstrate neurite outgrowth, similar to those in* **B** *plated on vitronectin. Magnification, ×100.*

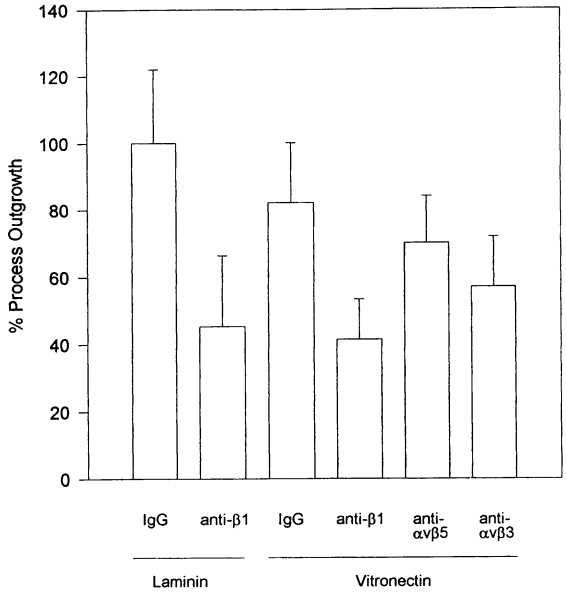

Figure 9. *Integrin αvβ5 fails to mediate neurite outgrowth of retinoic-acid-differentiated neuroblastoma cells on vitronectin. Retinoic-acid-differentiated (1 μmol/L, 6 days) SK-N-SH cells were harvested with buffered EDTA, resuspended in serum-free adhesion assay buffer, and plated onto 96-well plates previously coated with vitronectin, laminin, or ovalbumin in the presence of neutralizing anti-integrin antibodies or mouse IgG (50 μg/ml) for 4 hours (37°C, 5% CO_2), as described in Materials and Methods.[17,18] The mean neurite outgrowth on laminin in the presence of mouse IgG and after subtracting out process outgrowth on ovalbumin was taken to be 100%, and all subsequent measurements were expressed as a percentage of this. As previously reported,[17,18] MAb anti-integrin β1 (P4C10) produced 55% inhibition of neurite outgrowth on laminin. MAb anti-integrin β1 also produced 50% inhibition of neurite outgrowth on vitronectin, whereas MAbs anti-integrin αvβ5 (P3G2) and anti-integrin αvβ3 (LM609) both failed to significantly inhibit neurite outgrowth on vitronectin. Adhesion to ovalbumin was subtracted out from each well. Results are the mean ± SEM of replicates of five for each condition.*

serum fail to differentiate. The relevance of our findings regarding vitronectin expression with neuroblastoma differentiation to normal neuronal physiology was underscored by our observation that vitronectin is expressed in mature adrenal gland medulla and peripheral ganglia cell neurons.

We also investigated expression of several other matrix proteins in neuroblastoma tumors of various stages and found that expression of laminin and fibronectin correlates with the stage of differentiation of neuroblastic tumors *in vivo*. These studies suggest that these extracellular matrix proteins, as well as vitronectin, may promote neurite outgrowth of ganglioneuroblastoma cells *in vivo*, as has been reported for neuroblastoma cells *in vitro*.[6,7] The increase in matrix protein expression detected in ganglioneuroblastoma as compared with undifferentiated neuroblastoma, suggests an expanding role for integrin receptor-mediated functions as neuroblastoma cells differentiate *in vivo*. These findings support the concept that increased integrin expression on neuroblastoma cells *in vivo* portends a less aggressive behavior.[23–25]

We demonstrate in this report that vitronectin attachment of retinoic-acid-differentiated neuroblastoma cells is mediated partly by integrin αvβ5 (60%), whereas the contribution of integrin αvβ5 to vitronectin attachment of undifferentiated neuroblastoma cells was much lower (20%). However, the expression of integrin αvβ5 protein was similar in undifferentiated and retinoic-acid-differentiated neuroblastoma cells *in vitro*. Several interpretations of these data are possible at this time. One possibility is that the greater adhesive strength of the undifferentiated neuroblastoma cell, as compared with the retinoic-acid-differentiated neuroblastoma cell, for vitronectin renders inhibition of vitronectin adhesion with anti-integrin MAbs more difficult. An alteration in the activity state of integrin αvβ5 with differentiation is also consistent with the data, as MAb anti-integrin β1 similarly inhibited vitronectin attachment of both cell types. Other investigators have shown that the activity state of integrin αvβ5 *in vitro* is dependent on the cell type and can be altered by stimulation with growth factors.[14–16,32,43,54] As differentiation of neuroblastoma cells occurs, the signals to integrin receptors from inside and outside the cell most likely are received in the context of a different co-stimulatory milieu, which could potentially alter the activity state of an integrin. One such change in milieu is the significant alterations in the extracellular matrix during differentiation. Other groups have reported that expression of integrin αvβ5 on other cell types (carcinoma and oligodendrocyte precursor cells) increases their capacity for differentiation.[55,56] The difference in the adhesive strength for vitronectin of the two cell types could be due to a different vitronectin receptor integrin repertoire expressed on the undifferentiated and the differentiated cells. The undifferentiated neuroblastoma cells express at least three different vitronectin receptors that mediate, at least in part, vitronectin attachment (αvβ5, another αv integrin, and a β1 integrin, which is probably not αvβ1). In contrast, on retinoic-acid-differentiated neuroblastoma cells, two or more vitronectin receptor integrins mediate vitronectin attachment (αvβ5 and a β1 integrin).

Interestingly, integrin αvβ5 was not necessary for retinoic-acid-differentiated neuroblastoma cell neurite outgrowth on vitronectin, as neutralizing MAb anti-integrin αvβ5 failed to significantly inhibit neurite outgrowth. Neutralizing MAb anti-integrin αvβ3 also failed to significantly inhibit neurite outgrowth. Neutralizing anti-integrin β1 antibody resulted in 50% inhibition of neurite outgrowth, however, indicating

that a β1 integrin contributes to neurite outgrowth on vitronectin. Two β1 integrins have been identified as vitronectin receptors, αvβ1 and α8β1,[14–16,26] and thus are potential candidates for mediating neurite outgrowth of these cells on vitronectin. Integrin αvβ1 is expressed on several neuroblastoma cell lines[22] (reviewed in Ref. 27), but to our knowledge, these cells have not been examined for expression of integrin α8β1; the latter receptor was only recently identified. Integrin αvβ8 recognizes soluble vitronectin only[57]; thus, its potential role in mediating vitronectin-directed neurite outgrowth or cell adhesion is unclear. To our knowledge, expression of αvβ8 on these cells has not been reported. The mechanism mediating the pheochromocytoma cell neurite outgrowth on vitronectin reported previously was not defined.[49] Our findings indicate that a β1 integrin(s) mediates neurite outgrowth of neuroblastoma cells on vitronectin and laminin.

In summary, we have identified a cell adhesion mechanism related to the metastatic potential of ganglioneuroblastoma cells *in vivo*. The absence or minimal expression of vitronectin by undifferentiated neuroblastoma cells *in vivo*, and the expression of vitronectin protein in two types of mature peripheral neurons (adrenal gland medulla and peripheral ganglia cells), suggests that vitronectin protein plays a developmental role in peripheral neurons. The ability of vitronectin to promote neurite outgrowth as well as adhesion may indicate that it has multiple functions in ganglioneuroblastoma tumor cells *in vivo*. The expression of a functional vitronectin receptor (αvβ5) on differentiated neuroblastoma cells *in vitro*, and *in vivo*,[27] suggests that this receptor may mediate vitronectin adhesion and/or serve to transduce a signal(s) from the matrix to the cell. Vitronectin expression may have additional consequences due to its other known functions, such as regulation of fibrinolysis.[58] We speculate that a combination of molecular events in neuroblastoma cells *in vivo*, which include synthesis of vitronectin, promotes neuronal differentiation and the nonmetastic phenotype.

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