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Stage-Specific Expression of Integrin αvβ3 in Neuroblastic Tumors

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The ligand specificity of the integrin cell adhesion receptors probably determines the ability of specific integrins to promote tumor cell proliferation and metastasis. Therefore, we compared the expression of integrin αvβ3, a promiscuous receptor that binds with high affinity to numerous cell matrix proteins, with the expression of integrin αvβ5 and the integrin β1 subunit (which pairs with multiple α subunits) in neuroblastic tumors at various stages of differentiation. Undifferentiated neuroblastoma tumors rapidly invade and metastasize, whereas ganglioneuroblastomas rarely metastasize. Differentiating neuroblastomas are associated with an intermediate prognosis. Paraffin sections of neuroblastic tumors at various stages of differentiation obtained at biopsy from 17 patients were hybridized with antisense integrin subunit-specific αv, β3, β1, and β5 riboprobes. All neuroblastic tumors and seven adrenal glands obtained at autopsy were analyzed immunohistochemically with antibodies directed toward the αv, β3, β1, and β5 subunits. The αv subunit was expressed in neuroblastic tumors independent of the stage of differentiation, although mRNA and protein expression were generally weak in ganglioneuroblastomas, and was also detected in adrenal gland medullae. The β1 subunit was detected in most neuroblastic tumors independent of the stage of differentiation as well as in adrenal gland medullae. In contrast, the β3 subunit, which was not expressed in adrenal gland medullae, was expressed at the protein and mRNA levels in undifferentiated neuroblastomas (six of seven and seven of seven, respectively) but was not expressed in neuroblasts or ganglion cells in ganglioneuroblastomas (one case weakly positive out of five). The β5 subunit was expressed at the protein (five of five) and mRNA (four of five) levels in the ganglion cells of ganglioneuroblastomas and, although mRNA for this subunit was detectable in undifferentiated tumors, the protein was not detectable. The expression of integrin αvβ3 in undifferentiated neuroblastomas may contribute to the rapid growth of these tumors and their tendency to metastasize. (Am J Pathol 1996, 148:1423–1434)

The mechanism by which neuroblastomas invade and metastasize is unknown. In other tumors, cell adhesion to extracellular matrix proteins has been shown to be necessary for cell proliferation, invasion, and metastasis.[1–4] Cell adhesion to extracellular matrix proteins is mediated through cell surface receptors known as integrins.[1,2,4] Integrins are transmembrane-spanning, heterodimeric receptors in which the ligand specificity is determined, in part, on the specific pairing of the α and β subunits. Typically, a specific β chain can pair with multiple α subunits. In the αv subfamily, however, the αv subunit can pair with any one of five β subunits (β1, β3, β5, β6, and β8) on nucleated cells.[1,2,4–13] Although the β1 subunit can pair with 10 other α subunits, the β3, β5, β6, and β8 subunits are known to pair only with the αv subunit. Integrin αvβ3 is a promiscuous receptor that binds with high affinity to multiple ligands including vitronectin, fibronectin, fibrinogen, von Willebrand factor, osteopontin, bone sialoprotein, collagen types I and IV, laminin, and thrombospondin.[1,2,14–16]

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In contrast, the other αv-containing integrins have a more limited ligand specificity. Specifically, integrin αvβ5 recognizes vitronectin and osteopontin,[1,2,4,7–9,17] integrin αvβ1 recognizes fibronectin and on some cell types vitronectin,[1,2,4–6] integrin αvβ6 recognizes fibronectin,[10] and integrin αvβ8 recognizes soluble vitronectin.[13] The expression of integrin αvβ6 is limited to epithelial cells.[10,18] The promiscuity of the ligand recognition demonstrated by integrin αvβ3 suggests that expression of this receptor on tumor cells might facilitate tumor cell attachment, migration, and invasion and thus could act as a marker of potentially metastatic tumors. Indeed, αvβ3 has been shown to be expressed on astrocytes of the malignant phenotype.[15,16]

Neuroblastic tumors, which are one of the most common tumors of childhood, are thought to be derived from residual, primitive, neuroectodermally derived cells and arise predominantly in the adrenal gland medulla, retroperitoneum, pelvis, and mediastinum.[19–21] The histological grading of these tumors suggests the prognosis. Undifferentiated neuroblastomas, which are composed of undifferentiated neuroblasts, are typically associated with a poor prognosis and rapidly invade and metastasize. 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. An intermediate histological grade indicated by undifferentiated neuroblasts and immature ganglion cells, termed differentiating neuroblastoma, is associated with an intermediate prognosis. The concept that the histological grades of the neuroblastic tumors represent stages in differentiation is reinforced by the ability of the undifferentiated neuroblastomas and differentiating neuroblastomas to differentiate into ganglioneuroblastomas *in vivo* either spontaneously or with treatment.[19–21]

In a recent study of neuroblastomas, αv and β3 subunit proteins were found to be expressed on 1 of 3 metastatic neuroblastomas and in the neuroblasts of 1 of 5 ganglioneuroblastomas,[22] whereas a second group reported αv and β3 protein expression in 1 of 6 neuroblastomas and 2 of 2 ganglioneuroblastomas.[23] As integrin αvβ3 could play a role in the aggressive invasion and metastasis characteristic of undifferentiated neuroblastomas, we investigated the mRNA and protein expression of integrin αvβ3 in 17 neuroblastic tumors, which were histologically characterized as to their stage of differentiation. In addition, we examined the mRNA and protein expression of integrin αvβ5 in the same tumors. We found down-regulation of expression of integrin αvβ3 in mature ganglion cells of ganglioneuroblastoma in comparison with its expression in undifferentiated and differentiating neuroblastomas.

Materials and Methods

Tissue Collection

Formalin-fixed, paraffin-embedded tissues from 20 peripheral neuroblastoma biopsy specimens and 7 adrenal gland autopsy specimens obtained from The Children's Hospital of Alabama were used for these studies. The neuroblastic tumor tissue was obtained from 17 patients; in 3 patients, 2 different biopsies were investigated. Neuroblastomas were classified into undifferentiated neuroblastoma, differentiating neuroblastoma, and ganglioneuroblastoma using the following criteria. For diagnosis as an undifferentiated neuroblastoma, neuroblasts constituted the exclusive component of the tumor. For diagnosis as a differentiating neuroblastoma, neuroblasts constituted the predominant component of the tumor (>50%), with only small foci of ganglion cells. For diagnosis as a ganglioneuroblastoma, the predominant histology was that of ganglion cells with associated Schwann cells and neural stroma but with neuroblasts constituting only a small component of the tumor.[21,24,25] Ganglion cells were defined as those cells with prominent nucleoli and defined cytoplasmic borders.[21,24,25] Adrenal gland medullae pheochromocytes were identified by histological criteria as well as by staining with rabbit anti-synaptophysin antibody on serial tissue sections.[21,24,25] Formalin-fixed tumor tissues obtained from consecutive cases over a 4-year period and in which formalin fixation was initiated in the operating room were used to optimize cellular mRNA preservation. Patients with undifferentiated neuroblastoma (6 male, 1 female) ranged in age from 4 days to 2 years, patients with differentiating neuroblastoma (2 male, 3 female) ranged in age from 8 months to 5 years, and patients with ganglioneuroblastoma (1 male, 4 female) ranged in age from 9 months to 3 years. These neuroblastic tumors typically occur in young children.[21,24,25] Adrenal glands were obtained at autopsy from infants and children from 1 month to 13 years of age.

cDNAs, Riboprobe Transcription, and In Situ *Hybridization*

Subcloning of the following constructs from the cDNAs, human αv,[26,27] β3,[28] β5,[8,9] and β1,[29,30] ver-

ification of their specificity, riboprobe transcription using SP6, T7, or T3 phage polymerase, and their use in riboprobe *in situ* hybridization has previously been described.[16,31,32] A rat fibronectin cDNA in pSP73 vector used for transcribing riboprobes was composed of the *Pst*I/*Bam*HI insert (bp 5361 to 6298) of the published DNA sequence[33] and was a gift from Dr. Mitchell Olman (The University of Alabama at Birmingham). The fibronectin construct was oriented in such a manner that transcription using the SP6 phage polymerase resulted in a riboprobe that was complementary to cellular RNA (antisense). [^{33}P]UTP (Dupont Chemical Co., Boston, MA) was incorporated into the riboprobes for signal detection.[16,31] Specific activity of the riboprobes was typically 1×10^8 cpm/μg.

In situ hybridization analysis was performed as described previously.[16,31] After hybridization, the tissue sections were processed with the stringency conditions previously described, exposed (1 to 3 weeks), and developed.[16,31] The hybridized sections were graded as positive if ≥4 silver grains were observed at ×40 magnification over a nucleus or perinuclear area in at least two exposures on two different hybridizations. The grading system used to assess the intensity of hybridization was 4 to 5 grains, weakly positive (wk+); 6 to 10 grains, positive (+); and >10 grains, strongly positive (++).[16,31] To determine the percentage of cells expressing a particular mRNA within tissue sections, positive cells in 20 random fields were counted under bright field microscopy at ×40 magnification.[16,31] Greater than 3% of the indicated cell type within 20 high power fields hybridized with a particular probe unless otherwise noted below the hybridization signal in Tables 1 to 4. As a positive control, serial sections were hybridized with antisense fibronectin riboprobe. The endothelial cells expressed fibronectin mRNA, consistent with reports of fibronectin expression in endothelial cell basement membranes *in vivo*.[15,34] As a negative control for stickiness of the riboprobes,[16,31,32] a random (sense) riboprobe of similar length was used, which failed to hybridize to serial tissue sections.

Immunohistochemistry

Mouse anti-integrin β3 subunit (AP3)[35] and anti-integrin αv subunit (LM412)[36] monoclonal antibodies (MAbs) as well as affinity-purified rabbit polyclonal anti-integrin α5β1[6] antibody, which recognizes the integrin β1 subunit paired with any α subunit (GIBCO BRL, Grand Island, NY), have been characterized previously and their usage in immunohistochemistry described.[15] Rabbit polyclonal anti-integrin β5 subunit IgG, directed toward a 20-amino-acid peptide in the cytoplasmic tail, was previously characterized.[7] Goat anti-rabbit and anti-mouse IgG horseradish-peroxidase-conjugated secondary antibodies were purchased (BioRad Laboratories, Richmond, CA). Antibody dilutions were as follows: MAb anti-integrin β3 subunit (1:200 of ascites), MAb anti-integrin αv subunit (1:500 of ascites), rabbit anti-integrin β5 subunit IgG (20 μg/ml), rabbit anti-integrin α5β1 antiserum (1:500), and normal rabbit serum IgG (20 μg/ml).

Immunohistochemistry was performed by the indirect immunoperoxidase method on 5-μm formalin-fixed, paraffin-embedded sections as described[31,37] after antigen retrieval, which was performed to enhance antigen detection.[37] Briefly, tissue slides were deparaffinized in xylene (three times for 15 minutes each at 37°C) and rehydrated through decreasing concentrations of ethanol, followed by pepsin (Sigma Chemical Co., St. Louis, MO) digestion (1 mg/ml in phosphate-buffered saline (PBS) for 1 hour at 22°C).[31,37] Subsequently, antigen retrieval consisting of two 5-minute microwave incubations (on high) in 0.01 mol/L citric acid, pH 6.0, followed by cooling was performed.[37] Tissue sections were then blocked in methanol containing 1.5% hydrogen peroxide, followed by blocking with 5% bovine serum albumin (BSA)/PBS and then reacted with primary antibody in 5% BSA/PBS (1 hour at 22°C). Unlike all other antibodies, rabbit anti-integrin β5 subunit IgG antibody was incubated with tissue sections at 37°C for 10 minutes. Tissue sections were then washed, reacted with secondary antibody (1:1000 dilution) in 5% BSA/PBS (1 hour at 22°C), washed, reacted with 3,3′-diaminobenzidine substrate, washed, and stained with 0.5% methyl green (Sigma). The positive control used was detection of expression of the β1 subunit protein by endothelial cells in tissue sections reacted with rabbit anti-α5β1 antibody, and the negative control was absence of staining with normal rabbit serum IgG.

The grading scale for analysis of immunohistochemical positivity was as follows: light brown staining, weakly positive (wk+); medium brown staining, positive (+); and dark brown staining, strongly positive (++).[31,37] To determine the percentage of cells expressing a particular antigen within tissue sections, positive cells in 20 random fields were counted under bright field microscopy at ×40 magnification.[31,37] A positive immunohistochemical signal (wk+, +, or ++) required ≥3% of the indicated cell type within 20 high power fields to express the antigen.

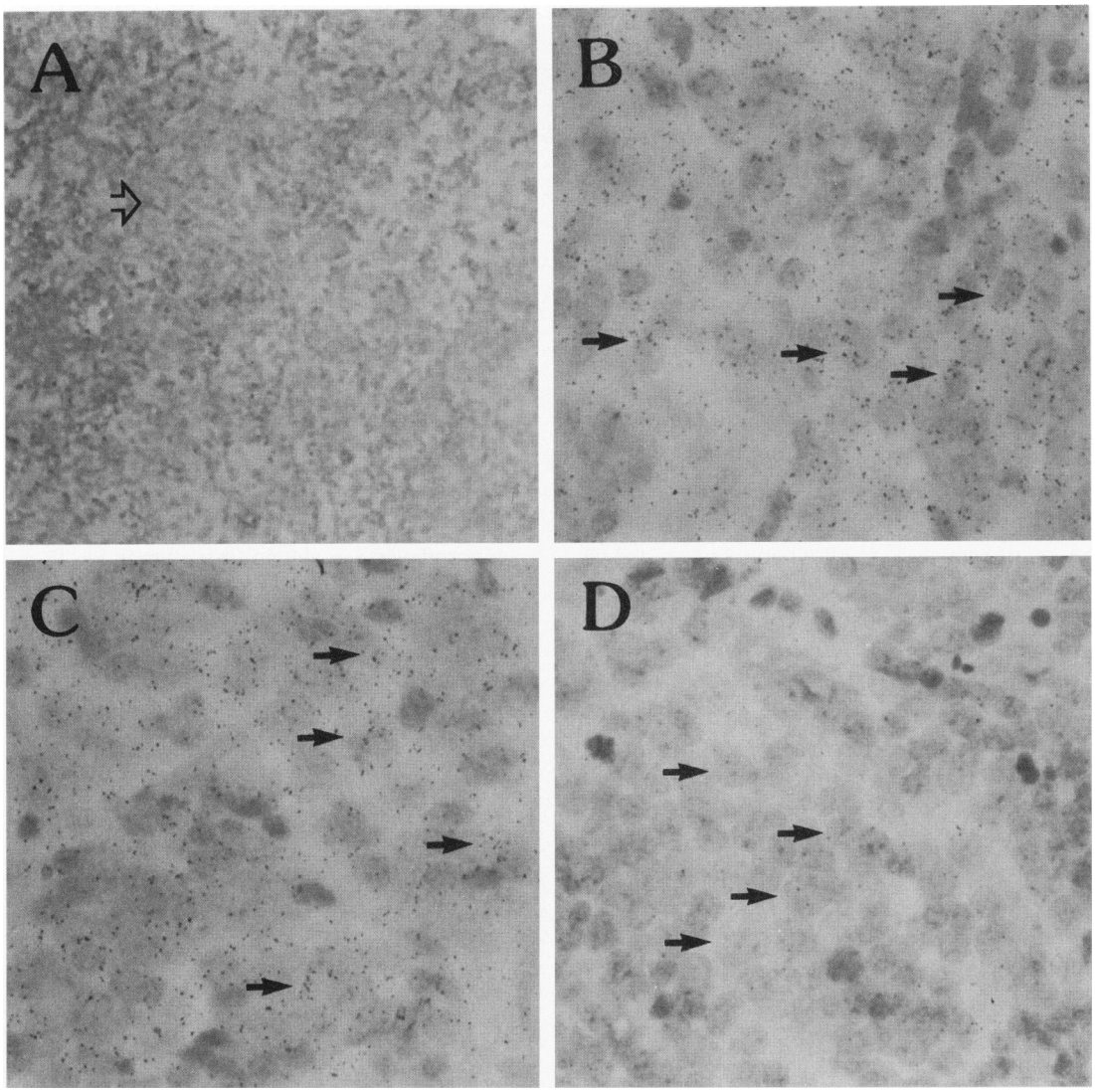

Figure 1. *mRNA expression of integrin αvβ3 in undifferentiated neuroblastoma* in vivo. *Deparaffinized sections of undifferentiated neuroblastoma (case 7, Tables 1 and 2) were hybridized with ^{33}P-labeled antisense αv, and β3 riboprobes, as described in Materials and Methods.*[16,31] A *and* B: *Hybridization with antisense αv riboprobe. The* open arrow *in* A *denotes the area of tumor shown at higher magnification in* B. B: *Clusters of nuclear and perinuclear grains indicate αv mRNA expression.* C: *Hybridization with antisense β3 riboprobe demonstrates clusters of nuclear and perinuclear grains, indicative of β3 mRNA expression.* D: *Hybridization with a sense riboprobe demonstrates no more than three nuclear or perinuclear grains, indicating absence of nonspecific hybridization.* Closed arrows *in* B, C *and* D *denote four undifferentiated neuroblastoma cells. Magnification, ×125 (*A*) and ×500 (*B *to* D*).*

Results

mRNA and Protein Expression of Integrin αvβ3 in Undifferentiated Neuroblastomas

To determine whether αv and β3 mRNAs are expressed in undifferentiated neuroblastomas *in vivo*, *in situ* hybridization on serial biopsy sections of seven tumors from different patients was performed.[16,31] The integrin subunit αv mRNA was demonstrated in seven of seven undifferentiated neuroblastomas and β3 mRNA in six of seven (Figure 1 and Tables 1 and 2). To determine the presence of αv and β3 proteins, serial sections of the same undifferentiated neuroblastoma biopsies were subjected to immunohistochemical analysis.[31,37] All seven undifferentiated neuroblastomas expressed integrin αv and β3 subunit proteins within neuroblasts (Figure 2, A and B, respectively, and Tables 1 and 2). As an internal control, mRNA and protein expression of integrin αvβ3 was detected on endothelial cells as expected and as reported for endothelial cells in culture.[14] These data confirm the *in situ* hybridization results and indicate that undifferentiated neuroblastoma cells express the integrin αvβ3 heterodimer. As neuroblastomas commonly arise in the adrenal gland medulla, adrenal glands from seven infants and chil-

Table 1. *Integrin αv Subunit Expression in Neuroblastic Tumors*

Tissue	mRNA/Protein		
	Neuroblasts	Ganglion cells	Pheochromocytes
Undifferentiated neuroblastoma			
Case 1	wk+/++		
Case 2	++/+		
Case 3	+/++		
Case 4	wk+/+		
Case 5	++/+		
Case 6	++/+		
Case 7	+/+		
Differentiating neuroblastoma			
Case 1a	+/wk+	wk+/wk+	
Case 1b	wk+/wk+	wk+/+	
Case 2	+/wk+	wk+/wk+	
Case 3	+/+	wk+/+	
Case 4	++/++	wk+/++	
Case 5	+/++	wk+/+	
Ganglioneuroblastoma			
Case 1	−/wk+	wk+/wk+	
Case 2	−/wk+	wk+/wk+	
Case 3	−/wk+	wk+/wk+	
Case 4	wk+/wk+	wk+/wk+	
Case 5	−/wk+	−/+	
Adrenal medullae			
Case 1			NH/wk+
Case 2			NH/wk+
Case 3			NH/+
Case 4			NH/wk+
Case 5			NH/+
Case 6			NH/+
Case 7			NH/−

In situ hybridization and immunohistochemical analysis were performed as described in Materials and Methods.[16,31,37] NH, not hybridized with indicated antisense riboprobe; NR, not reacted with indicated antibody. *In situ* hybridization analysis 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 to 10 grains over the nucleus or perinuclear area; ++ (very positive hybridization signal), >10 grains over the nucleus or perinuclear area. The grading scale for the immunohistochemical analysis was as follows: no color detected, negative (−); light brown staining, weakly positive (wk+); medium brown staining, positive (+); and dark brown staining, very positive (++). Percentage of ganglion cells in the differentiating neuroblastomas was as follows: case 1a, 8%; case 1b, 50%; case 2, 10%; case 3, 8%; case 4, 10%; and case 5, 50%. Two biopsies were performed on case 1 of the differentiating neuroblastomas at 80 months of age (case 1a) and 15 years of age (case 1b). Cases 3 and 4 of the ganglioneuroblastomas had second biopsies diagnosed as ganglioneuroblastomas that also failed to express β3 mRNA. In the ganglioneuroblastomas, >80% of tumor cells were ganglion cells.

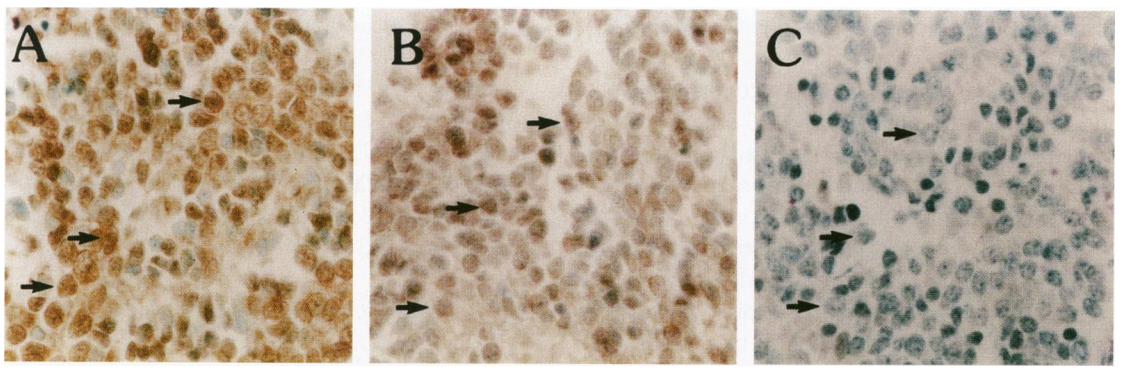

Figure 2. *Protein expression of integrin αvβ3 in undifferentiated neuroblastoma* in vivo. *Deparaffinized sections of undifferentiated neuroblastoma (case 3, Tables 1, 2, and 4) were reacted with anti-integrin antibodies followed by the immunoperoxidase technique, as described in Materials and Methods.*[31,37] **A:** *MAb anti-integrin αv subunit demonstrated strong staining of neuroblastoma cells.* **B:** *MAb anti-integrin β3 subunit also demonstrated staining of neuroblastoma cells.* **C:** *In contrast, rabbit anti-integrin β5 subunit IgG failed to react with the tumor cells.* **Arrows** *indicate three undifferentiated neuroblastoma cells in each panel. Magnification, ×500.*

Table 2. *Integrin β3 Subunit Expression in Neuroblastic Tumors*

Tissue	mRNA/Protein		
	Neuroblasts	Ganglion cells	Pheochromocytes
Undifferentiated neuroblastoma			
Case 1	−/++		
Case 2	++/+		
Case 3	+/+		
Case 4	+/+		
Case 5	+/+		
Case 6	++/+		
Case 7	+/++		
Differentiating neuroblastoma			
Case 1a	+/−	−/−	
Case 1b	+/wk+	wk+/wk+	
Case 2	−/−	−/−	
Case 3	wk+/+	−/+	
Case 4	+/+	wk+/+	
Case 5	+/wk+	wk+/wk+	
Ganglioneuroblastoma			
Case 1	−/−	−/−	
Case 2	−/−	−/−	
Case 3	−/−	−/wk+	
Case 4	−/−	−/−	
Case 5	−/−	−/−	
Adrenal medullae			
Case 1			NH/−
Case 2			NH/−
Case 3			NH/−
Case 4			NH/−
Case 5			NH/−
Case 6			NH/−
Case 7			NH/−

See Table 1 footnotes for details.

dren were subjected to immunohistochemical analysis with MAbs specific for αv and β3 subunits. In six of seven adrenal glands, the αv subunit was detected in medulla cells; however, the β3 subunit could not be detected in any of the adrenal gland medullae (Tables 1 and 2). The latter finding suggests that the αv subunit is pairing with an alternative β subunit in the adrenal gland medulla cells and is consistent with the general absence of integrin αvβ3 in mature ganglion cells of ganglioneuroblastoma.

mRNAs of two additional β subunits (β1 and β5) that also pair with the αv subunit were examined. In four of six undifferentiated neuroblastomas that were hybridized, mRNAs of the β1 and β5 subunits were demonstrated (Tables 3 and 4). Consistent with previous reports of β1 subunit protein in these tumors,[22,23] β1 protein was detected in six of seven cases of undifferentiated neuroblastoma (Table 3). In contrast to the generally coordinate detection of αv, β3, and β1 subunit mRNAs and proteins, β5 subunit protein was detected in only one of four cases that expressed β5 subunit mRNA (Table 4). Cell membrane solubilization with either acetone (10 minutes at 4°C) or 0.5% Triton X-100 (5 minutes at 22°C) before reaction with the rabbit anti-β5 subunit antibody failed to result in detection of the β5 protein in the three of four undifferentiated neuroblastomas in which β5 mRNA was expressed in the absence of protein (Table 4). In the adrenal gland medullae, β1 subunit protein was detected in all six cases subjected to immunohistochemical analysis, whereas the β5 subunit protein could not be detected in any of the cases.

In the neuroblastic tumor intermediate in the spectrum of differentiation (differentiating neuroblastoma), integrin αv and β3 mRNAs and proteins were expressed in most tumors (five of five and four of five, respectively; Tables 1 and 2); however, mRNA and protein of the β3 subunit was only weakly expressed in two cases (Table 2). The expression of integrin β1 and β5 subunit mRNAs and proteins was similar to that described above in undifferentiated neuroblastoma (Tables 3 and 4).

Absence of Integrin αvβ3 Expression in Mature Ganglion Cells of Ganglioneuroblastoma

To determine whether integrin αvβ3 expression was affected by the *in vivo* differentiation process, the

Table 3. *Integrin β1 Subunit Expression in Neuroblastic Tumors*

Tissue	mRNA/Protein		
	Neuroblasts	Ganglion cells	Pheochromocytes
Undifferentiated neuroblastoma			
Case 1	−/+		
Case 2	++/++		
Case 3	NH/wk+		
Case 4	wk+/+		
Case 5	−/wk+		
Case 6	wk+/+		
Case 7	wk+/−		
Differentiating neuroblastoma			
Case 1a	NH/−	NH/wk+	
Case 1b	wk+/wk+	wk+/wk+	
Case 2	−/wk+	−/wk+	
Case 3	wk+/wk+	−/wk+	
Case 4	wk+/+	wk+/wk+	
Case 5	wk+/+	wk+/+	
Ganglioneuroblastoma			
Case 1	−/−	wk+/+	
Case 2	wk+/wk+	wk+/+	
Case 3	wk+/wk+	wk+/+	
Case 4	−/wk+	wk+/+	
Case 5	−/−	wk+/+	
Adrenal medullae			
Case 1			NH/+
Case 2			NH/+
Case 3			NH/++
Case 4			NH/NR
Case 5			NH/++
Case 6			NH/+
Case 7			NH/wk+

See Table 1 footnotes for details.

most differentiated of the metastasizing neuroblastic tumors (ganglioneuroblastoma) were subjected to *in situ* hybridization with antisense αv and β3 riboprobes and immunohistochemical analysis with MAbs anti-integrin αv subunit and anti-integrin β3 subunit. In ganglion cells in all five cases of ganglioneuroblastoma, αv subunit protein was detected (weakly in four of five; Figure 4A), and mRNA of the αv subunit was detected in four of five cases (Table 1). In contrast, in ganglion cells in all five ganglioneuroblastomas, mRNA of the β3 subunit could not be detected and the β3 subunit protein was weakly detected in only one case, thus limiting formation of the integrin αvβ3 heterodimer in ganglioneuroblastoma (Figure 3, C and D, Figure 4C, and Table 2). Furthermore, in a patient previously diagnosed with an undifferentiated neuroblastoma (included in Tables 1 to 4 as an undifferentiated neuroblastoma), *in vivo* differentiation into a ganglioneuroblastoma occurred and was diagnosed 3 years later. In this second biopsy, mRNA expression of the integrin β3 subunit could not be detected, indicating loss of integrin αvβ3 expression with histological differentiation (data not shown). In Schwann cells of all five ganglioneuroblastomas, mRNA and protein of the β3 subunit could not be detected. These data taken together with the data in undifferentiated neuroblastoma suggest integrin αvβ3 expression is down-regulated as neuroblastoma cells differentiate *in vivo* into the mature ganglion cells of ganglioneuroblastoma (Tables 1 and 2).

Expression of the αv subunit in the absence of the β3 subunit in ganglion cells of ganglioneuroblastoma suggests the αv subunit is likely pairing with the β1, β5, or β8 subunit; therefore, we investigated the mRNA and protein expression of the β1 and β5 subunits. Currently, no antibody directed toward the β8 subunit is available. We found expression of the β1 subunit mRNA and protein in ganglion and Schwann cells of all five ganglioneuroblastomas (Table 3), consistent with and extending the work of previous investigators.[22,23] Interestingly, mRNA and protein of the β5 subunit was also demonstrated in ganglion cells in four of five ganglioneuroblastomas (Figure 3B, Figure 4B, and Table 4); however, mRNA and protein of the β5 subunit could not be detected in Schwann cells. These data indicate that the β1 and β5 subunits are available to pair with the αv subunit in ganglion cells of ganglioneuroblastomas.

Table 4. *Integrin β5 Subunit Expression in Neuroblastic Tumors*

Tissue	mRNA/Protein		
	Neuroblasts	Ganglion cells	Pheochromocytes
Undifferentiated neuroblastoma			
Case 1	wk+/−		
Case 2	++/wk+		
Case 3	NH/−		
Case 4	++/−		
Case 5	+/−		
Case 6	−/−		
Case 7	−/−		
Differentiating neuroblastoma			
Case 1a	NH/−	NH/++	
Case 1b	wk+/−	+/wk+	
Case 2	wk+/−	−/−	
Case 3	+/−	−/−	
Case 4	++/−	+/−	
Case 5	+/−	+/−	
Ganglioneuroblastoma			
Case 1	+/wk+	+/+	
Case 2	++/−	++/+	
Case 3	+/−	+/+	
Case 4	−/−	−/+	
Case 5	+/−	+/+	
Adrenal medullae			
Case 1			NH/−
Case 2			NH/−
Case 3			NH/−
Case 4			NH/−
Case 5			NH/−
Case 6			NH/−
Case 7			NH/−

See Table 1 footnotes for details.

Discussion

Tumor cell invasion and metastasis is a complex process known to require tumor cell adhesion to matrix proteins as well as tumor cell protease secretion, matrix protein digestion, tumor cell migration, and growth factor expression.[1–4] In this report, we have focused on identifying a potential *in vivo* tumor cell adhesion mechanism that would allow undifferentiated neuroblastoma cells to metastasize widely. We demonstrate expression of integrin αvβ3 mRNA and protein on undifferentiated neuroblastoma cells *in vivo* and a decrease in expression as undifferentiated neuroblastoma cells differentiate into mature ganglion or neuronal cells. Our data suggest integrin αvβ3 expression may be a useful *in vivo* marker of the undifferentiated neuroblastoma cell. Previously, no marker to detect these cells has been reported. Additional support for our observation of a change in expression of this integrin related to neuronal differentiation is that adrenal gland medulla cells, which are specialized neurons, and normal adult neocortical neurons fail to express integrin αvβ3 *in vivo*.[15,16] Altered expression of an integrin cell adhesion mechanism with cell differentiation also has been reported during embryogenesis.[38]

This apparent down-regulation of integrin αvβ3 parallels the markedly decreased invasive and metastatic behavior of ganglioneuroblastoma as compared with undifferentiated neuroblastoma and differentiating neuroblastoma. As integrin αvβ3 is a promiscuous receptor recognizing a large number of matrix proteins as ligands, expression of this receptor probably enables the cell to attach and migrate toward matrix proteins in the endothelial cell basement membrane as well as those in serum. This concept is supported by the work of other investigators,[39] which demonstrated that integrin αvβ3 expression paralleled malignant melanoma tumor progression. In addition, neutralizing anti-integrin αvβ3 antibody has been shown to inhibit metastasis in an animal model of malignant melanoma (reviewed in Refs. 1 and 2).

Discrepancies exist in the literature regarding integrin αvβ3 expression in neuroblastoma.[22,23] This is probably due to differences in the methodology of tumor classification. For example, Favrot et al[23] classified neuroblastoma tumors into two categories, neuroblastoma or ganglioneuroblastoma, and identification of Schwann cells and neural stroma were not required for classification as a ganglioneuroblas-

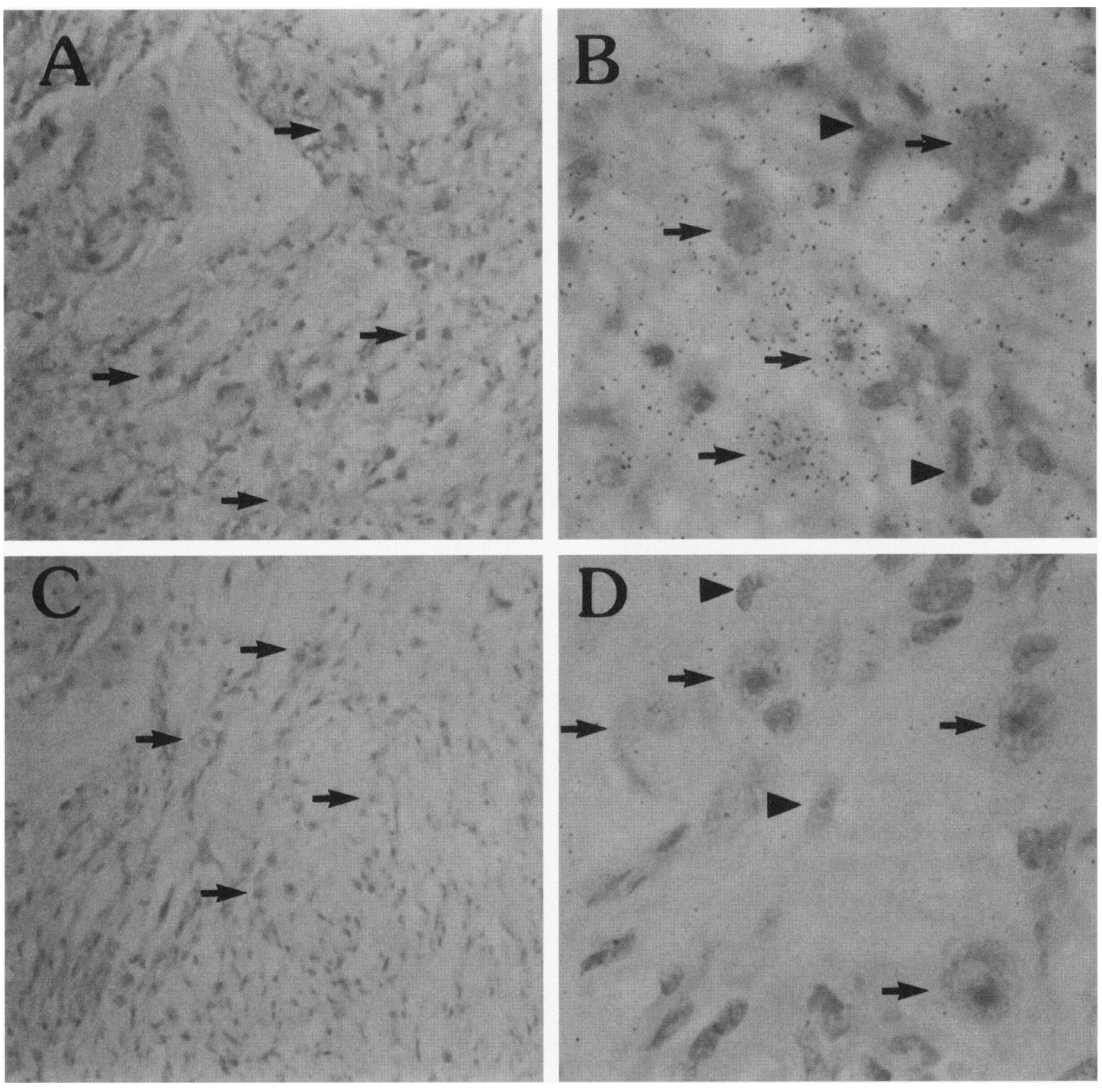

Figure 3. *Ganglion cells express mRNA of the β5 integrin subunit but not of the β3 integrin subunit. Deparaffinized sections of ganglioneuroblastoma (case 2, Tables 2 and 4) were hybridized with ^{33}P-labeled antisense β5 and β3 riboprobes, as described in Materials and Methods.*[16,31] **A** *and* **B**: *Hybridization with antisense β5 riboprobe demonstrated clusters of nuclear and perinuclear grains over ganglion cells, indicating β5 mRNA expression.* **C** *and* **D**: *In contrast, hybridization with antisense β3 riboprobe demonstrated no more than three nuclear or perinuclear grains over ganglion cells, indicating undetectable β3 mRNA expression. Magnification, ×125 (***A** *and* **C**) *and ×500 (***B** *and* **D**).

toma. The classification of neuroblastic tumors used for our study included an intermediate stage, differentiating neuroblastoma, and identification of Schwann cells and stroma (neural extracellular matrix) was necessary for the diagnosis of ganglioneuroblastoma.[24,25] Based on this difference in classification, we conclude that some tumors diagnosed as ganglioneuroblastoma by the previous group would have been diagnosed as differentiating neuroblastoma in our study. Our observation that ganglion cells in four of five differentiating neuroblastomas expressed integrin αvβ3 protein is consistent with the study of Favrot et al,[23] and the immunohistochemical findings of Mechtersheimer and colleagues[22] support our study. Evidence for biological differences in the ganglion cells of differentiating neuroblastoma *versus* ganglioneuroblastoma include a neural stroma surrounding the ganglion cells of ganglioneuroblastoma and a close anatomic relationship of ganglion cells and Schwann cells in ganglioneuroblastoma, which bears resemblance to that of normal peripheral neurons.[19–21,24,25]

An unexpected finding in this study was the consistent expression of the β5 subunit mRNA and protein only in ganglion cells of ganglioneuroblastoma. To our knowledge, this is the first reported example of integrin β5 subunit protein expression in human neuronal cells *in vivo*. The lack of coordinate detection of β5 subunit mRNA and protein in neuroblasts could be due to a cryptic epitope or different post-

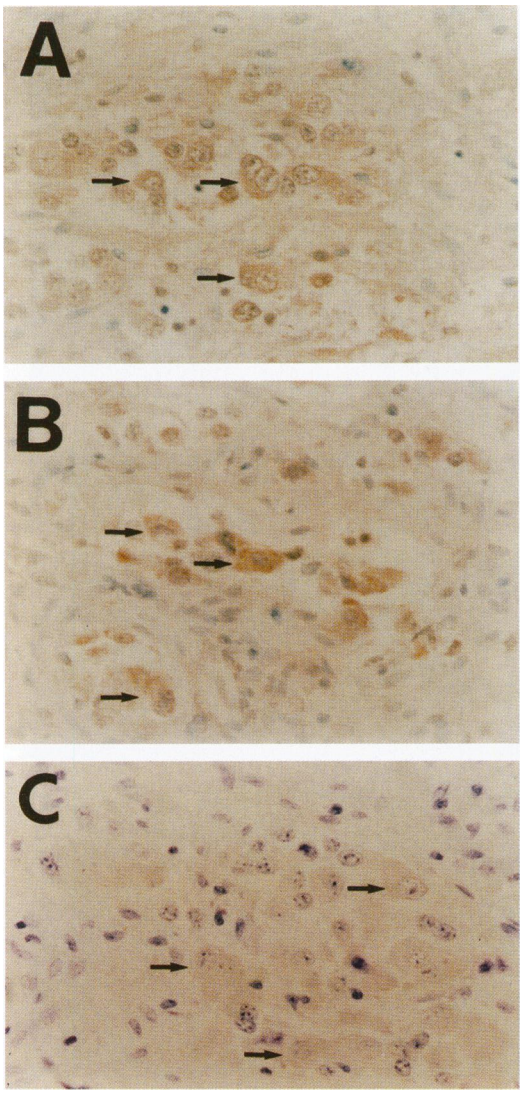

Figure 4. *Ganglion cells express protein of the αv and β5 integrin subunits* in vivo. *Deparaffinized sections of ganglioneuroblastoma (case 2, Tables 1, 2, and 4) were reacted with anti-integrin antibodies followed by the immunoperoxidase technique as described in Materials and Methods.*[31,37] **A:** *MAb anti-integrin αv subunit demonstrated weakly positive staining of ganglion cells.* **B:** *Rabbit anti-integrin β5 subunit IgG also demonstrated staining of ganglion cells.* **C:** *In contrast, MAb anti-integrin β3 subunit IgG failed to react with ganglion cells.* **Arrows** *denote three representative ganglion cells in each panel. Magnification, ×500.*

translational processing of the protein in these cells. The β5 subunit has been reported to only pair with the αv subunit, suggesting expression of integrin αvβ5 in ganglion cells of ganglioneuroblastoma. Integrin subunit expression in the absence of heterodimerization is restricted to a cytoplasmic vesicle pool,[36] which is inconsistent with the pattern of rabbit anti-β5 IgG immunohistochemical staining observed in ganglion cells. Integrin αvβ5 expression in ganglion cells of ganglioneuroblastoma is further supported by our co-localization by immunofluorescent analysis of the αv and β5 subunits on the cell membrane of retinoic-acid-differentiated neuroblastoma cells *in vitro* (C. L. Gladson, unpublished observation).

In summary, our data demonstrate mRNA and protein expression of integrin αvβ3 in undifferentiated neuroblastoma cells *in vivo* and a decreased expression or down-regulation when these cells differentiate into mature ganglion cells. Integrin αvβ3 expression by undifferentiated neuroblastoma cells probably contributes to their rapidly invasive and metastatic biological behavior. This report opens the door for a larger study comparing integrin αvβ3 expression with other known markers of poor prognosis in neuroblastoma, such as N-c-*myc* amplification, older age, stage, and DNA index.[19,21] Our data suggest that αv-integrin-mediated, ligand-dependent cell adhesion mechanism(s) in neuroblastoma cells varies with the state of differentiation.

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