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Insulin-Like Growth Factor II in the Pathogenesis of Human Neuroblastoma

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Insulin-like growth factor II (IGF-II) acts as an autocrine growth factor for many* in vitro *tumor cell lines including neuroblastoma. To examine the role of IGF-II in tumor biology we have analyzed a total of 56 primary neuroblastoma tumor samples for the presence of IGF-II using a combination of mRNA and protein analysis. A group of 21 samples was examined for the presence of IGF-II mRNA by slot blot and a separate group of 37 samples was examined for IGF-II immunoreactivity. IGF-II was detected in 48% of the total tumor specimens analyzed. IGF-II immunoreactivity was observed in cells resembling developing neuroblasts and was confined to the cytoplasm and proximal neurites. The appearance of IGF-II mRNA and protein did not correlate with tumor prognostic features including stage, histology, or N-myc amplification. These data suggest that the expression of IGF-II is not confined to a specific stage of the disease but may have a broader role in the pathogenesis of neuroblastoma. (Am J Pathol 1995, 147:1790–1798)

Cancer is the major cause of death in children between the ages of 1 and 15 years.[1] Neuroblastoma is the second most common solid tumor in childhood[2] and accounts for 10% of all juvenile cancers.[1] Neuroblastoma survival rates correlate with patient age, stage of disease, tumor histology, and N-*myc* DNA amplification.[3–5] Although N-*myc* amplification correlates with rapid disease progression, it alone is not sufficient to cause cellular transformation.[6–10] Additionally, although a number of chromosomal abnormalities have been described in neuroblastoma, no specific genetic defect has been identified that is responsible for the development of this disease.[11–13] Collectively, these observations suggest that multiple mechanisms contribute to the tumorigenesis of neuroblastoma.

Autocrine production of insulin-like growth factors has been implicated in the genesis and progression of several cancers including neuroblastoma.[14–21] Our work has focused on the autocrine production of insulin-like growth factor-II (IGF-II) by a cloned human neuroblastoma cell line (SH-SY5Y) as one event in neuroblastoma carcinogenesis.[22–25] IGF-II is a potent mitogen, abundant during nervous system development[26–29] with the highest expression in neural crest derivatives, brain, choroid plexus, and leptomeninges.[26–28,30] *In vitro*, IGF-II acting via the type I IGF receptor, stimulates neurite outgrowth, DNA synthesis, and cell proliferation in both cultured neuroblasts and neuroblastoma cell lines.[31,32] IGF-II mediates autocrine growth of cloned human neuroblastoma cells[15,22–24] and several additional cancers, including Wilm's tumor,[33] rhabdomyosarcoma,[34] and breast cancer.[17,35,36]

Autocrine production of IGF-II may support cellular transformation by either enhancing uncontrolled cellular proliferation[14,15,22,37] or increasing cellular resistance to programmed cell death (PCD) or apoptosis.[38,39] We speculated that the pattern of IGF-II expression in primary tumors would aid in defining its function in the genesis and/or progression of neuroblastoma. The current study was designed to determine whether IGF-II gene and protein expression were detectable in neuroblastoma tumor specimens and whether expression correlated with other prognostic features of this cancer.

We report that IGF-II expression in neuroblastoma is equally distributed among all clinical and histological stages. In each case, IGF-II protein is confined

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to differentiating neuroblasts and is never visualized in anaplastic small round neural cells. These data suggest a fundamental role for autocrine production of IGF-II in neuroblast survival and implicate a role for IGF-II in cellular resistance to apoptosis.

Materials and Methods

Tumor Specimens, Histology, and N-myc Amplification

Tumor specimens were obtained from the Department of Pathology at the University of Michigan Hospitals (Ann Arbor, MI) or Children's Human Tissue Network (Columbus, OH). A total of 57 primary tumor specimens were examined. Paraffin sections from 36 patient samples were analyzed for IGF-II immunohistochemistry. A group of 21 separate patient samples were analyzed for IGF-II gene expression. All specimens were staged according to the Evan's classification:[3,4] (1) stage I tumors were confined to the organ or structure of origin; (2) stage II tumors extended in continuity beyond the organ or structure of origin but not across the midline; (3) stage III tumors extended in continuity beyond the midline; (4) stage IV tumors were large primaries with remote disease involving skeleton, organs, soft tissues, or distant lymph nodes; and (5) stage IV-S tumors were specimens that would otherwise be classified as stage I or II but with remote disease confined to only one or more of the following sites: liver, skin, or bone marrow. Pathological diagnosis and histology were determined according to the Shimada classification.[5] The number of specimens representing clinical stages I to IV were 4, 12, 13, and 22, respectively, whereas 6 tumors were graded as stage IV-S. N-*myc* amplification (more than three copies) was determined by Southern blot analysis as previously described.[40]

Slot Blot Analysis

RNA was isolated from frozen tumor specimens by acid guanidinium thiocyanate-phenol-chloroform extraction.[41] Total RNA was directly applied to nylon Nytran membranes (Schleicher and Schuell, Keene, NH) utilizing a slot blot apparatus (Schleicher and Schuell). To eliminate false positive IGF-II signaling secondary to nonspecific Northern hybridization, we utilized two doses of total RNA, 50 and 500 ng. If a signal was observed at 500 ng, but not 50 ng, we attributed this to nonspecific cross-hybridization and we did not consider the sample positive for IGF-II gene expression. Membranes were successively hybridized with [^{32}P]dCTP-labeled (0.1×10^8 to 10×10^8 cpm/μg) cDNA probes for human IGF-II (bases 1 to 854 of a cDNA encoding exons 7 and 8 and part of 9 excised with *Pst*I)[42] and chicken β-actin (1.8 kb excised with *Pst*I).[43] Individual Nytran membranes were hybridized by stripping and reprobing, as described in the manufacturer's instructions (Schleicher & Schuell).

Immunohistochemistry

Paraffin sections from 36 untreated patients were processed for IGF-II immunohistochemistry. Sections were deparaffinized in xylene, hydrated through ethanol to phosphate buffer (0.1 mol/L, pH 7.3), and rinsed in H_2O_2 (0.5%) to remove endogenous peroxidase activity followed by 2% nonfat dry milk in Triton X-100 to reduce nonspecific adherence of antisera. Sections were incubated in IGF-II primary antiserum (monoclonal, IGF-II, 1:500, Upstate Biochemical Inc., NY[44]) in a humid chamber for 16 to 24 hours at 22°C. After primary antiserum, sections were incubated with biotinylated horse anti-mouse immunoglobulin G (Vector Laboratories, Burlingame, CA) followed by avidin conjugated to horseradish peroxidase (Vector). The chromogen was developed in 3,3′-diaminobenzidine (Sigma Chemical Co., St. Louis, MO) in the presence of H_2O_2.[45,46] A brown precipitate indicates the presence of antigen/antibody/horseradish peroxidase complexes. The sections were counterstained in Ehrlich's hematoxylin.

The IGF-II antiserum is a monoclonal antiserum raised against human IGF-II.[47] The cross-reactivity of the antiserum was determined by IGF-II tracer-binding inhibition in radioimmunoassays. The results are as follows: human IGF-II, 100%; multiplication stimulating activity II, identical to rat IGF-II, 100%; human IGF-I, 10%; human epidermal growth factor, <0.01%.[44] To control for specificity, the primary antiserum was preabsorbed with IGF-II (5 μg/ml at 4°C overnight). Serial adjacent sections from the same patient were treated in parallel with normal antiserum and preabsorbed antiserum. Immunoreactive cells were not detected in sections incubated with preabsorbed antiserum.

Results

IGF-II mRNA and N-myc Amplification

Twenty-one tumor samples were probed for IGF-II mRNA. The N-*myc* copy number of these samples had previously been determined. Fifteen samples

Table 1. *IGF-II Immunostaining and N-myc Copy Number in Primary Neuroblastoma Tumors*

Patient	Age (months)	Stage (Evans)	N-*myc*	IGF-II mRNA
1	67	I	1	−
2	1	I	1	+
3	27	I	1	+
4	NA	II	1	−
5	NA	II	1	−
6	2	II	1	+
7	11	II	1	+
8	NA	II	1	+
9	NA	III	1	+
10	11	III	1	+
11	60	III	150	+
12	6	III	1	+
13	NA	III	200	−
14	NA	IV	1	−
15	NA	IV	1	−
16	NA	IV	10+	+
17	NA	IV	>1	+
18	14	IV	1	+
19	53	IV	1	+
20	10	IV	1	+
21	NA	IV	6–8	+

NA, information not available.

(71%) expressed IGF-II mRNA, whereas six samples (29%) had no detectable IGF-II mRNA (Tables 1 and 2). Of the fifteen IGF-II-positive samples, eleven (73%) had an N-*myc* copy number of one whereas four (27%) had an N-*myc* copy number greater than one. A similar pattern of N-*myc* expression was noted in the tumors that contained no detectable IGF-II mRNA. Five (83%) of these samples had an N-*myc* copy number of one, whereas only one (17%) had an N-*myc* copy number greater than one (Tables 1 and 2). A positive correlation could not be demonstrated between N-*myc* copy number and expression of IGF-II mRNA.

IGF-II Immunohistochemistry

Of the 37 primary tumor specimens examined, 12 (32%) contained IGF-II immunoreactive cells (Table 3). The morphology of the IGF-II immunoreactivity cells was similar to that demonstrated by sympathetic neuroblasts (Figure 1, B–F). The cells were relatively small, contained an eccentric nucleus, and many extended neurites. These cells were not the typical N or S cells found within the tumors (Figure 1A) and may represent a population of maturing cells. IGF-II-positive varicosities were not apparent; therefore, no attempt was made to identify axons and dendrites. The IGF-II reaction product was localized within the cytoplasm and proximal neurites (Figure 1B); no nuclear staining was observed. The IGF-II reaction product was granular throughout the cell body and neurites. IGF-II immunoreactivity cells

Table 2. *Comparison of IGF-II Gene Expression with N-myc Gene Amplification in Untreated Neuroblastoma*

	N-*myc* copy number		
	1	>1	Total
IGF-II mRNA$^+$	11	4	15
IGF-II mRNA$^-$	5	1	6
Total	16	5	21

Fisher's exact test P = 1.0000, indicating no correlation between N-*myc* copy number and IGF-II gene expression.

Table 3. *IGF-II Immunoreactivity, Disease Stage (Evans), and Histology (Shimada) in Primary Neuroblastoma Tumors*

Patient	Age (months)	Stage (Evans)	Class (Shimada)	IGF-II (immunohisto-chemistry)
22	NA	I	†F	+
4	16	II	UF	+
23	1	II	F	−
24	9	II	F	−
25	6	II	F	−
26	92	II	F	−
27	19	II	F	+
28	14	II	F	−
29	16	III	F	−
30*	18	III	UF	−
31*	18	III	UF	+
32	30	III	UF	−
33	18	III	UF	−
34	12	III	F	−
35	24	III	UF	−
36	10	III	F	−
37	26	IV	UF	−
38	96	IV	UF	−
39	18	IV	UF	+
40	29	IV	UF	−
41	108	IV	UF	−
17	24	IV	UF	−
42	36	IV	BMM†	−
43	2	IV	F	+
44	144	IV	UF	−
45‡	96	IV	UF	+
46‡	96	IV	UF	−
47	NA	IV	F	+
48	NA	IV	NA	+
49	NA	IV	NA	−
50	1	IV-S	F	−
51	1	IV-S	F	+
52	3	IV-S	F	−
53	20	IV-S	F	+
54	2	IV-S	F	−
55	3	IV-S	F	−
56	14	NA	UF	+

NA, not available.
*Same patient; 30, pre-chemotherapy, and 31, post-chemotherapy.
†Bone marrow metastasis.
‡Same patient; 45, pre-chemotherapy, and 46, post-chemotherapy.
F = favorable; UF = unfavorable.

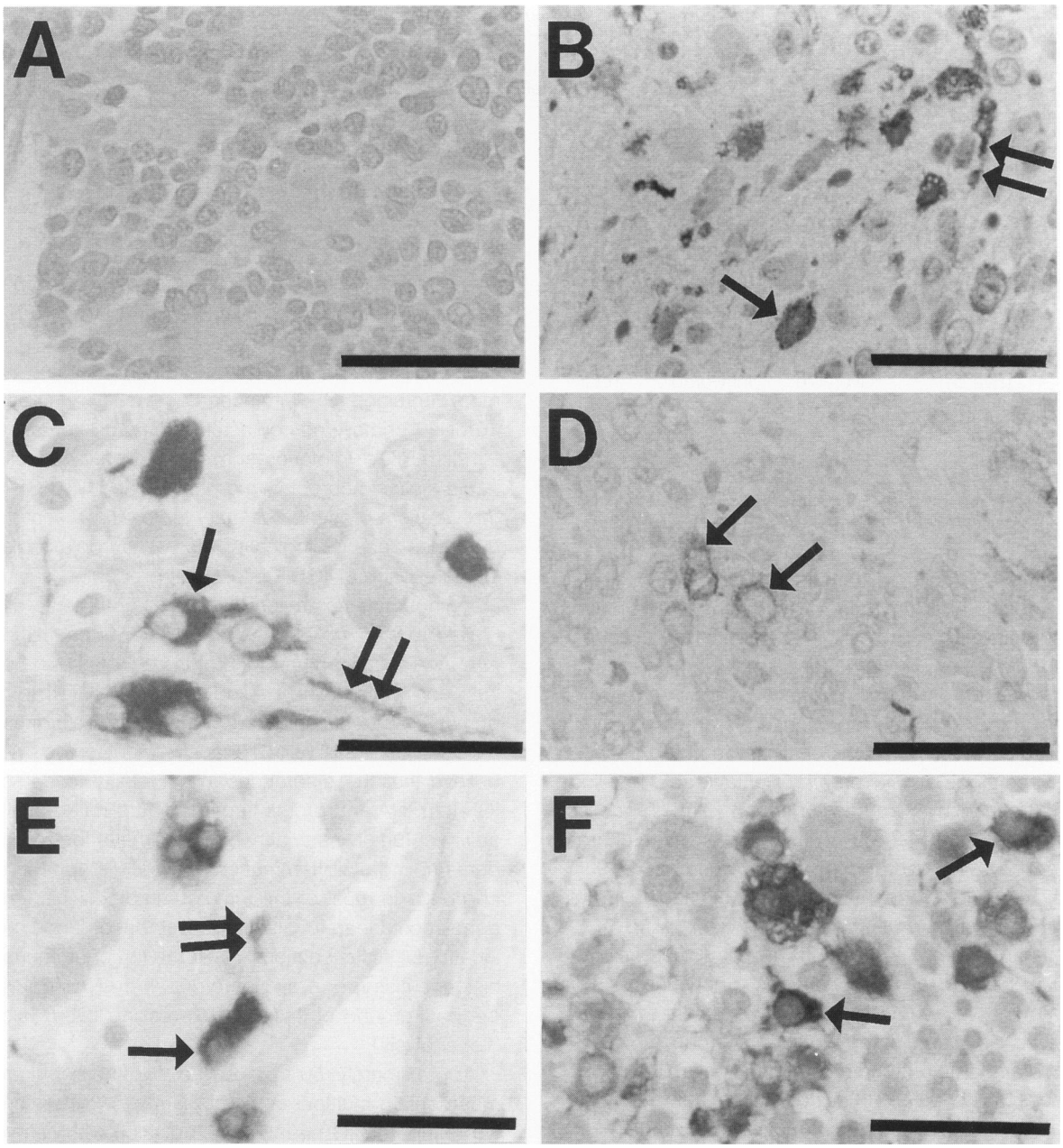

Figure 1. A: *An example of N cells from a neuroblastoma tumor. These cells are small and round with little cytoplasm and do not contain immunoreactive IGF-II.* B *to* F: *IGF-II-ir cells* (arrow) *and neurites* (double arrow) *from stage I* (B), *stage II* (C), *stage III* (D), *stage IV* (E), *and stage IV-S* (F) *neuroblastomas.*

were observed singly, in small clusters of 2 to 5 cells or in larger groups of up to 15 cells.

Two types of neuronal cells were present in all tumors: small, round undifferentiated cells, designated N cells and larger, differentiating neuroblasts with clearly definable neuritic processes.[48,49] Neuroblastoma tumors also contain large epithelial S cells.[50] N and S cells did not exhibit IGF-II immunoreactivity (Figure 1, A–F). Mature cell types, including fibroblasts, neurons, and Schwann cells, within the tumors were also negative for IGF-II (data not shown).

IGF-II Immunohistochemistry Versus *Tumor Stage (Evans) and Histology (Shimada Class)*

The location and morphology of IGF-II-immunoreactive cells within neuroblastoma tumors were similar across tumor stage and histology (Figure 1, B–F). Of

Table 4. *Comparison of IGF-II Immunoreactivity with Neuroblastoma Stage and Histology*

	Stage (Evans)			Histology (Shimada)		
	I, II, IV-S	III, IV	Total	Favorable	Unfavorable	Total
IGF-II$^+$	6	6	12	6	5	11
IGF-II$^-$	9	16	25	12	11	23
Total	15	22	37	18	16	34

Stage (Evans), Fisher's exact test $P = 0.4879$; histology (Shimada), Fisher's exact test $P = 1.0000$, indicating no correlation between stage or histology and IGF-II immunoreactivity.

Table 5. *Comparison between Neuroblastoma Histology and Stage*

Stage (Evans)	Histology (Shimada)		
	Favorable	Unfavorable	Total
I, II, IV-S	13	1	14
III, IV	5	14	19
Total	18	15	33

Fisher's exact test $P = 0.0002$, indicating a correlation between stage and histology.

the tissue sections examined for histology, 53% had favorable histology and 47% demonstrated unfavorable histology (Tables 3 and 4). Within this group of specimens, 40% were stage I, II, or IV-S representing a good prognosis, whereas 60% were classified as stage III or IV representing a poor prognosis (Tables 3 and 4). A correlation was noted between Evans stage and favorable or unfavorable histology as defined by Shimada. Fourteen (74%) of the advanced (stage III and IV) tumors were also characterized by unfavorable histology, whereas 13 (93%) of stage I, II, and IV-S tumors exhibited favorable histology (Table 5).

Fifteen tumor specimens were classified as stage I, II, or IV-S; of these, six (40%) contained IGF-II-immunoreactive cells. Twenty-two tumors were classified as stage III or IV. In this group, six (27%) contained IGF-II-immunoreactive cells. It was not possible to establish a correlation between tumor grade and IGF-II immunoreactivity. Favorable or unfavorable histology was also unrelated to IGF-II immunoreactivity. Six (33%) of favorable and five (31%) of unfavorable tumors contained IGF-II immunoreactivity (Tables 3 to 5).

Discussion

The production of and requirement for exogenous growth factors varies as cells undergo transformation.[51] The current study was designed to determine the pattern of IGF-II expression in untreated neuroblastoma tumors as a means of better understanding the role of IGF-II in neuroblastoma carcinogenesis. IGF-II mRNA and protein expression were compared with disease features prognostic for neuroblastoma including clinical stage, histology, and N-*myc* DNA amplification.

In our study, 12 of 37 primary neuroblastoma tumors contained IGF-II-immunoreactive cells whereas 16 of 20 primary neuroblastoma tumors expressed IGF-II mRNA. Immunoreactive IGF-II-positive cells had a distinct neuroblast morphology and, like IGF-II mRNA, were present in primary tumors representing all stages and histological grades of neuroblastoma. The presence of IGF-II in a small proportion of primary neuroblastoma tumors is in agreement with El-Badry and colleagues[15] who reported IGF-II mRNA in 2 of 8 primary neuroblastoma tumors. Suzuki and colleagues[52] failed to detect IGF-II in 5 primary neuroblastomas but did report IGF-II immunoreactivity in 20 of 20 pheochromocytomas. We believe that the differences in our ability to detect IGF-II mRNA and protein are related to limited availability of tumor material, which may have led to undersampling. IGF-II-immunoreactive cells appeared in groups in most of the tumors sampled. This tendency could lead to false negatives based upon the location of tissue sections within the tumor (immunohistochemistry) or a dilution of IGF-II mRNA against the background of cells not expressing IGF-II (slot blot analysis).

Approximately 50% of aggressive neuroblastomas contain multiple copies of N-*myc*[6–9]; therefore we compared IGF-II mRNA expression with N-*myc* amplification. Although IGF-II gene expression was detected in 71% of primary tumors, only 24% had an N-*myc* copy number greater than one. In the current study, N-*myc* was amplified in only 4 of the 21 tumor samples; therefore it is not possible to detemine the interaction of IGF-II and N-*myc* gene products. Although we could not establish a positive correlation between N-*myc* amplification and IGF-II gene expression, both factors are likely important but independent mediators of neuroblastoma cell growth.

The uniform expression of IGF-II independent of neuroblastoma stage, histology, or N-*myc* amplification implies a potential role for IGF-II throughout the progression of the disease. IGF-II may enhance au-

tocrine and/or paracrine tumor growth, increase the rate of cell survival, or possibly modulate neuronal differentiation. We have previously reported that autocrine IGF-II production by human neuroblastoma SH-SY5Y cells supports their unregulated growth.[14,22,37] These cells express both IGF-II and type I IGF receptor mRNA and protein.[14,37] IGF-II secreted into the media acts via the type I IGF receptor to increase DNA synthesis and cell number,[14] and blocking the type I IGF receptor with specific antibodies inhibits IGF-II-stimulated cell growth. Similar results have been reported for human SK-N-AS neuroblastoma cells,[15] breast cancer cells,[17,35,36] and rhabdomyosarcoma,[19,34] implying that these tumors are also capable of utilizing IGF-II in an autocrine loop to maintain unregulated growth. The uniform expression of IGF-II across all neuroblastoma tumors suggests IGF-II may function similarly *in vivo* to promote cellular growth. Alternatively, IGF-II may function in a paracrine manner to support surrounding neuroblastoma cells.[21] Neuroblastoma tumors contain a number of distinctive cell types within the neural crest lineage.[50] IGF-II, synthesized and secreted by neuroblasts, could enhance the growth of neighboring cells expressing the type I IGF receptor. This phenomenon has been demonstrated in a subset of breast tumors in which the epithelial cells express the type I IGF receptor and undergo mitogenesis in response to the IGF produced by the surrounding stromal cells.[17]

The differentiated phenotype of the IGF-II-immunoreactive neuroblasts in the primary neuroblastoma tumors also implies a potential role for IGF-II in the control of neuronal maturation.[54,55] The IGFs have been demonstrated to promote differentiation in several *in vitro* cell lines including oligodendroglia,[56] myoblasts,[57] and neuroblastoma cells.[55,58–60] In these instances, cells stop dividing and assume a mature cell-specific phenotype. IGF-II, acting as a differentiating factor within neuroblastoma, could contribute to maturation of individual tumor cell types. In support of this concept, expression of a different but related family of growth factor receptors, the neurotrophin Trk receptors, correlates with cellular differentiation in primary neuroblastoma tumors.[61–65] Several *in vitro* neuroblastoma cell lines also express Trk receptors.[66–69] As in primary tumors, the functional state of the Trk receptors varies across cell lines and conditions[66,69,70] but correlates with a more differentiated tumor state. Activation of Trk receptors by brain-derived neurotrophic factor reduces cellular proliferation and increases differentiation leading to the spontaneous regression of some neuroblastoma tumors.[66,69]

Another possible role for IGF-II in neuroblastoma may be the prevention of PCD. Tumor growth is not only caused by accelerated cellular proliferation but also by an accompanying decrease in PCD, also known as apoptosis.[38,39] Whether or not a cell proceeds to maturity or succumbs to PCD is often dependent on the availability of specific growth factors.[71] Several growth factors including IGF-II have been shown to protect cells from PCD during normal development and in models of injury.[38,39] In the Tag2 transgenic mouse, IGF-II expression promotes tumor formation whereas mice null for the IGF-II gene produce smaller tumors containing a fivefold greater number of apoptotic cells.[72] We have recently transfected human non-IGF-II-producing neuroblastoma cells with IGF-II and preliminary results indicate that IGF-II protects transfectants from PCD (Singleton, in press).

In conclusion, although it is known that IGF-II is a potent autocrine growth factor in several *in vitro* tumor cell lines, including ones derived from human neuroblastoma, the role of IGF-II in the context of primary tumor biology is more complex. Although approximately one-half of the 56 neuroblastoma tumors we examined expressed IGF-II, there was no direct correlation between the presence of IGF-II mRNA and protein with tumor prognostic features. Expression of IGF-II is not confined to a specific stage of the disease and may have a broad role in the pathogenesis of neuroblastoma. Alternatively, the presence of IGF-II within neuroblastoma tumors may be a permissive factor and not directly related to disease progression. We have discussed several possibilities, including roles for IGF-II in the cellular transformation, differentiation, and/or rescue of neuroblastoma cells from PCD. Additional investigation of primary tumors is required to delineate which of these possibilities accurately describes IGF-II's role in the pathogenesis of neuroblastoma.

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