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IGF2 Expression is a Marker for Paraganglionic/ SIF Cell Differentiation in Neuroblastoma

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Neuroblastoma is a childhood tumor of the sympathetic nervous system. Observations in the Beckwith-Wiedemann syndrome suggest that sympathetic embryonal cells with an abundant expression of the insulin-like growth factor 2 gene (IGF2) may be involved in the genesis of low-malignant infant neuroblastomas. We have therefore compared the cell type-specific IGF2 expression of the human sympathetic nervous system during early development with that of neuroblastoma. An abundant expression in normal sympathetic tissue was specific to extra-adrenal chromaffin cells, ie, paraganglia and small intensely fluorescent (SIF) cells, whereas sympathetic neuronal cells were IGF2-negative. A subpopulation of neuroblastomas expressed IGF2, which correlated with an early age at diagnosis, an extra-adrenal tumor origin, and severe hemodynamic signs of catecholamine secretion. Histologically IGF2-expressing tumors displayed a lobular growth pattern, and expression was restricted to the most mature and least proliferative cells. Typically, these cells were morphologically and histochemically similar to paraganglia/SIF cells and formed distinct ring-like zones in the center of the lobules around a core of apoptosis-like tumor cells. The similarities found between IGF2-expressing neuroblastoma cells and paraganglia/SIF cells in terms of histological features, anatomical origin, and age-dependent growth suggest a paraganglionic/SIF cell lineage of most infant tumors and also of extra-adrenal tumors diagnosed after infancy. Furthermore, since paraganglia/SIF cells undergo postnatal involution, the same cellular mechanism may be responsible for spontaneous regression in infant neuroblastoma. (Am J Pathol 1995, 146:833–847)

Neuroblastoma is an embryonal tumor of early childhood originating from the developing sympathetic nervous system.[1] Two major tumor types have been well described.[1–3] One prognostically unfavorable type usually affects children more than 2 years of age, with invasive growth at diagnosis, and a biological marker profile including a near-diploid DNA content, chromosome 1p deletions, an amplified N-*myc* gene, and low expression of the c-*trk* proto-oncogene, coding for the high-affinity receptor for nerve growth factor (NGF). The second type has an excellent prognosis, is usually diagnosed at an age of less than 18 months, grows locally, has a hyperdiploid DNA-content, and expresses the c-*trk* proto-oncogene abundantly but lacks 1p deletions and N-*myc* amplification. Moreover, these two forms differ significantly in their sites of origin. Aggressive tumors are usually localized to the adrenal area, whereas prognostically favorable infant tumors are much more frequently found at extra-adrenal sites,[4,5] suggesting an origin from the sympathetic trunk or from prevertebral sympathetic structures in the retroperitoneum. Histological signs of maturation are more frequent in prognostically favorable infant tumors than in the older age group, when criteria such as nuclear enlargement, increased cytoplasm, cell processes,[5–7] and presence of fibrillar material (neuropil) are used.[8] However, the majority of these infant tumors are still mor-

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phologically primitive,[5–7] although biochemical evidence (c-*src* splice variant, neuron-specific enolase, and synaptophysin expression) indicates a more mature phenotype than in aggressive neuroblastoma.[9] Some neuroblastomas display more overt evidence of maturation because of their content of ganglion-like cells, which have a more abundant cytoplasm and a more pronounced nuclear enlargement with a distinct nucleolus. Such evidence of neuronal differentiation is usually accompanied by the presence of Schwann cell-like elements. These tumors, frequently referred to as ganglioneuroblastomas,[5–7] are usually associated with a diagnosis after infancy and have a better prognosis than histologically immature tumors of the older age group.[5,7] Neuroblastoma has the highest frequency of spontaneous regression of all human cancers,[10] which is primarily associated with infant disease, particularly metastatic tumors of the 4S type.[1] However, in widespread disease of children more than 1 year old at diagnosis, spontaneous regression is a rare event (reviewed in ref 1). This unique capacity of neuroblastoma may also explain why nonradical surgery usually is curative in regional infant disease,[11] and why clinically silent tumors (neuroblastoma *in situ*) are a frequent autopsy finding in neonates who have died from nonneoplastic causes.[12]

The constituents and hormone/neurotransmitter production of the sympathetic nervous system are schematically shown in Figure 1. Cells of the sympathetic nervous system include neuronal, paraganglionic, small intensely fluorescent (SIF), and adrenal chromaffin cells. Paraganglia are discrete encapsulated neuroendocrine structures adjacent to sympathetic ganglia.[13] These structures appear during early sympathetic organogenesis and are of significant size during prenatal development, the largest of which is called the "organ of Zuckerkandl", located along the abdominal aorta.[14] In addition, small collections of microscopically and histochemically similar cells are found within all sympathetic ganglia.[15] These cells are denoted SIF cells, a term based upon their intense formaldehyde-induced catecholamine fluorescence. These purely neuroendocrine, paraganglia-like SIF cells are denoted type II, as opposed to another type of SIF cell (type I), which shows mixed neuroendocrine/interneuron features and exists as solitary cells within sympathetic ganglia.[16] In the fetus and the neonate, the predominant source of catecholamine production and release is these extra-adrenal sympathetic neuroendocrine cell types,[17,18] whereas the adrenal medulla is poorly developed both in size[14,15] and function.[17] During childhood, however, the size of paraganglia and the number of SIF cells decrease significantly.[15] Postnatal paraganglionic involution is observed in several mammalian species,[14,18,19] and in the rat this is an early event occurring during the first weeks of life, involving signs of chromaffin cell degeneration such as nuclear irregularity and pyknosis.[19] In contrast, adrenal chromaffin cell growth accelerates after birth and continues until puberty.[15] Catecholamines produced by sympathetic neuroendocrine cells are stored in granular vesicles. These organelles are rich in chromogranins[20] and are also responsible for the chromaffin reaction, specific for sympathetic neuroendocrine cells. Adrenal chromaffin cells differ from their extra-adrenal counterparts in that they produce adrenalin, which is the major catecholamine of the adrenal medulla from midgestation.[14]

Insulin-like growth factor 2 (IGF-2) exerts mitogenic, trophic, and differentiation-promoting effects on neuroblastoma-derived cell lines.[21–23] This growth factor may also mediate signals for cell survival of

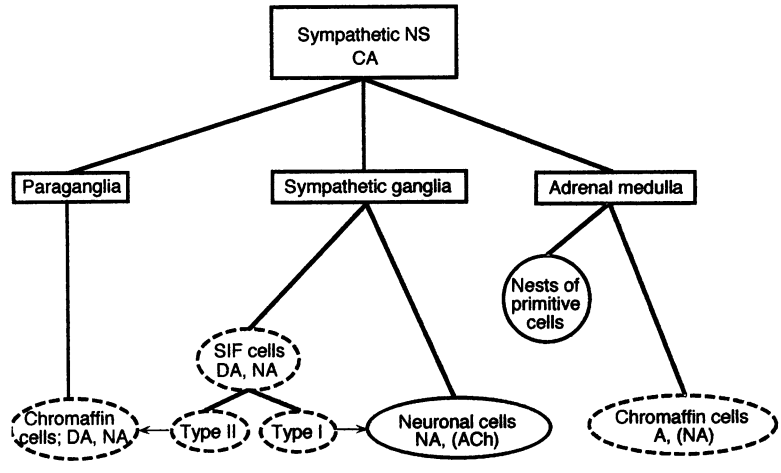


Figure 1. *Schematic representation of the constituents of the sympathetic nervous system and their neurotransmitter/hormone production. Neuroendocrine cell types are represented by dashed lines. Similarities in phenotype between SIF type II cells and paraganglia cells, as well as SIF type I cells and sympathetic neurons, are indicated by arrows. Neuroglial cells are omitted. The scheme is based on data presented in refs. 13 to 16. Abbreviations used: A, adrenalin; ACh, acetylcholine; CA, catecholamines; DA, dopamine; NA: noradrenalin; NS: nervous system. Minor production is shown within parentheses.*

embryonic sympathetic cells,[24] presumably counteracting programmed cell death (apoptosis), as is shown to be the case for IGF-1 in oligodendrocyte survival in fetal rat.[25] The study of transgenic mice deficient for *IGF2* expression indicates that regulation of prenatal growth is the major function of IGF-2.[26] An overactive *IGF2* gene has been suggested[27–30] as a mechanism for the fetal overgrowth[31] and predisposition for embryonal tumors[32] of the Beckwith-Wiedemann syndrome (BWS). A striking correlation between cell types with an abundant *IGF2* expression during normal development and the cell type-specific pattern of hyperplasia and tumor formation of the BWS has been observed.[30] The combination of paraganglionic hyperplasia[31] and an apparent predisposition for extra-adrenal infant neuroblastoma/ganglioneuroma[33–37] in this syndrome may suggest an extra-adrenal chromaffin origin of these tumors. The existence of an abundant *IGF2* expression in these normal and neoplastic cell types would provide further support for this hypothesis.

The purpose of this study was to evaluate the utility of *IGF2* expression as a lineage marker in neuroblastoma. During normal development of the sympathetic nervous system chromaffin cells with an extra-adrenal location displayed an abundant *IGF2* expression in contrast both to their adrenal counterparts and to sympathetic neurons. In neuroblastoma, *IGF2* expression was present primarily in extra-adrenal tumors, and *IGF2*-expressing tumor cells were morphologically and histochemically similar to paraganglia/SIF cells. We submit that this evidence of extra-adrenal chromaffin differentiation in neuroblastoma provides new insights into the marked clinical heterogeneity of this tumor. A paraganglionic/SIF cell lineage of a subset of neuroblastomas may thus explain differences in anatomical origin, age at diagnosis, endocrine activity, histology, and tendency for spontaneous regression and maturation.

Materials and Methods

Embryonic and Fetal Tissue Specimens

Tissue specimens from first- and second-trimester pregnancies were obtained at therapeutic terminations, early tubal pregnancies, and late spontaneous abortions with the permission of the local Medical Ethics Committee at Karolinska Hospital (93–216). Age was determined by ultrasound analysis, crown-to-rump length (18 to 220 mm), foot length, and anamnestic information. Developmental (postfertilization) age was used instead of gestational age. Eight conceptuses of the following ages were analyzed: 7.0, 7.5, 7.5, 10.5, 11.0, 13.0, 19.0, and 23 weeks. The first two and the last two specimens represented abnormal pregnancies but were accepted for evaluation of normal development as they appeared histologically normal, and given that results from analyses of these specimens were concordant with those from normal pregnancies.

Tumor Specimens

32 neuroblastomas, 1 ganglioneuroblastoma, and 2 ganglioneuromas were included in the study. They were chosen from a national study of children treated for these tumors in Sweden during a 7-year period (1986 to 1993). The international criteria for neuroblastoma diagnosis, staging, and response to treatment[3] were used for clinical classification of the tumors. Most low-stage (stages 1 to 2b) tumor specimens were collected before chemotherapy. Surgical biopsies were usually not taken in high-stage disease (stages 3 and 4) before treatment, but two such biopsies (cases 41 and 110) and four core needle biopsies (cases 105, 108, 115, 149) taken before treatment were included in the material. Because of the relative scarcity of extra-adrenal tumors diagnosed after 2 years of age in the total national material, a disproportionately large number of these tumors were sought in this study to better evaluate this tumor subtype. The specimens examined represented the primary tumor with one exception, in which a biopsy from a skin metastasis appearing during chemotherapy was utilized (case 165). Follow-up times for survivors ranged from 20 months to 6.5 years, and for children with fatal disease from 2 weeks to 17 months.

Tissue Processing

Tissue specimens were fixed in buffered formalin and routinely processed to paraffin. 5-μ thick sections were collected on silanized slides and stained with hematoxylin and eosin or used for *in situ* hybridizations and immunohistochemical stainings.

RNA Probes

A ^{35}S-labeled antisense riboprobe used for *in situ* hybridization analysis (see below) was made from a 680-bp Hinf1-Pst1 human *IGF2* cDNA insert, cloned into pGem-3 (SP6; ref. 38). The probe was transcribed from supercoiled plasmids, yielding a specific activity of approximately 250 Ci/mmol. A sense probe from the same plasmid, with a similar specific activity, was employed as negative control.

In situ *Hybridization Analysis*

Tissue sections were hybridized to riboprobes at 56 C overnight and washed stringently before RNAse treatment as described previously.[38] After application of NTBII (Eastman Kodak Co., Rochester, NY) photographic emulsion (diluted 1:1 in 2% glycerol in H_2O) and exposure for 3 to 7 days at 4 C, sections were counterstained with Mayer's hematoxylin and mounted.

Immunohistochemical Characterization

Antibodies with specificity for the following antigens were used in consecutive sections: neuron-specific enolase (NSE; polyclonal antibody, BioGenex Laboratories, San Ramon, CA), chromogranins A and B (monoclonal antibody, BioGenex Laboratories; polyclonal antibody, Milab, Malmö, Sweden) and Ki 67 (monoclonal Mib 1 antibody, Immunotech S.A., Marseille, France). These antibodies were chosen as markers for neuronal and neuroendocrine differentiation and proliferation, respectively. Human leukocyte common antigen (monoclonal CD45 antibody, Dakopatts, Glostrup, Denmark) was used for detection of retroperitoneal lymphatic cells during normal development. The avidin-biotin-peroxidase complex method with diaminobenzidine as chromogen was used according to the manufacturer's manual (ABC kit, Dakopatts).

Results

Morphological and Histochemical Characterization of Human Sympathetic Nervous System Development

Sympathetic Development from Week 8 to Week 23

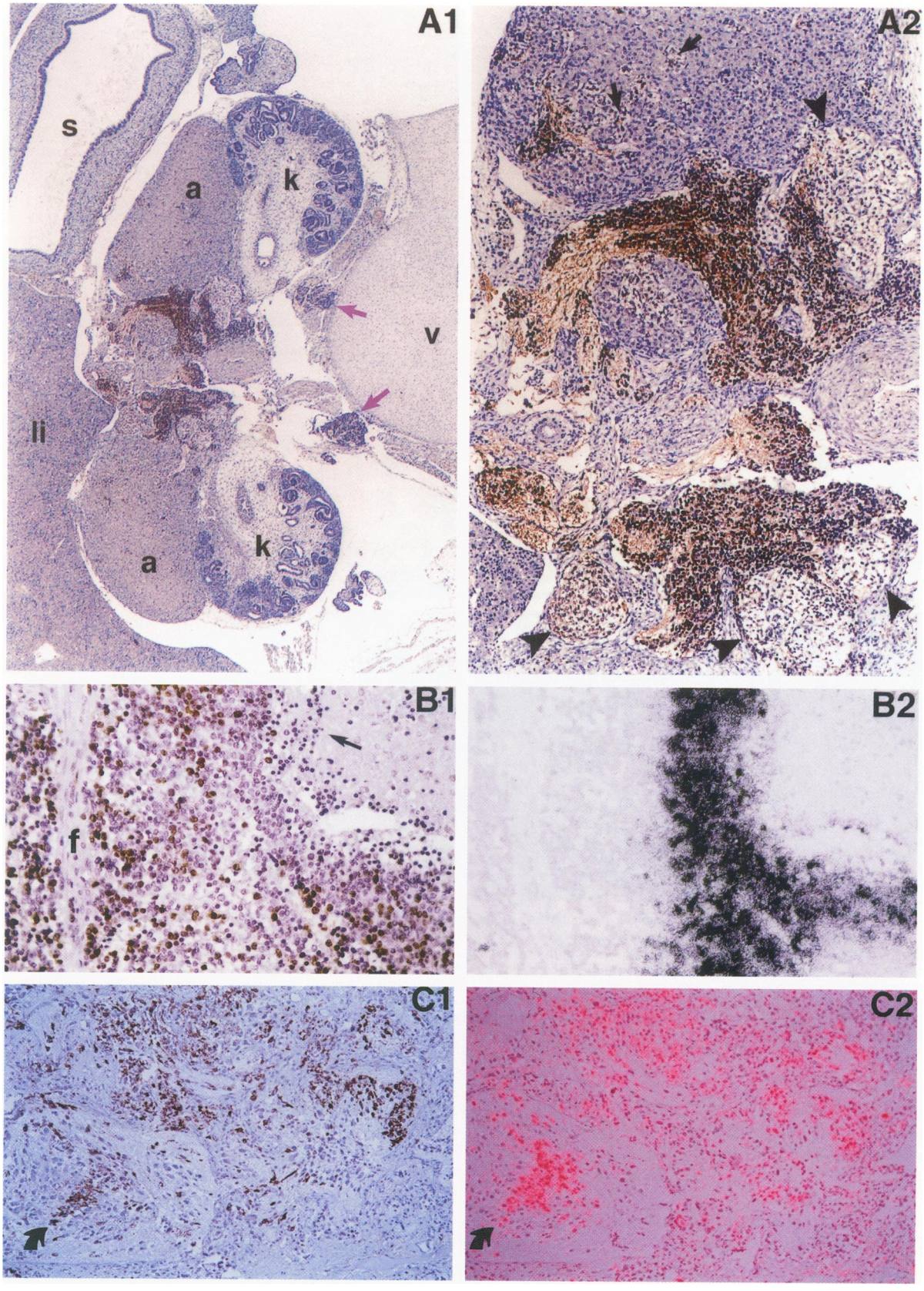
From week 8 of development, sympathetic cells were abundant in the retroperitoneal area, particularly at the adrenal level (Figure 2A). Chromaffin differentiation, represented by paraganglia, adrenal chromaffin cells (Figure 2A2), and SIF cells in the sympathetic trunk (Figure 3, A to C) was present already in the earliest specimens (8th week), whereas sympathetic neurons were present but morphologically primitive (Figures 2A2 and 3, C and D). During the period studied, paraganglia were of considerable size and particularly abundant in the juxta-adrenal area (Figure 2A), contrasting with relatively low numbers of adrenal chromaffin cells (Figure 2A2).

IGF2 *Expression*

Sympathetic neuronal cells did not express *IGF2* at any stage of development investigated (Figure 3, A, E, and G). Paraganglia, however, expressed the gene abundantly throughout development (Figure 3, A and G). Type II SIF cells (Figure 3, A and E), were also strongly *IGF2*-positive. A centralized location of such SIF cells within the sympathetic trunk was a consistent finding (Figure 3A). These cells were particularly prominent in the earliest specimens but were also present at later stages. Type I SIF cells appearing as solitary cells within sympathetic ganglia were identified from the 19th week onward. It was possible to demonstrate with reasonable certainty that these cells also expressed *IGF2* abundantly by superimposing photographs of consecutive sections utilized for *IGF2 in situ* hybridization and chromogranin immunohistochemistry (cf Figure 3, G and H).

Two types of adrenal medullary cells were seen. One type consisted of nests of primitive sympathetic cells, located mostly in the center of the gland with no detectable *IGF2* expression (Figure 3I). The other type consisted of chromaffin cells interspersed with fetal cortical cells, either on the medial side of the gland during early stages (until the 11th week; Figure 2A), or with a more central position during later stages (not shown). Whether *IGF2* expression in adrenal chromaffin cells was low or nonexistent could not be

Figure 2. *Immunohistochemical characterization of embryonic sympathetic development and tumor specimens.* (**A**) *Early sympathetic development in the retroperitoneal area. Abdominal cross-section at the adrenal level of a 7.5-week embryo. NSE immunoreactive (brown) structures represent sympathetic cells and peripheral nerves. Chromaffin differentiation is represented by paraganglia (arrowheads, A2) and scattered adrenal chromaffin cells (arrows, A2). Sympathetic neuronal cells are abundant but morphologically primitive (A2). Other symbols: a, adrenal; k, kidney; li, liver; s, stomach; v, vertebra; colored arrow, sympathetic trunk. Magnification: A1, 30×; A2, 75×.* **2B:** *Zones of proliferating,* IGF2*-expressing, and apoptosis-like cells in a neuroblastoma tumor lobule. Consecutive sections from a neuroblastoma of a newborn child with particularly abundant* IGF2 *expression and severe cardiovascular symptoms (case 157, Table 1). (B1) shows immunoreactivity for the cell cycle-specific Ki 67 antigen (brown) and apoptosis-like bodies toward the center of the lobule (arrow; same area shown in high-power view in Figure 4H), neighboring the zone of* IGF2*-expressing cells, illustrated by panel B2 (brightfield view). Note the slightly brighter nuclei of* IGF2*-expressing tumor cells. f, fibrovascular septum. Magnification, 200×.* (**C**) *Co-expression of chromogranin A+B and* IGF2 *in neuroblastoma. Consecutive sections from a malignant neuroblastoma of thoracic origin diagnosed at 3 years of age, causing severe hypertension (case 165, Table 1). (C1) shows chromogranin A+B immunoreactivity (brown). (C2) shows* IGF2 *expression in a composite darkfield (red) and brightfield (blue) view. A close to identical expression pattern is seen, particularly evident in the center of a nest of maturing cells (arrows). Magnification, 62×. All sections were counterstained with hematoxylin.*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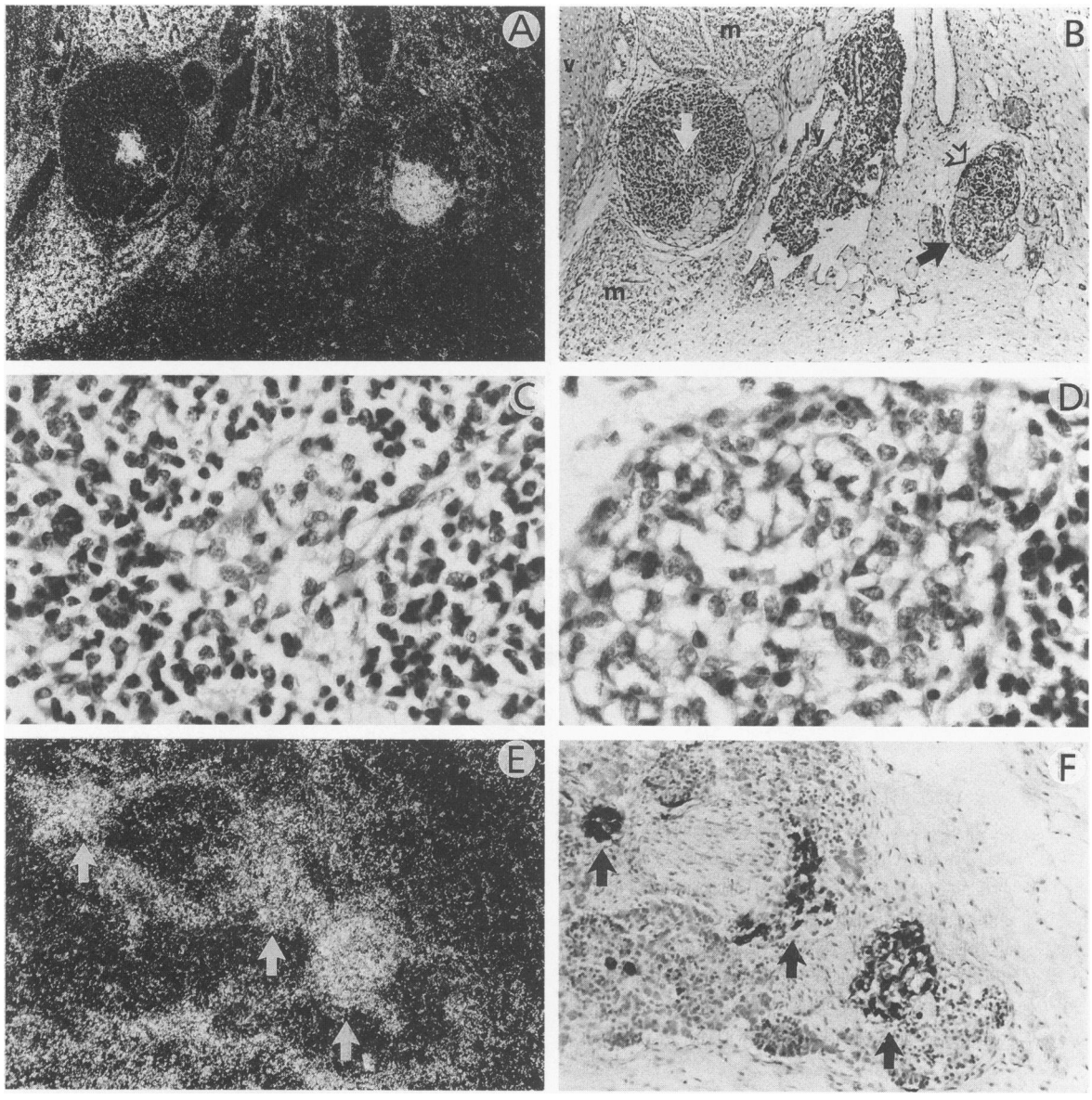
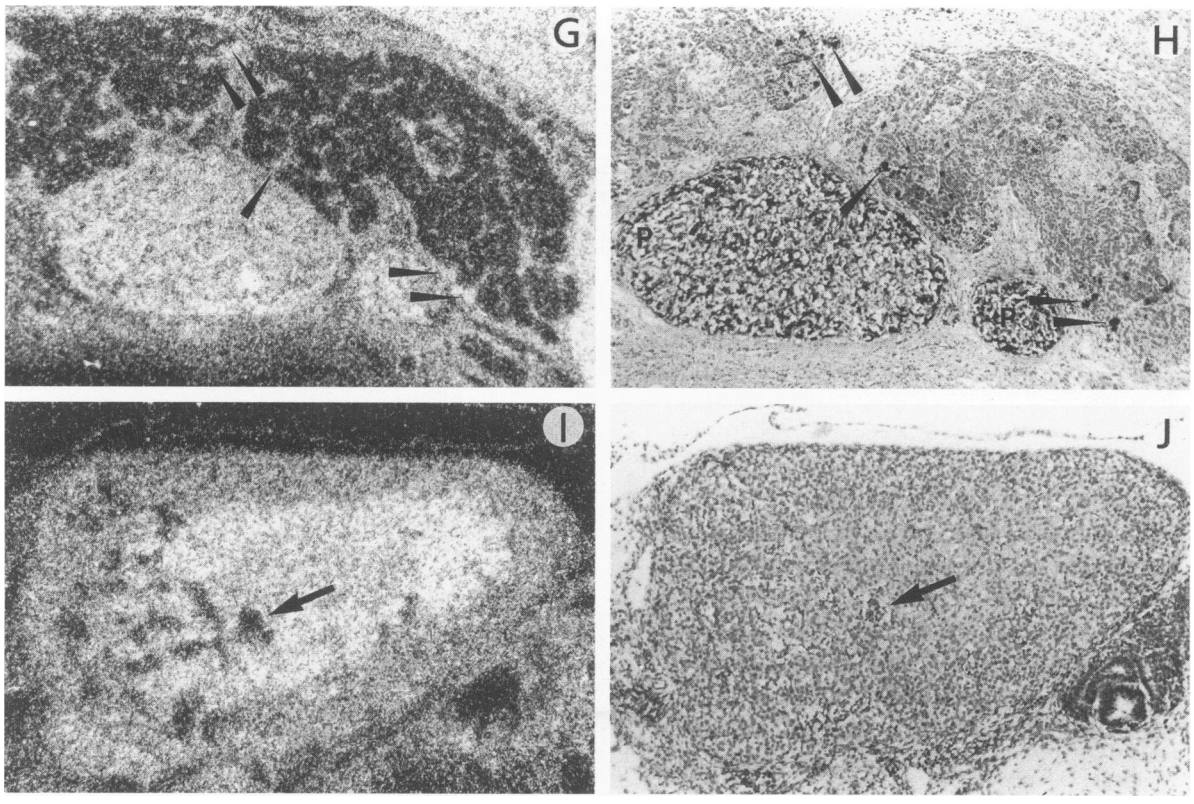


Figure 3. *Cell type-specific* IGF2 *expression in the developing sympathetic nervous system:* (**A, B**) *IGF2 is expressed in paraganglia and SIF type II cells of the sympathetic trunk, but not in sympathetic neuronal cells. Abdominal cross section of a 10.5-week fetus showing* (A) IGF2 in situ *hybridization (darkfield view) and (B) hematoxylin and eosin staining of a consecutive section. Symbols: ly, lymphoid tissue; m, muscle; black solid arrow, paraganglion; open arrow, sympathetic ganglion; white arrow, SIF cells in the center of the sympathetic trunk. Magnification, 33×.* (**C, D**) *Nuclear morphology of SIF cells* (**C**) *and paraganglia cells* (**D**) *compared with neighboring primitive sympathetic neurons. High-power magnifications (240×) from section shown in* (**B**). (**E, F**) *IGF2 expression in SIF type II cells of a juxta-adrenal sympathetic ganglion (19.5 weeks): Consecutive sections showing* IGF2 *expression* (**E**, *darkfield view) and chromogranin A+B immunoreactivity in hematoxylin counterstain* (**F**). *Arrows indicate SIF cells. Magnification, 63×.*

distinguished conclusively because of interfering signals from the strongly *IGF2*-positive cells of the fetal adrenal cortex (Figure 3I).

Immunohistochemical Characterization: Chromogranin A+B

The neuroendocrine nature of SIF cells and paraganglia was verified by intense chromogranin immunoreactivity (Figure 3, F and H). Adrenal chromogranin immunoreactivity was intense in the chromaffin cell type, whereas the nests of primitive sympathetic cells stained either negatively or weakly positively in areas of neuropil formation (not shown). Primitive sympathetic neuronal cells of sympathetic ganglia also stained negatively, with the exception of neuropil in the later stages (from 19 weeks; not shown). Morphologically more mature sympathetic neurons



(G,) *IGF2 expression in type I SIF cells. Other area of same sections as shown in* (E) *and* (F). *Type I SIF cells stain intensely for chromogranins (arrowheads, panel H).* IGF2 *expression of the same areas are indicated in panel G (darkfield view). P, paraganglion. Magnification, 33×.* (I, J). *Adrenal IGF2 expression. An adrenal gland of a 7.5-week embryo in a cross-abdominal section showing intense expression in cortical cells and low or nonexistent expression in chromaffin cells (mostly located on the medial/left side) and in a nest of primitive sympathetic cells (arrow).* (I) *Darkfield view.* (J) *Consecutive section stained with hematoxylin and eosin. (For further morphological information see Figure 2A2 showing NSE immunoreactivity of chromaffin cells in an adjacent section). Magnification, 40×.*

with increasing cytoplasm, nuclear enlargement, and distinct nucleoli were seen from the 19th week. These cells displayed weak chromogranin immunoreactivity (Figure 3F).

Other Markers

The neuronal character of retroperitoneal and adrenal sympathetic cells was confirmed by NSE immunoreactivity (Figure 2A). Cells of the lymphoid system were frequently identified in the retroperitoneal area at all stages of development (Figure 3B), but could be distinguished from the morphologically similar primitive sympathetic cells by a positive CD45 immunohistochemical reaction (not shown). Proliferation of primitive sympathetic neuronal, paraganglionic, and type II SIF cells was evident by a high cellular density of Ki 67 immunoreactivity (not shown).

IGF2 *Expression in Neuroblastoma*

Moderate *IGF2* expression of fibrovascular septa was identified in all specimens, but only those containing *IGF2*-expressing neuroblastic cells will subsequently be referred to as positive tumors. The data are summarized in Table 1.

Extra-Adrenal Tumors

Two distinct clinical forms were seen. All 13 tumors diagnosed before 2.5 years were locally growing and, with one exception, prognostically favorable, whereas most (4/5) older children presented with advanced tumor growth at diagnosis and died from tumor progression. One newborn child died from cardiogenic shock and hepatic thrombosis, presumably because of massive tumor secretion of catecholamines (case 157, Table 1). One 3.5-year old child with multiple skeletal tumor foci is in complete long-term remission (case 111, Table 1). *IGF2* expression was a frequent finding in both of these age groups (9/13 and 3/5 respectively, Table 1). In most tumors characteristic ringlike patterns of *IGF2* expression were found (Figure 4A), corresponding to zones of cells in tumor areas with a lobular pattern (Figure 4B). These zones had a central position within lobules and surrounded a core of cellular debris (cf.

Table 1. IGF2 *Expression in Neuroblastomas and Ganglioneuromas Diagnosed in Sweden 1986–1993 in Relation to Clinical Data and to Presence of a Lobular Histological Pattern and Apoptosis-Like Bodies*

Age	Site	Case no.	Stage	Outcome*	*IGF2* expression	Lobular pattern	Apoptosis-like bodies
Extra-adrenal tumors (age-sequenced)							
0	Pararenal	148	1	NED	+	+	–
0	Pararenal	157	3	DOD†	+	+	+
1 month	Thoracic	85	3	NED	–	–	–
1 month	Pelvic	118	1	NED	+	–	–
2 months	Pararenal	84	1	NED	+	+	–
2 months	Pararenal	124	1	NED	+	+	+
3 months	Thoracic	51	2b	NED	+	+	+
7 months	Pelvic	128	3	NED	+	+	+
12 months	Thoracic	146	3	NED	–	–	–
12 months	Para-adrenal	153	3	NED	–	+	–
13 months	Para-adrenal	78	3	NED	+	+	+
18 months	Thoracic	87	2a	NED	–	–	–
2.5 years	Pelvic	138	2b	NED	+	+	–
3 years	Thoracic	165	4	DOD	+	+	–
3.5 years	Abd, paravertebral	111	4‡	NED	–	+	+
4 years	Thoracic	130	4	DOD	–	–	–
5 years	Thoracic	69	3	DOD	+	+	+
6.5 years	Thoracic	41	4	DOD	+	–	–
Adrenal tumors (age-sequenced)							
0	Adrenal§	39	4S	DOD	+	+	+
0	Adrenal	115	4S	DOD	–	–	–
0	Adrenal§	125	4S	NED	+	+	+
0	Adrenal§	103	1	NED	–	+	–
10 months	Adrenal	106	4‖	NED	–	–	–
11 months	Adrenal	108	4‡	NED	(+)¶	–	–
21 months	Adrenal	135	3	DOD	+	+	+
3 years	Adrenal	149	3	DOD	–	–	–
3.5 years	Adrenal	68	4	DOD	–	–	–
3.5 years	Adrenal	105	4‡	NED	–	–	–
4.5 years	Adrenal	155	4	DOD	–	–	–
5 years	Adrenal	110	4‡	dead#	–	+	+
6 years	Adrenal	32	4	DOD	–	–	–
8.5 years	Adrenal	127	2b	NED	+	+	+
11.5 years	Adrenal	126	4	DOD	–	–	–
Ganglioneuroma							
2.5 years	Thoracic	73		NED	–	–	–
11 years	Pararenal	59		NED	–	–	–

Follow-up times for survivors range from 20 months to 6.5 years.
*NED, No evidence of disease; DOD, dead of disease.
†Cardiogenic shock at birth.
‡Skeletal involvement.
§Histologically verified.
‖Hepatic involvement.
¶Few scattered cells.
#Died from septic shock 12 months after diagnosis, while in autopsy-verified complete remission.

Figures 4, A and B, and 2B). Within the lobules, tumor cells displayed a gradient of increasing morphological maturation toward the center (Figures 2B and 4, C to E). Determinants of increasing maturation were increasing nuclear size with diminished basophilia and increasing cytoplasm.[5–8,39] *IGF2*-positive cells were therefore the most mature of the lobule, typically displaying a more abundant cytoplasm and larger and brighter nuclei with punctate basophilia (Figure 4E). Usually the morphological differences between *IGF2*-positive and *IGF2*-negative tumor cells were subtle (eg, Figure 2B). The nuclear morphology of *IGF2*-expressing neuroblastoma cells was reminiscent of the normal fetal paraganglia and SIF cells (cf. Figures 3, C and D, and 4E). However, clear evidence of ganglionic differentiation with abundant neuropil formation, more prominent nuclear enlargement, and a distinct nucleolus was found in two *IGF2*-positive tumors with a lobular pattern (exemplified in Figure 4, F and G). Both tumors were diagnosed after infancy. The density of *IGF2*-expressing tumor cells varied considerably, both between specimens and between different areas within a specimen, depending on the extent of lobule formation. *IGF2*-negative tumors were less mature, usually with condensed nuclei (not shown) and lack of a lobular histological pattern (Table 1).

Adrenal Tumors

Three of the six infant tumors clinically assigned to an adrenal origin were stage 4S, with massive hepatic

tumor growth causing death from respiratory problems in two of the children (Table 1). The other four infants remain alive despite distant tumor growth in three cases. Two of the 4S tumors expressed *IGF2* in paraganglion/SIF cell-like maturing cells within lobular pattern tumor areas, as described above. Intraadrenal tumor growth was histologically verified in both. The remaining infant tumors were morphologically immature, lacking the lobular pattern, and did not express *IGF2*, with the exception of one case (case 108, Table 1) where scattered cells were positive.

In contrast, most (6/9) children diagnosed with adrenal tumors after infancy died from tumor progression. Only two tumors of this older age group expressed *IGF2* (Table 1). One was diagnosed shortly after infancy (case 135), and the other was a prognostically favorable stage 2 tumor (case 127). Both of these tumors contained the lobular pattern characteristic of most extra-adrenal neuroblastomas (Table 1). One ganglioneuroblastoma, defined as a mixture of neuroblastic elements with varying degrees of ganglionic maturation and Schwann cells,[7,39] was tested (case 155). This tumor neither expressed *IGF2* nor displayed a lobular arrangement of the maturing cells (Table 1).

IGF2 *Expression in Ganglioneuroma*

Ganglioneuroma is a benign tumor, histologically resembling the mature features of ganglioneuroblastoma.[7,39] No *IGF2* expression was identified in two such tumors (Table 1).

Immunohistochemical Characterization of Neuroendocrine Differentiation and Proliferation in Neuroblastoma

Sections consecutive to those analyzed for *IGF2* expression were used for immunohistochemical analyses. *IGF2*-expressing cells were readily identifiable in these sections by their increased size, central location in tumor lobules, and their nuclear morphology (Figure 2, B and C). Chromogranin immunoreactivity was principally specific to *IGF2*-expressing cells, although present with varying intensity (Figure 2C), whereas *IGF2*-negative tumor cells were also chromogranin-negative. Chromogranin-positivity was also seen in the central areas of tumor lobules associated with cellular debris (not shown). A more detailed inspection revealed that this part of the tumor lobule consists of apoptosis-like cellular remnants. Morphological evidences of apoptosis were present in a zone of cells with small, condensed, and occasionally fragmented nuclei adjacent to the zone of *IGF2*-expressing cells (Figures 2B and 4H). The DNA of these cells was fragmented as determined by the TUNEL terminal DNA nick end labeling technique (J. C. Hoehner, F. Hedborg, H. Jernberg-Wiklund, H. L. Olsen, S. Påhlman, Int J Cancer, in press). Invariably, these apoptosis-like bodies did not express *IGF2* (Figure 2B). The bulk of the cellular debris area consisted, however, of cell remnants devoid of nuclei (Figures 2B and 4H). Foci of apoptosis-like bodies were only observed in tumors with a lobular pattern, most of which expressed *IGF2* (10/12; Table 1).

The spatial distribution of proliferating tumor cells was determined immunohistochemically by detection of the cell cycle-specific Ki 67 protein. A particularly large proportion of tumor cells were positive in aggressive tumors. Ki 67 immunoreactivity was most prominent among tumor cells with fibrovascular stroma contact, whereas cellular zones with *IGF2* expression had a low fraction of Ki 67-immunoreactive cells (Figure 2B). Hence, *IGF2*-expressing cells do not appear to be proliferative to any great extent.

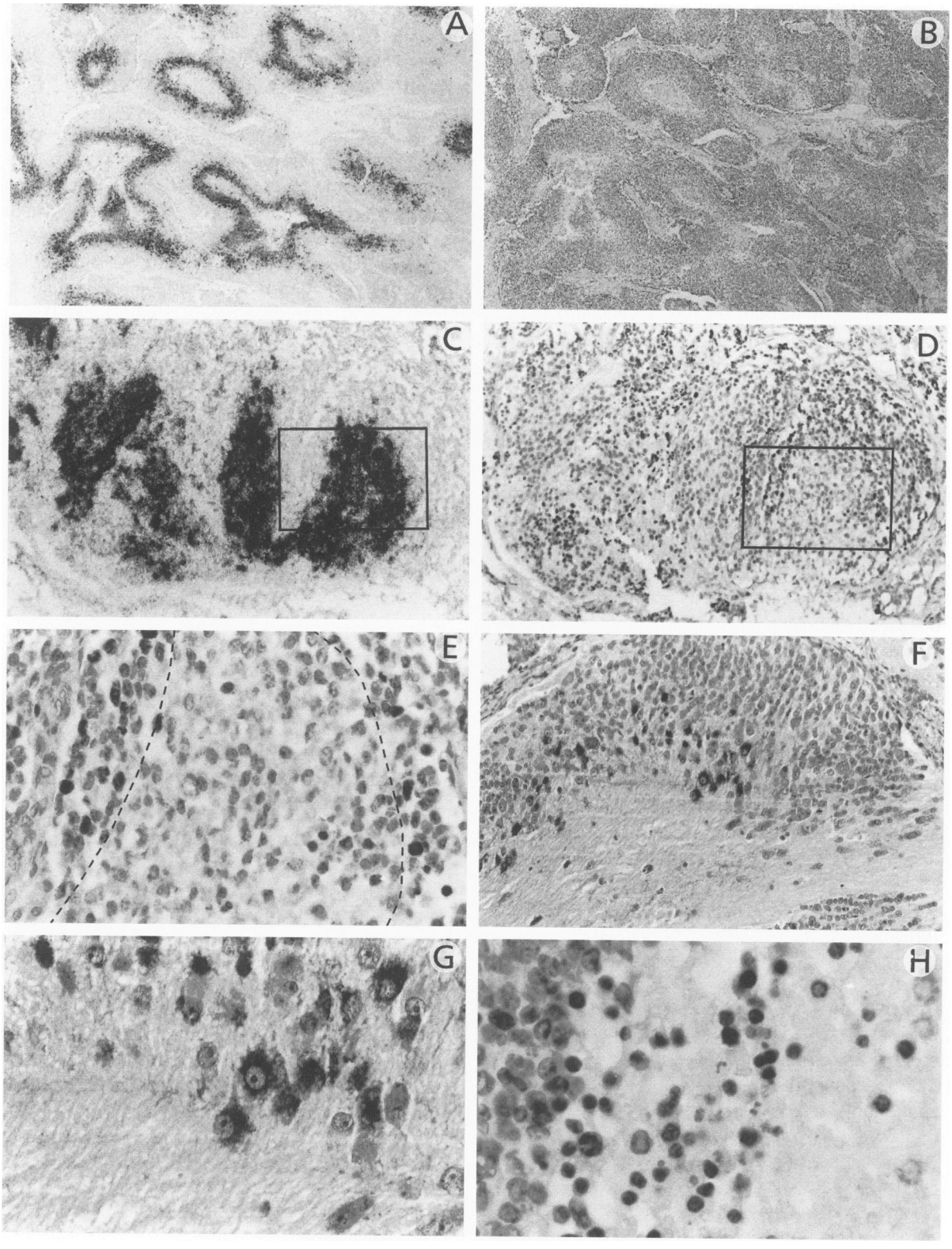
Correlation Between IGF2 *Expression in Tumor Tissue and Clinical Signs of Endocrine Tumor Activity*

Severe cardiovascular symptoms, a relatively rare occurrence with neuroblastoma,[40] were found in five cases, three of them diagnosed during infancy (cases 78, 118, and 157; Table 1) and the other two at 21 months and 3 years of age (cases 135 and 165). These tumors were particularly rich in *IGF2*-expressing cells. Four of these children required treatment for hypertension. The fifth tumor was congenital; the child suffering unexpected asphyxia at birth (case 157; Table 1) died a few days later with a clinical picture of cardiogenic shock in combination with hepatic vascular thrombosis. The content of *IGF2*-expressing cells in this tumor was the highest found (Figure 4A).

Discussion

Specificity of IGF2 *Expression in the Sympathetic Nervous System during Development*

Whereas *IGF2* expression was absent in sympathetic neuronal cells at all stages of differentiation, paraganglia and SIF cells did express this gene at high levels. In contrast, *IGF2* expression in chromaffin cells of the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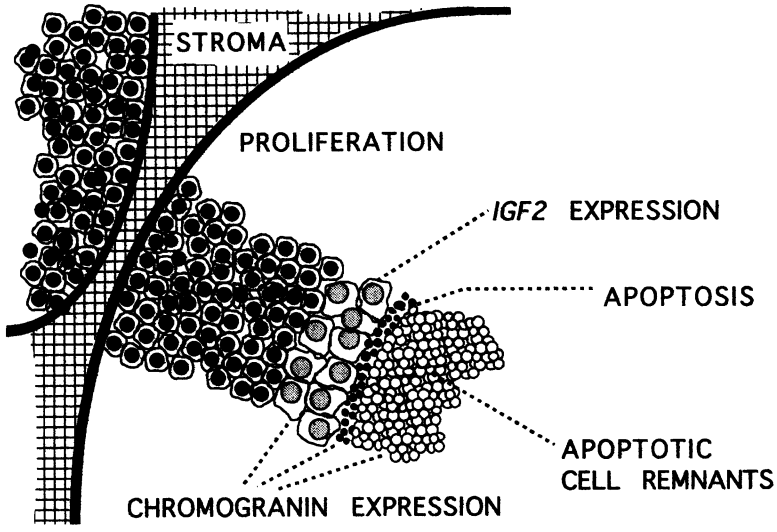

Figure 5. *Schematic representation of functional zones in an* IGF2*-expressing neuroblastoma lobule (cf. Figure 2B).*

adrenal medulla was negligible, as previously reported.[41] Another type of adrenal medullary cell, primitive sympathetic cells, was present in small nests and showed no detectable expression. In the developing sympathetic nervous system high-level *IGF2* expression is therefore specific for extra-adrenal neuroendocrine cells.

Are There Two Separate Lineages in Neuroblastoma?

Observations such as spontaneous maturation of neuroblastoma into benign ganglioneuroma,[42–45] the neuronal morphology of ganglioneuroblastomas,[7,39] neurite formation,[46,47] and other neuronal characteristics of neuroblastoma and derived cell lines[46–48] are fundamental to the traditional view that differentiation in neuroblastoma continues along a neuronal lineage. The results of this investigation reveal, however, that a subset of neuroblastomas express *IGF2* as a putative sign of neuroendocrine differentiation. This feature of differentiation in neuroblastoma provides a new perspective on its pathology. The two most consistent morphological signs linked to *IGF2* expression in neuroblastoma were a lobular pattern of cellular growth and moderately enlarged and pale nuclei with a punctate pattern of basophilia. When using the terminology suggested by Joshi et al[7,39] such tumors would be classified as "poorly differentiated" and "differentiating" neuroblastomas. However, a greater emphasis was placed on ganglion cell-like nuclear features as determinants for differentiation in this classification system. We therefore believe that this and other systems for describing cellular maturation in neuroblastoma (reviewed in ref. 7) are confusing and would benefit considerably by introducing a distinction between neuronal and neuroendocrine phenotypes and that *IGF2* expression is a useful tool in this respect.

The prenatal abundance of paraganglia and SIF cells, contrasting with their postnatal decline, suggests an important developmental role of these cells. In view of the poorly developed adrenal medulla during fetal life, it is likely that the fetal sympathetic endocrine functions are exerted predominantly by the extra-adrenal chromaffin tissue. It has been shown that paraganglia are the major source of catecholamines during fetal asphyxia.[17,18] If neoplasias were to develop from these prenatally active cell types, one might predict features such as a congenital or infantile tumor appearance in extra-adrenal locations, a high endocrine activity with a lack of

Figure 4. *Cell type-specific* IGF2 *expression in neuroblastoma:* (**A, B**) IGF2 *is expressed in characteristic ringlike patterns in tumors with a lobular histology. Consecutive sections of a congenital pararenal neuroblastoma (case 157; see also Figure 2B).* (**A**) *IGF2 expression (brightfield view)* (**B**) *Ki 67 immunohistochemistry; here used for illustration of the lobular histological pattern. Magnification, 12×.* (**C–E**) *IGF2 expression correlates to areas with morphological maturation. Consecutive sections from a prognostically favorable pelvic infant neuroblastoma (case 128; Table 1).* (**C**) *IGF2 expression (brightfield view; 58×).* (**D**) *Consecutive section stained with hematoxylin.* (**E**) *High-power magnification (180×) of boxed area in* (**D**) *showing nuclear morphology. Zone of IGF2-expressing cells is indicated (dashed lines). Note similarity in nuclear morphology compared to normal fetal SIF and paraganglia cells (cf. Figure 3,* **C** *and* **D**). (**F, G**) *Occasional* IGF2*-expressing tumors display ganglion cell-like features. A prognostically favorable pelvic neuroblastoma, diagnosed at 2.5 years (case 138) with a lobular pattern coexpressing chromogranin A+B* (**F**) *and IGF2 (not shown). Magnification, 58×.* (**G**) *High-power magnification (180×) of* (**F**)*, showing nuclear morphology and neuropil.* (*H*) *Morphological evidence of apoptosis in the central area of an* IGF2 *positive tumor lobule. Nuclear condensation and fragmentation and cell remnants without nuclei are seen adjacent to a zone of* IGF2*-expressing tumor cells. High-power view (360×) of area indicated in Figure 2B1. All specimens are counterstained with hematoxylin.*

adrenalin in the profile of secreted catecholamines, a tendency for spontaneous postnatal regression, and histological features resembling paraganglia/SIF cells. Because prognostically favorable and stage 4S infant neuroblastomas meet all these criteria we suggest that these tumors, as well as extra-adrenal tumors of older children, constitute a subgroup of neuroblastomas that may be viewed as embryonal paraganglia/SIF-omas. However, these neuroendocrine neuroblastomas differ phenotypically from adult paragangliomas in that neuroblastomas express the neuronal c-*src* splice variant c-*srcN*[48] and bcl-2 (J. C. Hoehner, F. Hedborg, H. Wiklund-Jernberg, L. Olsen, Påhlman S; Int J Cancer, in press), while adult paragangliomas do not (ref. 48; J. C. Hoehner, unpublished data). Thus prognostically favorable infant and extra-adrenal neuroblastomas do show neuronal characteristics, suggesting a derivation from a progenitor cell with a mixed neuroendocrine and neuronal phenotype. This progenitor cell may have the capacity to mature either along a neuronal or a neuroendocrine lineage, as also discussed below.

Based on the bipotential properties of paraganglia and SIF cells, which both have a capacity for *in vitro* and *in vivo* transdifferentiation into a neuronal phenotype when exposed to nerve growth factor (NGF),[49–51] one could infer that low-malignant neuroblastomas, which are shown to express high transcript levels of c-*trk*, the high-affinity NGF receptor gene,[52,53] may have the same capacity for transdifferentiation from a paraganglionic/SIF cell phenotype into tumors with a ganglionic morphology. This speculation is consistent with the clinical types of neuroblastoma reported to undergo spontaneous maturation into ganglioneuroma. Goldman et al[43] reviewed eight cases of maturing neuroblastomas, all of which were extra-adrenally located and seven of which were diagnosed before 2 years of age. Spontaneous maturation into ganglioneuroma is also reported in 4S tumors (reviewed in refs. 43 and 44), here shown to be of the same neuroendocrine phenotype as other prognostically favorable infant tumors. Observations in ganglioneuroma, such as its typically extra-adrenal location,[45] the relatively late age at diagnosis,[45] and its association to the BWS[33,35] also favor the hypothesis that this benign tumor represents a maturational remnant from infant tumors. Interestingly, the small number of *IGF2*-expressing tumors with a ganglion cell-like morphology found in this investigation were diagnosed after infancy, consistent with an age-dependent conversion of phenotype. However, despite the ganglionic features of these tumors, they would rather be classified as "differentiating neuroblastomas" than "ganglioneuroblastomas" when applying the terminology suggested by Joshi et al,[7,38] since no Schwann cell component was seen.

Do Some Neuroblastomas, Clinically Assigned to an Adrenal Origin, in Fact Originate from the Juxta-Adrenal Sympathetic Tissue?

It is likely that the abundant juxta-adrenal sympathetic tissue during development would be a significant contributor to the same types of neuroblastomas as found in other extra-adrenal parts of the sympathetic nervous system. Such a tumor origin may, however, be difficult to distinguish clinically from a true adrenal medullary origin. This may explain why suprarenal growth is not an infrequent finding in low-stage infant tumors. A true adrenal medullary growth for 4S tumors was histologically confirmed in this study despite the presence of the extra-adrenal chromaffin differentiation marker *IGF2* expression. This apparent paradox would be reconciled by assuming that the adrenal siting of 4S tumors, instead of being the true site of origin, is the result of organ-specific metastatic spread, which is a prominent feature of this tumor form.

Clinical Implications

IGF2 expression is not a reliable predictor of a favorable outcome; however, it is associated with prognostically favorable tumors. It may rather provide improved biological characterization of an individual tumor, eg, in evaluation of endocrine activity. It is conceivable that the site-dependent phenotypical differences in aggressive neuroblastomas of older children, as indicated by their differences in *IGF2* expression, may have therapeutic implications. For example, targeted therapy with radiolabeled catecholamine metabolites could be more efficacious in extra-adrenal tumors because of the higher content of catecholamine-storing granular vesicles in tumors with a neuroendocrine phenotype.

The Tumor Lobule: a Clue to the Function of IGF2 *in Neuroblastoma?*

Analogous to the organoid *"Zellballen"* pattern of adult paragangliomas,[13] most *IGF2*-expressing neuroblastomas were organized into lobules surrounded by thin fibrovascular septa. Distinct functional zones of proliferation, cell maturation, *IGF2* expression, and cell death could be defined in these lobules (Figure 5). Somewhat surprisingly, in view of the putative role

of IGF-2 as an autocrine growth factor in neuroblastoma cell lines,[21–23] *IGF2*-expressing cells were nonproliferative. As *IGF2*-expressing cells were the most mature, a differentiation-promoting role of IGF-2 may be inferred instead, consistent with other cell line data.[22] Alternatively, *IGF2* expression could be the effect, rather than the cause, of cell maturation, as has been shown with retinoic acid-induced *in vitro* differentiation.[54] Furthermore, as *IGF2* expression defined a border to apoptosis-like cells, a role for this growth factor in the process of programmed cell death may be implicated. Nevertheless, the presence of proliferation in the periphery of tumor lobules followed by maturation and subsequent apoptotic cell death toward the center may be pertinent clues to the unique pattern of growth, maturation, and spontaneous regression that is associated with these tumors.

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