Expression of the Neurotrophin Receptor p75NTR in Medulloblastomas Is Correlated with Distinct Histological and Clinical Features: Evidence for a Medulloblastoma Subtype Derived from the External Granule Cell Layer

JENS BÜHREN, ARNDT H.A. CHRISTOPH, MD, ROLF BUSLEI, MD, STEFFEN ALBRECHT, MD, OTMAR D. WIESTLER, MD, AND TORSTEN PIETSCH, MD

Abstract. Medulloblastomas (MBs) are primitive neuroectodermal tumors (PNET) of the cerebellum. They represent the most frequent malignant pediatric brain tumors, but their origin still remains unresolved and controversial. MB cells correspond to different stages of neural development and differentiation as illustrated by their expression of neuronal and glial markers. In the present study, we examined the expression pattern of the common low-affinity neurotrophin receptor p75NTR in a series of 167 MBs by immunohistochemistry. While p75NTR was present in only 17% of classic MBs (CMB), we found expression of p75NTR in all desmoplastic (nodular) MBs (DMB) examined, and in 71% of those MBs with a significant desmoplastic component. Furthermore, both desmoplastic histology and p75NTR expression were present preferentially in those tumors of adolescents and adults that are frequently located laterally in the cerebellar hemispheres. In DMBs, p75NTR was expressed predominantly in the proliferative, reticulin-rich areas, which may show coexpression of GFAP. In the pale islands of DMB, p75NTR was expressed only weakly or was absent. The expression pattern showed an inverse relation to that of the synaptic vesicle protein synaptophysin that was predominant in p75NTR negative classic MBs. Since the neurotrophin receptor p75NTR is expressed in cells of the external granule cell layer (EGL) of the fetal cerebellum, our findings suggest that progenitor cells of the EGL are the cellular origin of a distinct subset of MB, namely the desmoplastic variant and MBs with a significant desmoplastic component.

Key Words: Desmoplastic; Differentiation; Granular layer; Medulloblastoma; p75 neurotrophin receptor; PNET; Primitive neuroectodermal tumor.

INTRODUCTION

Medulloblastomas (MB) are cerebellar primitive neuroectodermal tumors of high malignancy, first described by Bailey and Cushing (1). Their molecular pathology is still poorly understood. Medulloblastomas are the most common malignant brain tumors of childhood and express markers of CNS progenitor cells or of neuronal and/or glial lineages and therefore reflect different stages of neuronal or glial development and differentiation (2–9). Until today the origin of MBs remains controversial. In early (10) and later (11) hypotheses, an origin of MBs from the cells of the external granule cell layer (EGL) of the fetal cerebellum was postulated. Rorke proposed that MBs should be included in the group of tumors designated as primitive neuroectodermal tumors (PNETs) (12, 13). These tumors, as described by Hart and Earle (14), are all believed to arise from a common neuroepithelial stem cell of the subependymal matrix layer, regardless of their location in the CNS.

A variant of MB, first described by Foerster and Gagel (15) as "circumscribed arachnoidal sarcoma of the cerebellum", is the desmoplastic MB (DMB), which is characterized by the coexistence of undifferentiated tumor cells separated into files and rows by a dense intercellular network of reticulin fibers and distinct reticulin-free islands of lower cellularity with more differentiated cells. Recent studies uncovered significant differences in molecular cytogenetics and expression of the calcium-binding protein Calbindin-D_{28k} between classic MB and the desmoplastic variant of MB (16–18).

Neurotrophic factors and their receptors play a crucial role in differentiation and maintenance of the developing neuronal progenitor cell. Physiologically, growth and differentiation of cerebellar granular cells are regulated by the action of the neurotrophins BDNF and NT-3 and the coordinated expression of their specific receptors TrkB and TrkC, respectively (for review, see 19). The p75 neurotrophin receptor (p75NTR, formerly called low-affinity nerve growth factor receptor) is a member of the tumor necrosis factor-receptor family. It contains a death domain and is involved in both survival and apoptosis of neural cells (20, 21). p75NTR has been detected in the EGL of the developing rodent (22, 23) and human (24, 25) cerebellum, but its role in cerebellar development has not yet been determined.

More recently, several studies have examined the expression of neurotrophin receptors in PNETs (26–30) with a focus on the Trk receptors (9, 31, 32). In the present study we examined the expression and distribution pattern of the common low-affinity neurotrophin receptor p75NTR in a large series of cerebellar PNETs and 10 MB cell lines by immunohistochemistry and correlated its expression with histology, differentiation, and patients' age.

TABLE 1
Distribution of Medulloblastoma Subtypes

Tumor	Abbre-viation	n	Mean age
Classic medulloblastoma	CMB	106	10 years
Desmoplastic MB	DMB	17	20 years
MB with desmoplastic component	MDC	17	21 years
MB with desmoplastic reaction	MDR	21	12 years
Medullomyoblastoma	MMB	3	7 years
Cerebellar neuroblastoma	CNB	3	3 years
		167	12 years

Our results strongly suggest the existence of distinct sub-types of MBs originating from different progenitor cell populations of the cerebellum.

MATERIALS AND METHODS

Tumor Samples

A total of 167 medulloblastomas and other central primitive neuroectodermal tumor specimens were selected for this study (Table 1). The samples were taken from the files of the Department of Neuropathology and the Brain Tumor Reference Center, both at the University of Bonn Medical Center. Tumor tissue was fixed in 4% buffered formalin and routinely processed for paraffin embedding. Twenty tumors were snap-frozen in liquid nitrogen directly after surgery and stored at $-80°C$. For classification, tumor samples were studied using a panel of routinely applied histological and immunohistochemical stains: hematoxylin and eosin (HE), Gomori (reticulin fibers), synaptophysin, neuron-specific enolase (NSE), the embryonal variant of the neural cell adhesion molecule (eNCAM), the proliferation marker Ki-67 (Mib-1), glial fibrillary acidic protein (GFAP), and, if required for differential-diagnostic purpose, PAS, Bodian, Nissl, vimentin, and desmin.

All samples were classified by 2 neuropathologists according to the WHO classification of brain tumors (33). The cases were divided into the following groups: 1) Classical MB (CMB; 106 cases, 63%): These cases had the prototypical appearance of "small blue cell tumors," and were composed of small, undifferentiated cells lacking intercellular reticulin fibers. 2) Desmoplastic MBs (DMB; 17 cases, 10%): These tumors were biphasic and contained both a highly cellular component with a dense intercellular network of reticulin fibers, as well as hypocellular, reticulin-free nodules ("pale islands"). By definition, this pattern had to be present throughout the entire tumor. 3) MBs with desmoplastic component (MDC; 17 cases, 10%): In these tumors, most areas had the appearance of CMB, but foci typical of DMB were also present. 4) MB with desmoplastic reaction (MDR; 21 cases, 13%): These tumors had significant desmoplasia (as assessed on the reticulin stain) but did not show the typical biphasic growth pattern of DMB. Most cases of MDR were located superficially and showed obvious leptomeningeal invasion. 4) Medullomyoblastomas (MMB; 3 cases, 2%): This diagnosis was made only if rhabdomyoblastic differentiation was obvious on the H&E stain by the presence of large tumor cells with eosinophilic fibrillary cytoplasm and was confirmed by desmin immunohistochemistry. 5) Cerebellar

neuroblastomas (CNB; 3 cases, 2%): These tumors showed very large reticulin free pale islands. Cells in the islands were well differentiated and resembled immature granule cells (Fig. 7a). They were frequently arranged in parallel files similar to those found in neuroblastomas. The mitotic activity was very low in these islands, as determined with the proliferation marker Ki-67. They expressed the neuronal markers synaptophysin, NSE, eNCAM, and NeuN (Fig. 7d–g). These tumors were classified as "cerebellar neuroblastoma" (CNB) according to Rubinstein (34). The WHO classification (33) includes them in the group of DMBs.

Fetal Cerebellar Samples

Four fetal brains were obtained at autopsy; gestational ages were 17, 20, 28, and 40 weeks. They were fixed in formalin and routinely processed into paraffin. Paraffin sections of cerebellum were treated in the same manner as paraffin sections of tumors.

Primary Antibodies

The primary antibody ME 20.4 against the $p75^{NTR}$ (mouse monoclonal from the clone Hb 8737 [ATCC]) is an IgG monoclonal antibody which was generated by immunizing mice with WM 245 melanoma cells (38). This monoclonal antibody (MAb) is directed against epitopes near the NGF binding site because it interferes with the binding of ^{125}I-labeled NGF (35). A membranous staining pattern is considered specific and reflects binding of the antibody to the extracellular domain of $p75^{NTR}$ (38). Monoclonal antibodies against the following additional antigens were used: synaptophysin, NSE, eNCAM, Ki-67, and the neuronal nuclear antigen NeuN (36). A polyclonal antibody was used to detect the glial marker GFAP (Table 3).

Immunohistochemistry

Paraffin sections were cut at 4 µm, mounted on slides suitable for the capillary gap method (Fisher Scientific; Pittsburgh, PA), and air-dried in an incubator overnight at 37°C. Before staining the sections were deparaffinized in xylene, rehydrated in a graded alcohol sequence, and boiled for 2×5 minutes (min) at 600 W in citrate buffer solution (pH 6.0) in a microwave oven (Siemens; Munich, Germany). Immunostaining was carried out on a DAKO Tech Mate™ staining apparatus (DAKO; Glostrup, Denmark) using the reagents for the indirect streptavidin-peroxidase method provided by the manufacturer. Finally, the slides were dehydrated in graded alcohols and embedded in Corbit-Balsam (Hecht; Kiel, Germany).

Frozen tumor tissue was cut on a cryotome (25°C) at 5 µm, the sections were mounted onto slides (SuperFrost™; Menzel, Germany), dried, fixed in cold acetone for 10 min, air-dried for 30 min, wrapped in aluminium foil, and stored at $-80°C$. For immunostaining, sections were thawed for 2 hours (h) and then incubated in a moist chamber at room temperature with blocking solution (phosphate-buffered saline [PBS] with 5% non-fat dry milk [NFDM; Bio-Rad, Heracles, CA] and 2% normal rabbit serum [DAKO]) for 30 min. Nonspecific binding of avidin and/or biotin reagents was reduced by treatment with avidin and biotin solution (Avidin/Biotin blocking kit [Vector; Burlingame, CA]) for 15 min each. After rinsing with PBS, the slides were

TABLE 2
Primary and Secondary Antibodies Used for Immunostaining

Antibody	Species/Clonality	Dilution	Manufacturer
Anti-desmin (D33)	mouse monoclonal	1:50	DAKO
Anti-GFAP (Z0334)	rabbit polyclonal	1:400	DAKO
Anti-Ki-67 (Mib-1)	mouse monoclonal	1:250	Dianova, Hamburg, Germany
Anti-eNCAM (735)	mouse monoclonal	1:200	Dr. Gerardy-Schahn; MHH, Hannover; Germany
Anti-NeuN (Mab60)	mouse monoclonal	1:500	Dr. R.J. Mullen; Salt Lake City, UT; USA
Anti-NSE (H14)	mouse monoclonal	1:150	DAKO
Anti-p75^{NTR} (M 20.4/Hb 8737)	mouse monoclonal	Undiluted*	ATCC
Anti-synaptophysin (SY 38)	mouse monoclonal	1:20	DAKO
Anti-vimentin (V9)	mouse monoclonal	1:10	DAKO
Secondary antibody anti-mouse lg, biotinylated	rabbit polyclonal	1:167	DAKO
Secondary antibody anti-rabbit lg, biotinylated	swine polyclonal	1:167	DAKO
Secondary antibody anti-mouse lg, FITC-conjugated	horse polyclonal	1:150	Vector
Secondary antibody anti-rabbit lg, texas red-conjugated	goat polyclonal	1:150	Vector
Secondary antibody solution anti-mouse lg and anti-rabbit lg, biotinylated (TechMate™)	goat polyclonal	Undiluted	DAKO

* Hybridoma culture supernatant.

TABLE 3
Expression of p75^{NTR} in Cerebellar PNETs

Tumor	n	Negative	p75^{NTR}+	p75^{NTR} ++	p75^{NTR} +++
CMB	106	88 (83%)	12 (11%)	3 (3%)	3 (3%)
DMB	17	0 (0%)	3 (18%)	6 (35%)	8 (47%)
MDC	17	5 (29%)	5 (29%)	1 (6%)	6 (35%)
MDR	21	17 (81%)	1 (5%)	1 (5%)	2 (10%)
MMB	3	1 (33%)	1 (33%)	1 (33%)	0
CNB	3	0	1 (33%)	2 (67%)	0
	167	111 (67%)	23 (14%)	14 (8%)	19 (11%)

incubated with primary antibody against p75^{NTR} (undiluted hybridoma culture supernatant of ME 20.4 from the clone Hb 8737 [American Type Culture Collection ATCC; Rockville, MD]) overnight at 4°C. Unbound primary antibody solution was removed with filter paper followed by several rinses with PBS and PBS containing 0.05% Triton X-100 (Sigma; Deisenhofen, Germany). The sections were incubated at room temperature with the secondary antibody (rabbit anti-mouse biotinylated [DAKO], 0.6% in PBS with 2% NFDM) and avidin-biotin-horseradish peroxidase complex (DAKO) for 30 min each. The slides were developed using diaminobenzidine tetrahydrochloride solution (5% in tris buffer solution pH 7.5), washed with deionized water, counterstained with hematoxyline (Merck; Darmstadt, Germany), dehydrated in graded alcohols, and embedded in Corbit-Balsam (Hecht).

Double Immunofluorescence

Tumor sections were deparaffinized in graded alcohols. Rehydration in deionized water and PBS was followed by microwave treatment as described above. Sections were incubated in a moist chamber at room temperature with blocking solution (PBS with 5% NFDM and 2% normal rabbit serum [DAKO] and normal horse serum [DAKO]) for 30 min. After rinsing with PBS, the primary antibodies against p75^{NTR} and against GFAP (1:400 in PBS containing 0.1% bovine serum albumin [BSA] and 0.02% sodium azide [Merck]) were applied for 45 min at room temperature. Primary antibody solution was removed with filter paper followed by 2 rinses with PBS and 1 rinse with PBS containing 0.1% BSA and 0.02% sodium azide. The sections were incubated at room temperature with the fluorochrome-conjugated secondary antibodies (horse anti-mouse immunoglobulin, fluorescein isothiocyanate [FITC]-conjugated [Vector] and goat anti-rabbit immunoglobulin, Texas red-conjugated [Vector]) for 45 min. Antibodies were applied at a concentration of 10 µg/mL (1:150), diluted in PBS with 0.1% BSA and 0.02% sodium azide. After several rinses with PBS, sections were embedded in Fluoromount medium (Southern Biotechnologies Association; Birmingham, UK) and viewed and documented on a BH2 fluorescence photomicroscope (Olympus).

Evaluation of Immunostaining and Statistical Analysis

The evaluation of the immunostaining was performed by 2 observers. Both intensity of the immunostain as well as the distribution and the percentage of the labeled cells were scored semiquantitatively as follows: Negative immunostain (−); faint immunoreactivity, (focal or widespread, +); moderate immunoreactivity (focal or widespread, ++) or intensive focal staining (less than 50% of the cells intensively labeled, ++); strong immunoreactivity (more than 50% of the cells intensively labeled, +++). For statistical analysis of correlations between p75^{NTR} staining and other features, we performed a χ^2-test. Differences of the mean ages between groups of different immunoreactivity within the MB subgroups were checked by an independent Student's t-test.

Only correlations with a p value < 0.05 were considered significant.
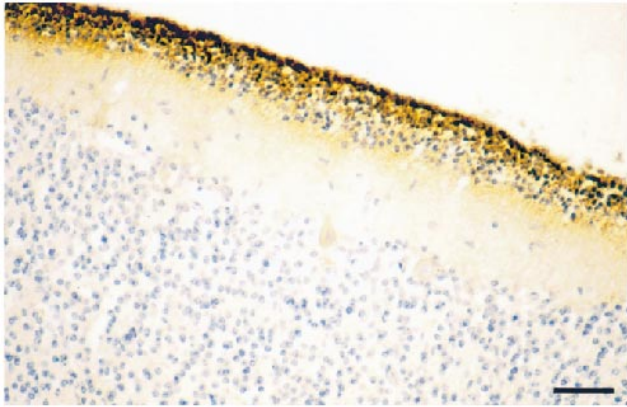
Fig. 1. p75^{NTR}-expression in the EGL of a 28w fetal cerebellum. The receptor is expressed both by the proliferative (outer) and by the premigratory (inner EGL) cells. Scale bar, 100 μm.

RESULTS

Fetal Cerebellum

The pattern of p75^{NTR} expression was similar in the 4 specimens of human fetal cerebellum (gestational age 17 w, 20 w, 28 w, 40 w) that were tested. p75^{NTR} was expressed both by the proliferative and by the premigratory cells of the EGL. (Fig. 1) and it was also expressed by Purkinje cells and in the dentate nucleus. The granule cells in the IGL showed no expression of p75^{NTR}.

Classic Medulloblastomas (CMB)

Of the 106 tumors (63%) in this series classified as CMB, 88 (83%) did not express p75^{NTR} (Fig. 2), while 18 (17%) were positive (12 of these showed only faint immunoreactivity [Table 3]). This is significantly less than other MB subtypes, of which 62% were p75^{NTR} immunoreactive (p < 0.000000005). Synaptophysin immunoreactivity was inversely correlated with p75^{NTR} expression and was present in only 17% of the p75^{NTR} positive CMBs, but in 45% of p75^{NTR} negative CMBs (p <0.05). However, no other statistically significant correlations with expression or absence of other neuronal marker proteins like NSE, eNCAM and NeuN were found. The patients' age at operation ranged from 7 days to 59 years with a mean age of 10 years. Patients with p75^{NTR}-positive CMBs were significantly older than patients with p75^{NTR}- negative CMBs (mean age 18 vs 8 years; p < 0.01).

Desmoplastic Medulloblastomas (DMB)

All 17 DMBs examined expressed p75^{NTR} (Table 3) with focal or faint reactivity (+, Fig. 3A) in 3 cases
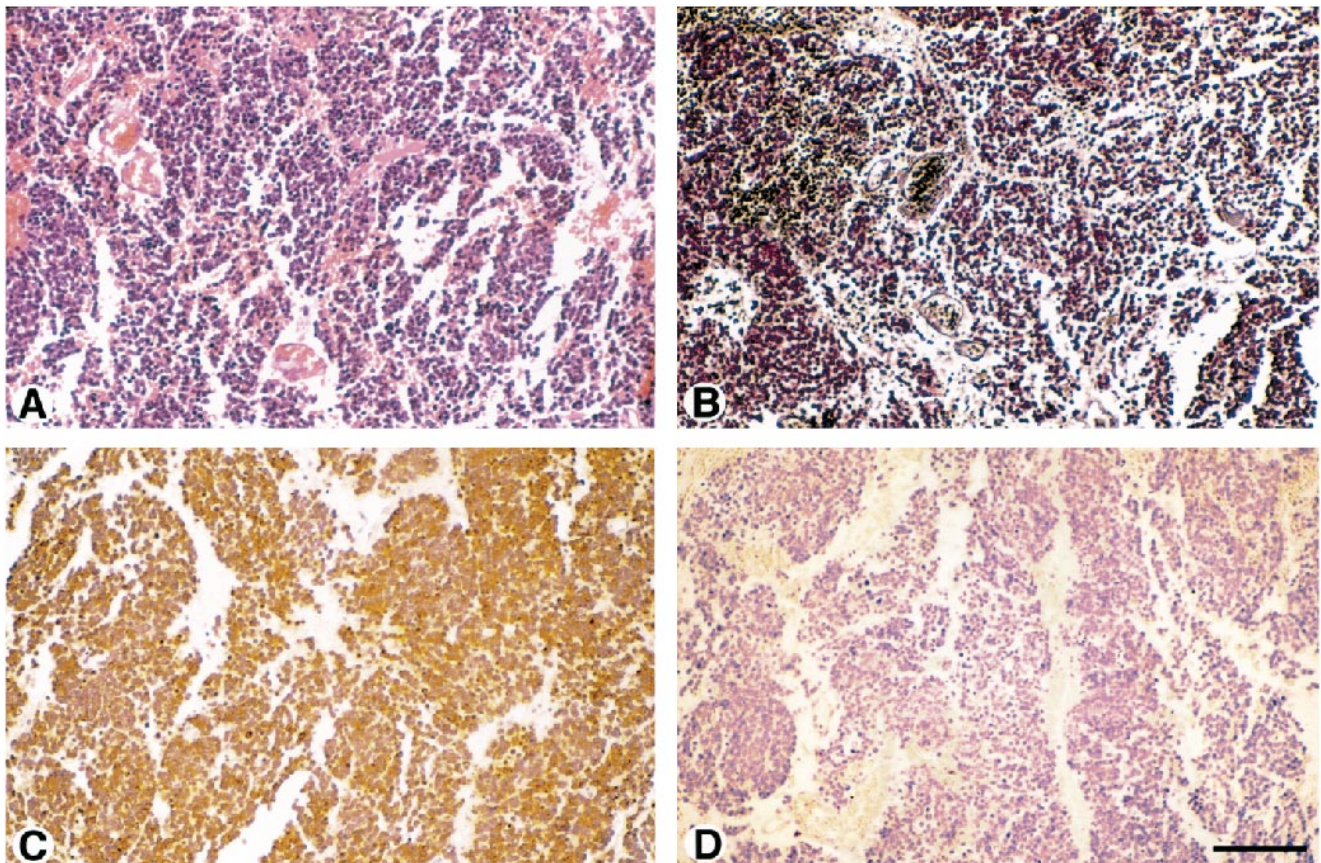
Fig. 2. Classic medulloblastoma. A: H&E stain. B: Gomori silver stain. C: Positive synaptophysin immunostaining. D: Negative p75^{NTR} immunoreaction. Scale bar, 100 μm.
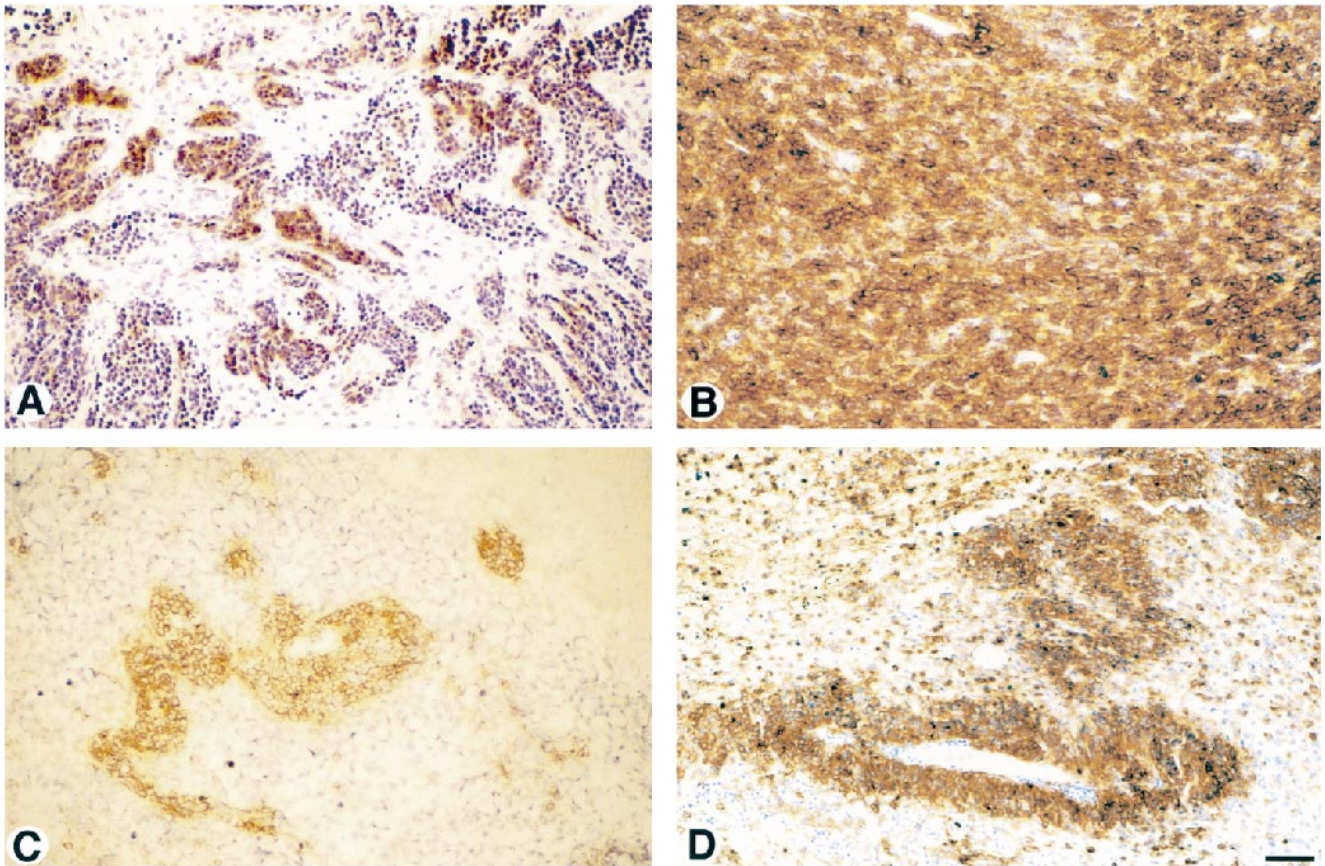
Fig. 3. Patterns of p75[NTR]-immunoreactivity in DMBs. A: Focal expression, B: Strong homogeneous immunoreactivity. C: Perivascular localization of p75[NTR]-positive cell clusters. D: Perivascular growth of p75[NTR]-positive tumor cells. Scale bar, 100 μm.

(18%) and strong or very strong immunoreactivity in the remaining 14 cases (82%) (Figs. 3, 4). Interestingly, in most cases, only faint immunoreactivity for p75[NTR] could be detected in the reticulin-free pale islands, whereas reticulin-rich areas were strongly labeled (Fig. 4). In contrast, neuronal markers like NSE and eNCAM were predominantly expressed in the islands (Fig. 4). In some tumors there was focal staining of scattered cell clusters. Typically, these clusters were located in perivascular areas (Fig. 3C). In other cases p75[NTR] positive tumor cells had a perivascular distribution (Fig. 3D). Although synaptophysin immunoreactivity was infrequent in DMBs, there was no statistically significant correlation between expression of p75[NTR] and the expression or absence of the neuronal markers synaptophysin, NSE, eNCAM, and NeuN.

Differences of patients' age could be found between the group of slightly (+) stained DMBs on the one hand (mean age 12 years) and the DMBs with higher levels of p75[NTR] expression (++ or +++) on the other hand (mean age 22 years). However, these findings were not statistically significant (p > 0.05).

Medulloblastomas with Desmoplastic Component (MDC)

Immunoreactivity for p75[NTR] was observed in 12 of the 17 MDCs (71%; p < 0.001 when compared with the other tumor subgroups), of which 7 showed intensive staining either focally (grade ++) or widespread (grade +++, Table 3). The staining pattern was similar to that seen in DMBs, however, nondesmoplastic areas showed immunopositivity, too. We could not find any significant correlation with expression or absence of the neuronal marker proteins synaptophysin, NSE, eNCAM, and NeuN. As in the other MB subgroups, there was a significant difference of mean age between p75[NTR]-negative and p75[NTR]-positive MDCs (12 years vs 24 years; p < 0.05).

Medulloblastomas with Desmoplastic Reaction (MDR)

The majority of the 21 MDRs examined were p75[NTR]-negative: in 17 tumor samples (81%) no immunoreactivity could be detected (Fig. 3); only 4 tumors (19%) were immunopositive for p75[NTR] to varying degrees (Table 3). In the group of p75[NTR]-positive MDRs the patients' mean age was higher than in the group of MDRs without
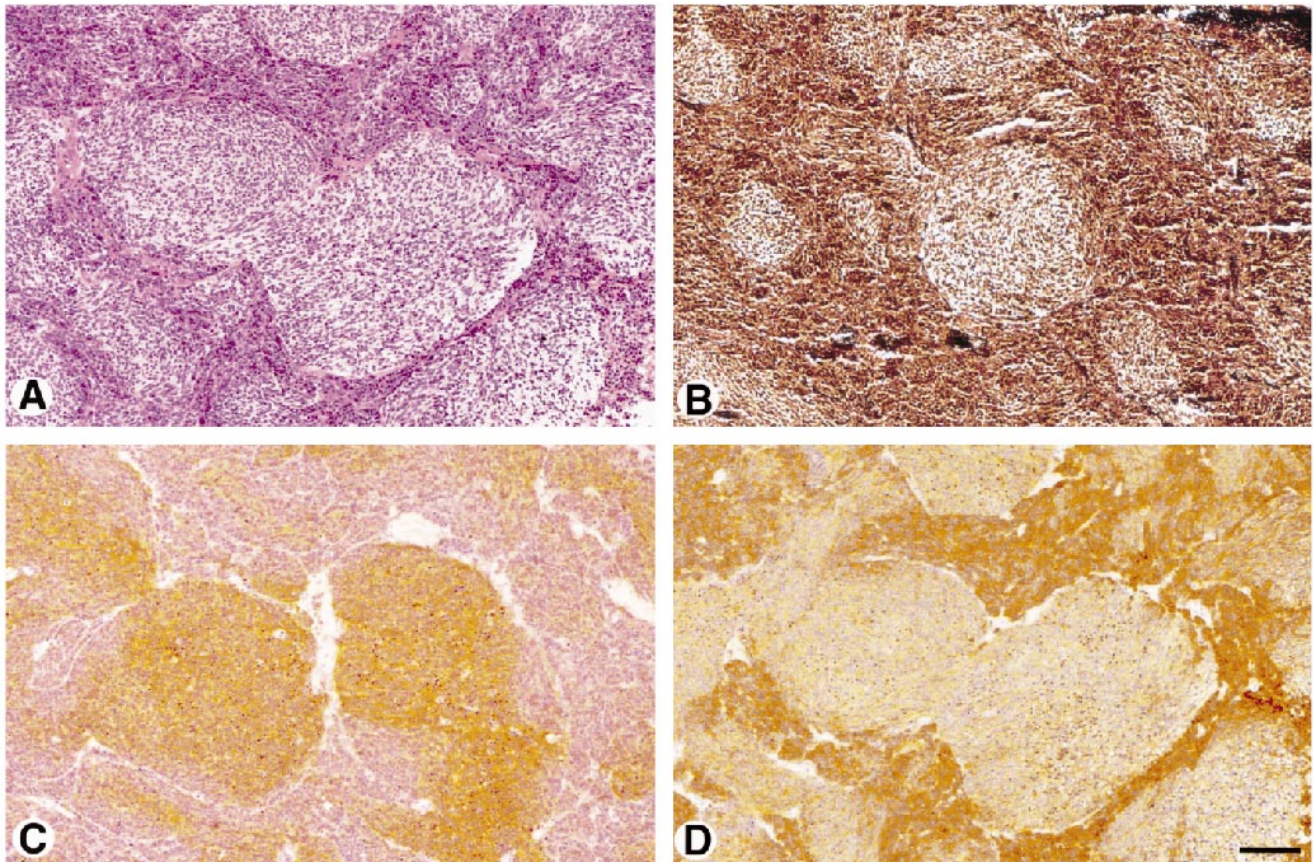
Fig. 4. Desmoplastic MB. A: H&E. B: Gomori silver stain. C: anti-NSE. D: p75^{NTR}-immunostaining: Intense expression in reticulin-rich areas, moderate staining in islands. Scale bar, 100 μm.

expression of p75^{NTR} (p75^{NTR}-negative tumors: 9 years; p75^{NTR}-positive tumors: 26 years), but these results did not reach statistical significance (p = 0.079).

Medullomyoblastomas (MMB)

Among the 3 MMB, 1 tumor was negative for p75^{NTR}; the other 2 showed faint or moderate immunoreactivity. Interestingly, 1 tumor showed colocalization of p75^{NTR} and GFAP on clusters of tumor cells.

Cerebellar Neuroblastoma (CNB)

All 3 cases of CNB were p75^{NTR}-positive. In 2 tumors a moderate p75^{NTR}-immunoreactivity could be detected, while the islands of highly differentiated cells were p75^{NTR}-negative (Fig. 6). The third case had faint immunoreactivity throughout the whole tumor.

GFAP Expression in Cerebellar PNETs

As proposed by Aguzzi (3), we considered GFAP-immunoreactive cells to be neoplastic only if immunoreactivity was restricted to the perikaryal area; cells with GFAP-immunoreactive stellate processes were considered reactive astrocytes. When these stringent criteria were applied, 21 (14%) of the 145 tumor samples tested showed GFAP expression. In the majority of the tumors, GFAP was only present focally. Of the GFAP-expressing tumors, 64% were also positive for p75^{NTR} (GFAP-negative tumors: 27%, p < 0.001). In some cases, there was colocalization of both antigens in identical cell clusters. The fraction of GFAP-expressing tumors was significantly higher among MBs with desmoplastic features (DMB and MDC): 38% vs 10% in nondesmoplastic (classic) MBs (p < 0.0005). However, there was no statistically significant correlation between p75^{NTR}-immunoreactivity and GFAP expression among CMBs.

Patients' Age and p75^{NTR} Expression

As shown above for separate MB subsets, the expression of p75^{NTR} was significantly correlated with higher age. These findings also apply to the entire series of 167 PNETs: Patients' mean age is 12 years; p75^{NTR}-negative tumors: 9 years, p75^{NTR}-positive tumors: 19 years (p < 0.000005); tumors with strong p75^{NTR} expression (+++): 25 years (p < 0.000001). The data are summarized in Table 4.

Tumor Location and p75^{NTR} Expression

In 72 cases we had access to the patients' clinical data. Only 3 of 51 of the midline tumors (6%) expressed
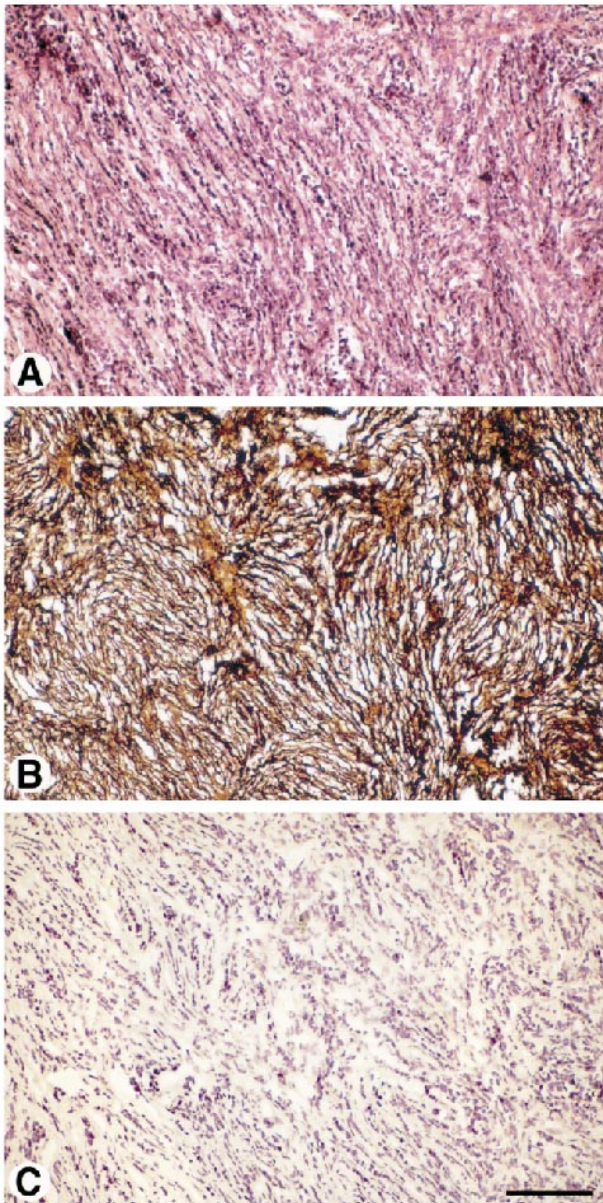
Fig. 5. MB with desmoplastic reaction. A: Indian filing on H&E. B: Gomori silver stain C: Negative p75[NTR]-immunostaining. Scale bar, 100 μm.

p75[NTR]. In contrast, 17 of the 21 tumors (81%) originating laterally in a cerebellar hemisphere expressed p75[NTR]; this difference is highly significant (p < 0.00000005).

DISCUSSION

Historical Review

The cell of origin of MB has been the subject of controversy for many years. Early hypotheses (10) favored the external granule cell layer of Obersteiner (Egl) as site of origin. An origin of MB from the EGL is supported by several cases of medulloblastoma adjacent to persistent and/or altered fetal EGL (37–39). These observations suggest that MB may arise from progenitor cells located in the EGL that have persisted after the first year of life. The second major hypothesis postulates that MB is derived from pluripotent stem cells of the ventricular (subependymal) matrix (VM). This cell type is believed to be present throughout the embryonal CNS and is capable of differentiating into either neuronal or glial cells. The occurrence of tumors with similar histopathologic features at other sites of the CNS, particularly supratentorially, would become explicable by this second hypothesis. These neoplasms had been described by Hart and Earle (14) as "primitive neuroectodermal tumors" (PNETs). Thus, Rorke proposed to classify MB as PNET of the cerebellum (12).

Subsequently, numerous immunohistochemical studies revealed different degrees of neuronal and/or glial differentiation in MBs/PNETs. Evidence of neuronal differentiation has been presented by several authors, including expression of NSE, synaptophysin, and class III ß-tubulin (2–5, 7). In an important immunohistochemical study on this subject (18), Katsetos et al demonstrated the expression of the calcium-binding protein calbindin-D_{28k} (calD) in some VM cells and in Purkinje neurons. In contrast, calD was absent in the EGL and its derivates. In MBs, calD was expressed in 80% of the CMBs examined, supporting the theory that MB cells might represent transformed VM cells (18). This led to the conclusion that the classic MBs might be derived from the VM cells located in the midline, as postulated by Rorke. On the other hand, calD was absent in all of the DMB specimens examined, including their pale islands. This strongly suggests that the desmoplastic MBs, which are often localized laterally in the cerebellar hemispheres rather than in the midline, might originate from the EGL of the fetal cerebellum, as proposed earlier by Kadin and Rubinstein (38).

While calD is absent in cells of the EGL, the neurotrophin receptor p75[NTR] (formerly referred to as low-affinity nerve growth factor receptor) is expressed in the EGL in both the developing rodent (22, 23) and human (24, 25) cerebellum, as well as in Purkinje neurons and in the dentate nucleus. The receptor is not expressed in postmitotic granule cells of the IGL and in VM cells. In the past, several studies (26–30) examined the expression of p75[NTR] in PNETs arising in different locations, but only one (30) suggested correlations between expression of p75[NTR] in MBs and possible derivation from the EGL.

Expression of p75[NTR] in Cerebellar PNETs

In the present study, we examined the expression and distribution pattern of p75[NTR] in a large series of cerebellar PNETs. We found p75[NTR] expressed in all of the MBs with desmoplastic features throughout the tumor (DMBs), and in 71% of MBs with a desmoplastic component (MDC). In contrast, the majority (83%) of classic (nondesmoplastic) MBs did not express the receptor. These findings led us, in agreement with Katsetos
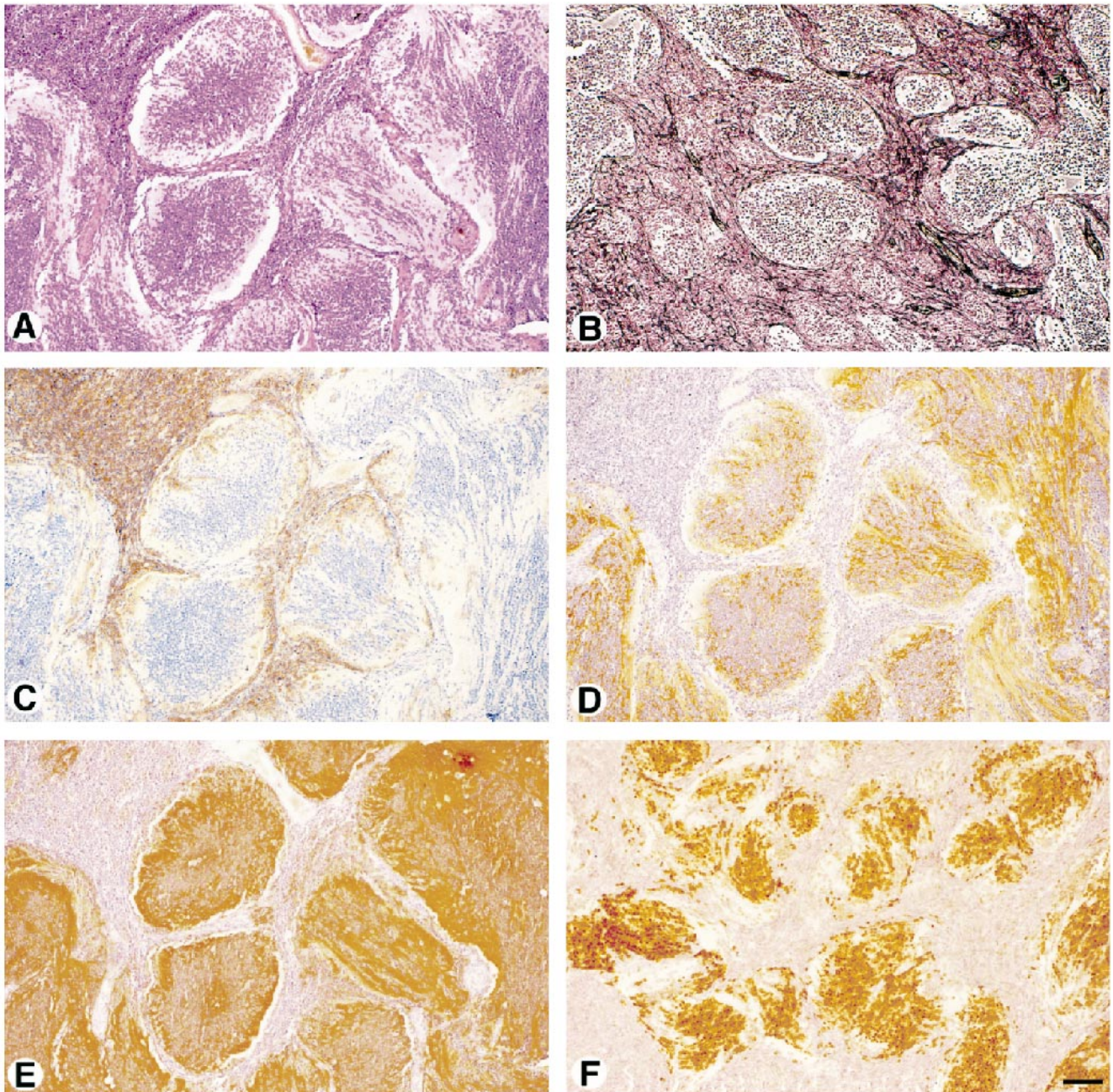
Fig. 6. Cerebellar neuroblastoma. A: H&E. B: Gomori silver stain. C: anti-p75^{NTR}. D: anti-synaptophysin. E: anti-NSE. F: anti-NeuN. Scale bar, 100 µm.

et al (18), to the conclusion that MBs of the "classic" type might be derived from pluripotent VM progenitor cells and, whereas MBs of the nodular (desmoplastic) type with pale islands originate from cells of the EGL. Obviously, the presence of p75^{NTR} in DMBs does not exclude an origin from VM cells with absolute certainty. We cannot rule out that the expression of p75^{NTR} in MB cells may be an epiphenomenon of malignant transformation. However, this would not explain the strong correlation between the desmoplastic (nodular) phenotype and the expression of p75^{NTR}.

The desmoplastic phenotype occurs predominantly in adolescents and adults (40–42). When p75^{NTR} expression was correlated with the patients' age, we found a significant (p < 0.000001) correlation between the expression of p75^{NTR} and patients' age, regardless of the MB subtype. This correlation was even stronger than the known association of desmoplastic phenotype with age (in our study: p < 0.00005). Similar results could be obtained for expression of p75^{NTR} and tumor location: In our study, the well-known correlation between desmoplastic histology and location in the cerebellar hemisphere reached a

TABLE 4
Patients' Mean Age in Cerebellar PNETs; MB Subgroups Listed By Grade of p75^{NTR} Immunoreactivity

Tumor	All	Negative	Positive	+	++	+++
CMB	10 y	8 y	18 y	18 y	13 y	23 y
DMB	20 y		20 y	12 y	23 y	22 y
MDC	21 y	12 y	24 y	24 y	25 y	27 y
MDR	12 y	9 y	26 y	8 y	15 y	41 y
MMB	7 y	9 y	7 y		7 y	
CNB	3 y		3 y	3 y	3 y	
all PNET	12 y	9 y	19 y	17 y	15 y	25 y

p value < 0.0000005. When expression of p75^{NTR} was correlated to the location, we found a 100-fold stronger level of significance ($p < 0.000000005$). Interestingly, 4 cases of p75^{NTR}-positive ''classic'' MBs had a nodular architecture with pale islands but lacked the typical reticulin pattern of DMB. A similar histological pattern has been described previously by Burger et al (2). Clinical information was available for 2 of these cases, and both were located laterally in a cerebellar hemisphere. This suggests that MBs, which show only a limited nodular appearance and lack a typical reticulin fiber network, may also derive from the EGL. In contrast, some superficial MBs induce a desmoplastic reaction due to leptomeningeal invasion but lack the typical nodular appearance of DMB; they were classified as ''MB with desmoplastic reaction'' (MDR) in our series. MDR only rarely expressed p75^{NTR} and did not seem to afflict adolescents or adults more frequently, as noticed for DMB/MDC. Because of their clinical features and their lack of p75^{NTR} expression, we postulate that MDRs do not represent a distinct entity and should be grouped together with classic MB.

As in the developing cerebellum, the biological function of p75^{NTR} in PNETs is unclear. During neural development, p75^{NTR} mediates apoptosis in several cell types (43). However, when D283 Med cells were engineered to express p75^{NTR} and treated with nerve growth factor (NGF), they did not show any reaction (44). Additional introduction of the TrkA receptor in the same cell line led to apoptosis when exposed to NGF (45). MBs frequently express the high-affinity neurotrophin receptors TrkA, TrkB, and TrkC (32); the specific function of the p75^{NTR} is believed to depend on the presence or absence of the Trk receptor (45). It remains to be determined whether the neurotrophins brain-derived neurotrophic factor (BDNF), or neurotrophin-3 (NT-3), which are necessary for granule cell development, have any effect on p75^{NTR}-expressing MB cells signalling via p75^{NTR} alone or in cooperation with the Trk receptors.

p75^{NTR} and Neuronal Differentiation in MBs

Although p75^{NTR} expression seems to characterize a distinct subtype of MBs, we also have evidence that this may be dependent on the degree of neuronal differentiation in these tumors. Some authors (3, 5) found expression of synaptophysin in DMBs predominantly in the pale, reticulin-free islands. However, most of the DMB/MDC in our series were completely negative for this protein or showed only faint reactivity. In contrast, in all of the cases of CNB that were examined, the pale islands were synaptophysin-immunoreactive with little or no p75^{NTR} expression, while the surrounding reticulin-rich areas had the reverse phenotype. The pale islands in DMB are regarded as foci of neuronal differentiation (34). Interestingly, this is similar to the physiological situation during cerebellar development: p75^{NTR} is present in the proliferative EGL, but absent in the postmitotic IGL; in contrast, synaptophysin, a specific synaptic vesicle protein, is absent in the EGL, but is expressed in the IGL. We also found a statistically significant negative correlation ($p < 0.05$) between synaptophysin immunoreactivity and p75^{NTR} expression in classic (nondesmoplastic) MBs, suggesting that p75^{NTR} expressing CMBs have a more immature phenotype.

Another neuronal differentiation marker, which characterizes mature neurons (e.g. granule cells) is the neuronal nuclear antigen NeuN (36). Its expression in MBs correlates with neuronal differentiation (A.H.A.C., unpublished data). In our panel of tumors, we could not detect any correlation between the expression of p75^{NTR} and NeuN; neither in the subgroup of classic nor in the subgroup of desmoplastic MBs.

p75^{NTR} and Glial Differentiation in MBs

Glial differentiation in PNET/MB, defined by the expression of GFAP, has been the subject of many studies (2, 3, 7–9). Herpers and Budka (46) found expression of GFAP exclusively in DMBs. We detected GFAP also in nondesmoplastic MB, but the observation that clusters of neoplastic GFAP-expressing cells could be detected more frequently in MBs with desmoplastic features (3) was confirmed by our study ($p < 0.0005$). In contrast to other authors (2, 3) , the GFAP-positive tumor cells were most frequently located in the reticulin-rich component, but not in the pale islands showing neuronal differentiation. Interestingly, we observed colocalization of p75^{NTR} and GFAP in clusters of neoplastic cells in some DMBs. GFAP expression in DMB might be interpreted as evidence of an origin from pluripotent progenitor cells. However, aberrant GFAP expression has also been detected after immortalization of EGL progenitors (47). GFAP expression in DMBs probably represents a similar aberration, especially since GFAP is only present focally in scattered cell clusters, while p75^{NTR} is expressed at high levels throughout the tumors.

PNETs Constitute a Heterogenous Group of Tumors

Although they share a similar ''small blue cell tumor'' histological appearance, several lines of evidence indicate

Ventricular matrix **EGL**

Cerebellar progenitor cells

(classic) medulloblastoma   **(desmoplastic) medulloblastoma**

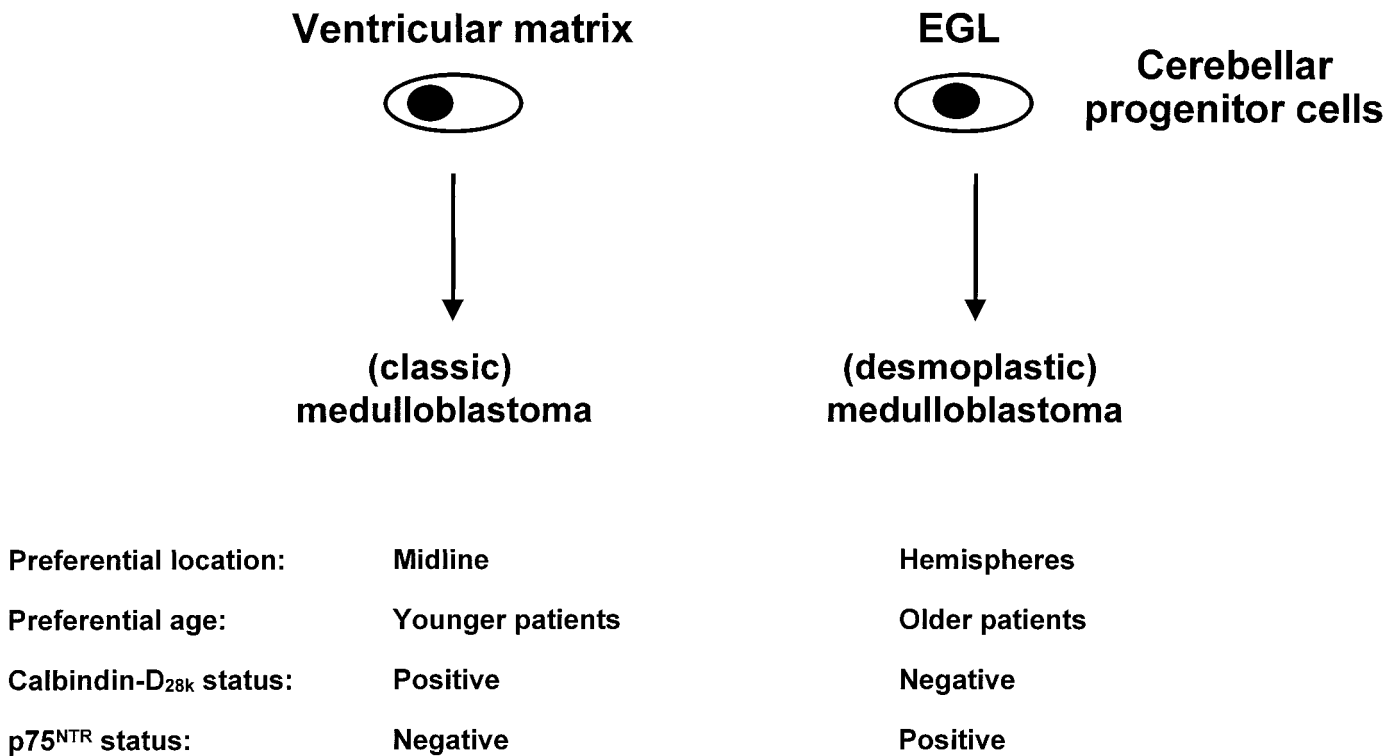|		
Preferential location:	**Midline**	**Hemispheres**
Preferential age:	**Younger patients**	**Older patients**
Calbindin-D$_{28k}$ status:	**Positive**	**Negative**
p75^{NTR} status:	**Negative**	**Positive**

Fig. 7. Schematic diagram on p75^{NTR} expression and origin of medulloblastomas.

that PNETs are in fact a heterogenous group of tumors, both clinically and biologically, thus putting into question the PNET concept. First, the clinical outcome of patients with PNET seems to be critically dependent on tumor location. In most studies, supratentorial PNET have a significantly worse prognosis compared with MBs even when taking into account the completeness of resection (48). Second, significant differences in cytogenetics, such as loss of the distal chromosome 17p only in MBs (49) or the expression of the basic helix-loop-helix transcription factor *HASH1* in supratentorial PNETs (50) have been detected. Third, the expression of certain granule cell lineage markers like the *PAX-6* gene (51), the zinc finger protein Zic (52), and the granule cell marker Hath-1 (53, R.B., unpublished data) in MBs point to an origin from specific progenitor cells located in the cerebellum and committed to a specialized neuronal lineage. These markers are expressed by granule cells in the cerebellum. Fourth, the results of Katsetos et al (18) and the findings of our study on p75^{NTR} point to different origins of the classic MB variant on one hand, and the desmoplastic MB variant on the other hand. Our data suggest that p75^{NTR} expression is typical of a subset of MBs that are derived from the EGL, tend to arise in the cerebellar hemispheres, occur in older patients, and have at least a focally nodular growth pattern with or without reticulin fibers (Fig. 7). In contrast, "classic" MBs that do not express p75^{NTR} and (a) lack desmoplastic features (CMB) or show only a desmoplastic reaction (MDR), (b) arise

in the cerebellar vermis, and (c) occur preferentially in children, may originate from the progenitor cells present in the subependymal matrix layer (Fig. 7). The unique tumor entity referred to as cerebellar neuroblastoma (CNB) might also be considered a derivative of the EGL.

Finally, recent molecular genetic data support the hypothesis of different molecular pathogenetic events leading to classic and desmoplastic MB: in addition to a higher frequency of loss of heterozygosity (LOH) at 9q (16) in DMBs, there was an association between desmoplastic features and inactivating mutations of the *PTCH* gene (17) located in this region. *PTCH* is the human homologue of the *Drosophila patched* gene. An important role for Ptch and its ligand Sonic hedgehog (Shh) in differentiation, growth, and migration of neural progenitors, including granule cell precursors, is assumed (54, 55). Inactivating germline mutations of *PTCH* exist in patients with the nevoid basal cell carcinoma syndrome (56). These patients are predisposed to develop MBs, again, predominantly those of the desmoplastic variant. Until now, only few of the downstream components in the *Shh/ Patched* pathway have been identified, and it remains to be seen whether the expression of p75^{NTR} in EGL neurons and DMBs is regulated by this pathway.

In summary, there are several lines of evidence indicating that PNETs in fact represent a heterogenous group of tumors with distinct biological and genetic features dependent on their site of origin and their different molecular pathogenesis.

REFERENCES

1. Bailey P, Cushing H. Medulloblastoma cerebelli. Arch Neurol Psychiatry 1925;14:192–223

2. Burger PC, Grahmann FC, Bliestle A, Kleihues P. Differentiation in the medulloblastoma. Acta Neuropathol (Berl) 1987;73:115–23

3. Aguzzi A, Wiestler OD, Kleihues P. Differenzierung im Medulloblastom: Immunhistochemische Untersuchung an 247 Fällen der Therapiestudie SIOP/GPO MED 84. Verh Dtsch Ges Path 1988;72:284–87

4. Molenaar WM, Jansson DS, Gould VE et al. Molecular markers of primitive neuroectodermal tumors and other pedriatic central nervous system tumors. Lab Invest 1989;61:635–43

5. Gould VE, Jansson DS, Molenaar WM et al. Primitive neuroectodermal tumors of the central nervous system. Lab Invest 1990;62:635–43

6. Trojanowski JQ, Tohyama T, Lee VM. Medulloblastomas and related primitive neuroectodermal tumors of childhood recapitulate molecular milestones in the maturation of neuroblasts. Mol Chem Neuropathol 1992;17:121–35

7. Mannoji H, Takeshita I, Fukui M, Ohta M, Kitamura K. Glial fibrilly acidic protein in medulloblastoma. Acta Neuropathol (Berl) 1981;55:63–69

8. Janss AJ, Yachnis AT, Silber JH et al. Glial differentiation predicts poor clinical outcome in primitive neuroectodermal brain tumors. Ann Neurol 1996;39:481–89

9. Tajima Y, Molina RP, Rorke LB et al. Neurotrophins and neuronal vs glial differentiation in medulloblastomas and other pediatric brain tumors. Acta Neuropathol 1998;95:325–32

10. Stevenson L, Echlin R. The nature and origin of some tumors of the cerebellum (medulloblastoma). Arch Neurol Psychiatr 1934;31:93

11. Rubinstein LJ. Cytogenesis and differentiation of primitive central neuroepithelial tumors. J Neuropathol Exp Neurol 1972;31:7–26

12. Rorke LB. The cerebellar medulloblastoma and its relationship to primitive neuroectodermal tumors. J Neuropathol Exp Neurol 1983;42:1–15

13. Rorke LB, Trojanowski JQ, Lee VM et al. Primitive neuroectodermal tumors of the central nervous system. Brain Pathol 1997;7:765–84

14. Hart MN, Earle KM. Primitive neurectodermal tumors of the brain in children. Cancer 1973;32:890–97

15. Foerster O, Gagel O. Das umschriebene Arachnoidalsarkom des Kleinhirns. Z Ges Neurol Psych 1939;164:565

16. Schofield D, West DC, Anthony DC, Marshal R, Sklar J. Correlation of loss of heterozygosity at chromosome 9q with histological subtype in medulloblastoma. Am J Pathol 1995;146:472–80

17. Pietsch T, Waha A, Koch A et al. Medulloblastomas of the desmoplastic variant carry mutations of the human homologue of drosophila patched. Cancer Res 1997;57:2085–88

18. Katsetos CD, Herman MM, Krishna L et al. Calbindin-D_{28k} in subsets of medulloblastomas and in the human medulloblastoma cell line D283 Med. Arch Pathol Lab Med 1995;119:734–43

19. Lindholm D, Hamnér S, Zirrgiebel U. Neurotrophins and cerebellar development. Perspect Dev Neurobiol 1997;5:83–94

20. Frade JM, Rodŕguez-Tébar A, Barde YA. Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature 1996;383:166–68

21. Frade JM, Barde YA. Nerve growth factor: Two receptors, multiple functions. BioEssays 1998;20:137–45

22. Yan Q, Johnson EM. An immunohistochemical study of the nerve growth factor receptor in developing rats. J Neurosci 1988;8:3481–98

23. Rocamora N, Garća-Landona FJ, Palacios JM, Mengod G. Differential expression of brain-derived neurotrophic factor, neurotrophin-3, and low-affinity nerve growth factor receptor during the postnatal development of the rat cerebellar system. Mol Brain Res 1993;17:1–8

24. Yachnis AT, Rorke LB, Lee VM, Trojanowski JQ. Expression of neuronal and glial polypeptides during histogenesis of the human cerebellar cortex including observations on the dentate nucleus. J Comp Neurol 1993;334:356–69

25. Chen EY, Mufson EJ, Kordower JH. Trk and p75 neurotrophin receptor in the developing human brain. J Comp Neurol 1996;369:591–618

26. Prior R, Reifenberger G, Wechsler W. Nerve growth factor receptor in tumors of the human nervous system. Immunohistochemical analysis of receptor expression and tumour growth fraction. Pathol Res Pract 1989;185:332–38

27. Baker DL, Molenaar WM, Pleasure S et al. Human central nervous system primitive neuroectodermal tumors expressing nerve growth factor receptors: CHP707m. Ann Neurol 1990;28:136–45

28. Baker DL, Reddy UR, Trojanowski JQ et al. Nerve growth factor receptor expression in peripheral and central neuroectodermal tumors, other pedriatic brain tumors, and during development of the adrenal gland. Am J Pathol 1991;139(1):115–22

29. Keles GE, Berger MS, Schofield D, Bothwell M. Nerve growth factor receptor expression in medulloblastomas and the potential role of nerve growth factor as a differentiating agent in medulloblastoma cell lines. Neurosurg 1993;32:274–80

30. Kokunai T, Sawa H, Tatsumi S, Tamaki N. Expression of nerve growth factor receptor by human primitive neuroectodermal tumors. Neurol Med Chir (Tokyo) 1994;34:523–29

31. Segal RA, Goumnerova LC, Kwon YK, Stiles CD, Pomeroy SL. Expression of the neurotrophin receptor TrkC is linked to a favorable outcome in medulloblastoma. Proc Natl Acad Sci USA 1994;91:12867–71

32. Washiama K, Muragaki Y, Rorke LB et al. Neurotrophin and neurotrophin receptor proteins in medulloblastomas and other primitive neuroectodermal tumors of the pediatric central nervous system. Am J Pathol 1996;148:929–40

33. Kleihues P, Burger PC, Scheithauer BW Histological typing of tumors of the central nervous system. New York: Springer, 1993

34. Bigner DD, McLendon RE, Bruner JM. Russel's and Rubinstein's pathology of tumors of the nervous system. 6th ed. London: Arnold, 1998

35. Ross AH, Grob P, Bothwell M et al. Characterization of nerve growth factor receptor in neural crest tumors using monoclonal antibodies. Proc Natl Acad Sci USA 1984;81:6681–85

36. Mullen RJ, Buck CR, Smith AM. NeuN, a neuronal specific nuclear protein in vertebrates. Development 1992;116:201–11

37. Scheinker IM. Zur Frage der Pathogenese und Pathologie der Medulloblastome. Monatsschr Psychiatr Neurol 1939;101:103

38. Kadin ME, Rubinstein LJ. Neonatal cerebellar medulloblastoma originating from the fetal external granular layer. J Neuropathol Exp Neurol 1970;29:583–600

39. Amacher AL, Torres QU, Rittenhouse S. Congenital medulloblastoma: An inquiry into origins. Childs Nerv Syst 1986;2:262–65

40. Hubbard JL, Scheithauer BW, Kispert DB et al. Adult cerebellar medulloblastomas: The pathological, radiographic and clinical disease spectrum. J Neurosurg 1989;70:536–44

41. Bloom HJ, Bessell EM. Medulloblastoma in adults: A review of 47 patients treated between 1952 and 1981. Int J Radiat Oncol Biol Phys 1990;18:763–72

42. Carrie C, Lasset C, Blay JY et al. Medulloblastoma in adults: Survival and prognostic factors. Radiother Oncol 1993;29:301–7

43. Van Der Zee CEEM, Ross, GM Riopelle RJ, Hagg T. Survival of cholinergic forebrain neurons in developing p75^{NTR}-deficient mice. Science 1996;274:1729–32

44. Pleasure SJ, Reddy UR, Venkatakrishnan G et al. Introduction of nerve growth factor (NGF) receptors into a medulloblastoma cell line results in expression of high- and low-affinity NGF receptors

but not NGF-mediated differentiation. Proc Natl Acad Sci USA 1990;87:8496–8500

45. Muragaki Y, Chou TT, Kaplan DR, Trojanowski JQ, Lee VM. Nerve growth factor induces apoptosis in human medulloblastoma cell lines that express TrkA Receptors. J Neurosci 1997;17: 530–42

46. Herpers MJHM, Budka H. Primitive neuroectodermal tumors including the medulloblastoma: Glial differentiation signaled by immunoreactivity for GFAP is restricted to the pure desmoplastic medulloblastoma (''arachnoidal sarcoma of the cerebellum'') Clin Neuropathol 1985;4:12–18

47. Gao WQ, Hatten ME. Immortalizing oncogenes subvert the establishment of granule cell identy in developing cerebellum. Development 1994;120:1059–70

48. Albright AL, Wisoff JH, Zeltzer P et al. Prognostic factors in children with supratentorial (nonpineal) primitive neuroectodermal tumors. A neurosurgical perspective from the Children's Cancer Group. Pediatr Neurosurg 1995;22:1–7

49. Burnett ME, White EC, Sih S, v. Haken M, Cogen PH. Chromosome arm 17p deletion analysis reveals molecular genetic heterogeneity in supratentorial and infratentorial primitive neuroectodermal tumors of the central nervous system. Cancer Genet Cytogenet 1997;97:25–31

50. Rostomily RC, Bermingham-McDonough O, Berger MC, Tapscott SJ, Reh TA, Olson JM. Expression of neurogenic basic helix-loop-helix genes in primitive neuroectodermal tumors. Cancer Res 1997;57:3526–31

51. Kozmik Z, Sure U, Rüedi D, Busslinger M, Aguzzi A. Deregulated expression of PAX-5 in medulloblastoma. Proc Natl Acad Sci USA 1995;92:5709–13

52. Yokota N, Aruga J, Takai S et al. Predominant expression of human Zic in cerebellar granule cell lineage and medulloblastoma. Canc Res 1996;56:377–83

53. Ben-Arie N, Bellen HJ, Armstrong DL et al. Math 1 is essential for genesis of cerebellar granule neurons. Nature 1997;390:169–72

54. Hahn H, Christiansen J, Wicking C et al. A mammalian patched homolog is expressed target tissues of sonic hedgehog and maps to a region with developmental abnormalities. J Biol Chem 1996;271: 12125–28

55. Wechsler-Reya RJ, Scott MP. Control of neuronal precursor proliferation in the cerebellum by Sonic Hedgehog. Neuron 1999;22: 103–14

56. Gorlin RJ. Nevoid basal-cell carcinoma syndrome. Medicine 1987;66:98–113