Apoptosis, Neuronal Maturation, and Neurotrophin Expression Within Medulloblastoma Nodules

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Abstract. Nodular/desmoplastic medulloblastomas are a well-established histopathological subtype containing reticulin-free nodules or “pale islands” that are comprised of cells with round “neurocytic” nuclei and abundant cytoplasm. Significant neuronal maturation occurs within nodules. We used immunohistochemistry to evaluate neuronal differentiation in the nodules of 6 of these tumors. The neuronal markers NeuN, synaptophysin, and MAP-2 were identified in the “pale islands” of all 6 nodular medulloblastomas examined, and high and medium molecular weight nonphosphorylated neurofilaments were detected in 2 of the 6 cases. We also observed collections of apoptotic cells within nodules. Given the known role of neurotrophin signaling in neuronal maturation and apoptosis, we analyzed immunohistochemically the distribution of neurotrophin receptors TrkA and TrkC and their primary ligands NGF and NT3 in 14 nodular medulloblastomas. TrkA and TrkC were detected in 13 and 10 cases, respectively, and were predominantly localized within nodules. NGF and NT3 were distributed diffusely with some nodular accentuation. The localized expression of Trk receptors within nodules of desmoplastic medulloblastomas suggests neurotrophin signaling is involved in the apoptosis and neuronal differentiation in medulloblastomas. We also examined expression of p53 and BCL-2 in these tumors; both were prominent in internodular regions but only weakly expressed within nodules. Trk receptors, p53, and BCL-2 are all expressed during development of the normal cerebellum. Interestingly, the immunohistochemical expression profile of these proteins in the differentiating nodules of medulloblastomas is in many ways similar to their expression in the developing cerebellum. Thus similar signaling pathways may be operational in cerebellar development and medulloblastoma tumor differentiation.

Key Words: Apoptosis; Desmoplastic; Medulloblastoma; Neurotrophin; Nodular; PNET; Trk.

INTRODUCTION

The nodular/desmoplastic medulloblastoma is a subtype well known for its content of nodular regions of reduced cellularity often referred to as “pale islands.” These distinctive structures are noted in advanced cases for their bland “neurocytic” nuclei and fine fibrillar “neuropil” that is created by the processes of the differentiating cells (1–3). Neuronal differentiation in these intranodular cells has been documented both ultrastructurally and immunohistochemically (1, 4, 5). Cells in the surrounding internodular tissue are less differentiated, cytologically more malignant, and mitotically more active. Apoptosis is often more prominent in the nodules than it is in internodular regions (4). Not surprisingly, given their degree of differentiation, nodules are associated with better outcomes (6–10). With regards to molecular analysis of nodular/desmoplastic medulloblastomas, it appears that mutations in the hedgehog pathway gene PTCH are much more common in this subtype than in “classic,” i.e. non-nodular, medulloblastoma (11).

Recent molecular studies of medulloblastoma prognostic factors, done independently of histopathological considerations, suggest that increasing levels of the neurotrophin receptor TrkC also are associated with a more favorable clinical outlook (12–14). The neurotrophins are a family of polypeptide signaling factors mediating neuronal proliferation, differentiation, and programmed cell death (15). Neurotrophins previously implicated in medulloblastoma pathobiology include nerve growth factor (NGF) and NT-3 (16, 17). Both of these bind with low affinity to the p75 neurotrophin receptor, while NGF preferentially binds with high affinity to the TrkA receptor and NT-3 preferentially binds with high affinity to the TrkC receptor.

Since neurotrophins induce cytologic differentiation and apoptosis, it seems reasonable that neurotrophin signaling may play a role in the evolution of the nodules in medulloblastomas. This prompted the present study characterizing the distribution of neuronal proteins, neurotrophins, and apoptosis-related proteins in nodular medulloblastomas.

MATERIALS AND METHODS

Histological Examination

The tumors reported herein were received as either surgical or consultation cases at the Johns Hopkins Hospital or obtained from the files of the Pediatric Oncology Group. All were selected as medulloblastomas of the desmoplastic/nodular subtype according to WHO criteria (3). The 14 patients included 8 boys and 6 girls, ages 7 months to 29 yr (mean age at diagnosis: 4 yr). Tumors were divided into 4 quartiles based on an estimate of the percentage of nodular tissue within each (1%–25%, 25%–50%, 50%–75%, and 75%–100%).
26%–50%, 51%–75%, or 76%–100%) as determined by review of the hematoxylin and eosin-stained sections.

**Immunohistochemistry**

For immunohistochemical studies, paraffin-embedded specimens were sectioned at 4 μm and deparaffinized. Trk receptor, BCL-2, p53, neurotrophin, and synaptophysin immunohistochemistry specimens were subjected to antigen retrieval by steaming (20 min at 80°C). Slides were then incubated at room temperature with antibodies directed towards Trk A (1:1,000 overnight, Santa Cruz Biotechnology sc-118, Santa Cruz, CA), Trk C (1:500 overnight, Santa Cruz Biotechnology sc-117), nerve growth factor-beta (1:2,000 overnight, Chemicon AB1528 SP, Temecula, CA), neurotrophin-3 (1:2,000 overnight, Chemicon AB1517P), BCL-2 (1:25 45 min, Dako Co, Carpinteria, CA), p53 (1:500 45 min, Dako Co, Carpen-teria, CA), or synaptophysin (1:50 45 min, Boehringer Mannheim, FRG). For MAP-2, NeuN and nonphosphorylated neurofilament (NF) immunohistochemistry, sections were preincubated in 3% H2O2 and 0.3% Triton before microwaving for antigen retrieval. Sections were microwaved twice for 5 min in Tris buffer (pH 7.6, MAP-2 immunostaining) or citrate buffer (pH 4.5, neurofilament immunostaining). Sections were then blocked in 3% horse serum (Chemicon) and incubated with antibodies directed at NeuN (1:500), MAP-2 (1:500, Sternberger SMI-52, Baltimore, MD) or NF (1:500, Sternberger SMI-32) overnight at 4°C as previously published (18). Antibodies were detected using the ABC method with diaminobenzidine serving as the chromogen. Immunopositivity was evaluated semi-quantitatively using the following scale: −, negative; +/−, weak; +, moderate; ++, strong.

**RESULTS**

**Neuronal Differentiation**

NeuN, MAP-2, and synaptophysin were expressed within nodules but were largely absent from internodular regions in all 6 cases examined (Table 1; Fig. 1A–C). Nodular immunopositivity for NF was present in 2 of 6 cases (Fig. 1D). Scattered small clusters of internodular cells most likely representing incipient nodules were sometimes weakly positive for all 4 neuronal markers. In addition, non-nodular regions of desmoplasia in Case 4 were moderately immunoreactive for NeuN and MAP-2. Immunoreactivity for BCL-2 and p53 was, however, much stronger in nodules than in inter nodular regions. Cells within nodules were positive for p53 in 11 cases whereas only 2 of 6 cases expressed BCL-2. p53-positive cells within nodules often had an irregular cytoplasmic border, whereas BCL-2 expression was more restricted to the periphery of nodules. Scattered small clusters of internodular regions were positive for p53 in 5 cases (Fig. 2A). Similar cells were also identified less frequently outside nodules. Internodular regions were strongly immunoreactive for BCL-2 in 11 of the 12 nodular medulloblastomas examined (Table 2; Fig. 2C). In 9 of these 11 cases, nodules also contained scattered cells positive for BCL-2 often concentrated in the periphery. BCL-2-positive cells within nodules had round to oval nuclei and moderate amounts of cytoplasm; they did not display the irregular cytoplasmic border of GFAP-positive astrocytic cells. In 2 cases (Case 8 and Case 9), diffuse BCL-2 staining encompassed both nodular and internodular regions. Strong to moderate internodular p53 immunoreactivity was detected in 5 of the 11 cases examined. Except for a region of diffuse positivity in case 9, cells within nodules were only weakly positive.

**Neurotrophin Expression**

Neurotrophin receptors (TrkA, TrkC) and ligands (NGF, NT-3) were analyzed immunohistochemically in 14 nodular medulloblastomas (Table 2). TrkA immunoreactivity was identified in 13 cases. Expression was restricted to differentiating nodules (Fig. 3A) in 8 cases, while TrkA was highly expressed within nodules and weakly and/or focally expressed in inter nodular areas in 5 cases. TrkC staining was identified in 11 cases, although in 3 of these it was weak. Again, the immunoreactivity was predominantly nodular (Fig. 3C), although faint, patchy staining of inter nodular regions was identified in 5 cases. NGF was expressed in 11 of the 14 nodular medulloblastomas, while NT-3 was strongly positive in all 6 cases examined (Fig. 3B, D). The expression pattern of NGF and NT-3 was diffuse, although higher levels of expression were frequently present within nodules.

**DISCUSSION**

In this report we provide evidence that neurotrophin signaling could play a role in the development of nodular/desmoplastic medulloblastomas—a subtype associated with neuronal differentiation and improved prognosis. We also investigated the extent of neuronal maturation in nodules and documented strong inter nodular expression of BCL-2 and p53, but only weak expression of these 2 proteins in nodules. Our data suggest that nodule formation is similar in many ways to normal granule cell maturation during cerebellar development.

While the histogenesis of medulloblastomas is controversial, the cerebellar external granule cell layer (EGL) and its remnants have long been candidate progenitors for at least some of these tumors (19). Recently developed animal models support this hypothesis. For example, knockout

**TABLE 1**

<table>
<thead>
<tr>
<th>Case</th>
<th>NeuN</th>
<th>MAP-2</th>
<th>Syn</th>
<th>NF</th>
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<td>++</td>
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</table>

Legend: Syn = Synaptophysin; NF = Nonphosphorylated neurofilaments.
Fig. 1. Neuronal maturation. Medulloblastoma nodules immunopositive for NeuN (A), MAP-2 (B), synaptophysin (C), and nonphosphorylated high and medium molecular weight neurofilaments (D). Magnification: A–D, × 160.

TABLE 2
Neurotrophin, BCL-2, and p53 Expression

<table>
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<tr>
<th>Case</th>
<th>% Nodular</th>
<th>Nodular apoptosis</th>
<th>Trk A</th>
<th>NGF</th>
<th>Trk C</th>
<th>NT3</th>
<th>BCL-2</th>
<th>p53</th>
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<td>++ I</td>
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Legend: D = diffuse; N = nodular; PN = predominantly nodular with weak diffuse; I = internodular; ND = not determined.
mice heterozygous for the PTCH gene develop abnormal proliferations of EGL cells and medulloblastoma-like tumors (20, 21). The EGL is a layer 6 to 8 cells deep and covers the perinatal cerebellum. Proliferating cells form the outer portion of the EGL, while the inner portion contains differentiating cells that migrate inwardly to populate the internal granule cell layer (IGL) (22). Extensive apoptosis occurs in the EGL, while lesser degrees of apoptosis are detected in the IGL (23). The EGL layer disappears following maturation of the cerebellum and the presence of EGL cells after the first 12 to 18 months of life in humans is considered abnormal.

In what appears to be similar fashion, substantial neuronal maturation occurs within medulloblastoma nodules. The markers we examined, NeuN, MAP-2, nonphosphorylated neurofilament proteins, and synaptophysin, cover a spectrum of neuronal maturation. In the cerebellum, NeuN is expressed early in EGL neuronal differentiation and is detected throughout the maturation process (24). MAP-2 and synaptophysin are expressed later in cortical neuronal maturation, while nonphosphorylated high and medium molecular weight neurofilament proteins are normally expressed in mature neurons (18, 24–26). Our observation that NeuN, MAP-2, and synaptophysin are expressed within the nodules of all 6 tumors examined, but nonphosphorylated neurofilaments are expressed in only 2 tumors suggests that significant neuronal maturation occurs in all cases, but that maturation is not always complete.

We also identified prominent apoptosis within nodules. In the majority of our cases either apoptotic bodies or condensed “lymphocyte-like” nuclei were concentrated in nodules as was reported by Katsetos and colleagues (4). In situ end labeling studies by others have confirmed that condensed or “lymphocyte-like” nuclei in medulloblastomas are indeed apoptotic cells, not lymphocytes (27, 28). Since medulloblastomas with increased apoptotic indices are clinically less aggressive, the apoptosis associated with nodular differentiation may partially account for the better clinical outcomes in patients with nodular/desmoplastic medulloblastomas (29).

Low levels of the anti-apoptotic factor BCL-2 in nodules provide one explanation for the enhanced nodular...
apoptosis. We identified internodular BCL-2 expression in 11 of the 12 desmoplastic medulloblastomas examined, but few BCL-2-positive cells within nodules. In a recent immunohistochemical study, Schiffer and colleagues also noted an absence of BCL-2 staining in medulloblastoma nodules (30). BCL-2 is expressed in proliferating neuronal precursors and immature neurons, and may play a role in neuronal maturation in addition to protecting neurons from apoptosis (31).

BCL-2 is present in both the external and internal granule cell layers in the developing cerebellum (23). However, just as BCL-2 levels appear to decrease with neuronal differentiation in desmoplastic medulloblastomas, BCL-2 levels decline during normal cerebellar maturation and are almost undetectable in adults (32). Cultures of cerebellar granule cells from mice lacking BCL-2 are more susceptible to apoptotic cell death than granule cells with normal BCL-2 levels (33). Furthermore, cerebellar granule neurons undergoing programmed cell death are immunonegative for BCL-2 (23). Thus the prominence of apoptosis within nodules could well be fostered by a lack of anti-apoptotic BCL-2.

We observed internodular p53 expression in 5 of 11 desmoplastic medulloblastomas. Mutations in p53 are relatively rare in medulloblastomas, occurring in only 5%–10% of cases (34, 35); however, p53 is detected immunohistochemically in up to 46% of cases (36). Expression of p53 is strong in the external granule cell layer of developing rat cerebellum, but is downregulated following differentiation and migration into the internal granule cell layer (37). The decreased p53 expression we observed in nodular medulloblastomas following neuronal differentiation may therefore reflect an adherence to this developmental program.

Expression of the neurotrophin receptors TrkA and TrkC was exclusively nodular in the majority of cases, although in some cases TrkA and TrkC were weakly expressed in non-nodular regions as well. The primary ligands for these receptors, NGF and NT-3, were also identified in most cases. Localization of NGF and NT-3 was less restricted, as would be expected for polypeptide factors known to diffuse more than 3 mm through neural tissue (38). In several cases, however, nodules were more strongly positive for NGF and NT-3 than surrounding regions of tumor, suggesting an intranodular source for

Fig. 3. Neurotrophin expression. The neurotrophin receptors TrkA (A) and TrkC (C) are localized to nodules while their cognate ligands NGF (B) and NT-3 (D) are more diffusely expressed. Magnification: A–D, ×160.
Fig. 4. Comparison of gene expression profiles during cerebellar development and medulloblastoma nodule formation. The layers of the developing cerebellum are in many ways similar to the nodular and internodular regions of medulloblastomas. The cerebellar external granule cell layer (EGL) contains mitotically active neuroblasts histologically similar to the pleomorphic internodular medulloblastoma cells, while the differentiated neurons of the internal granule cell layer (IGL) are similar to the tumor cells populating medulloblastoma nodules. BCL-2, p75NTR, and p53 are expressed in both the cerebellar EGL and medulloblastoma internodular regions, while synaptophysin, TrkC and p27kip1 are expressed in both the cerebellar IGL and medulloblastomas nodules. Right panel: BCL-2 is expressed transiently in the pediatric IGL, but no BCL-2 is detected in the mature adult cerebellum (a). Left panel: TrkC is also present in the differentiating neurons of the inner EGL layer (b).

The expression of neurotrophins and their receptors in medulloblastomas has been analyzed in previous studies. Washiyama and colleagues demonstrated immunopositivity for TrkA in 27%, TrkC in 48%, and NT-3 in 9% of 27 medulloblastomas (39). A later study by the same group identified TrkA and TrkC in 25% and 85% of 20 medulloblastomas, respectively (40). The ligands NGF and NT-3 were present in 30% and 15% of the 20 tumors, however no coexpression of TrkA and its ligand NGF was seen.

We observed coexpression of TrkA and NGF in 10 of 14 cases. Several factors could explain the increased prevalence of neurotrophin-positive medulloblastomas and common coexpression of TrkA and NGF expression in our cases. The antisera used in our study were different from those used by previous investigators. More importantly, we focus on the nodular/desmoplastic medulloblastoma subtype, and hypothesize that neurotrophin signaling plays a role in nodular differentiation. In previous studies, the histologic subtype of the medulloblastomas was not reported and the number of nodular lesions examined may have been small. Nodules are present in 20%–30% of all medulloblastomas, but tumors with extensive nodularity are rare.

Interestingly, while TrkC mRNA is abundant in medulloblastomas, TrkA mRNA has not been detected. No TrkA transcripts were identified in a total of 47 medulloblastoma surgical specimens analyzed by Northern
blotting and rtPCR (12, 14). However, TrkA and NGF mRNA was detected in all 6 medulloblastoma cell lines examined by another group (41). The discrepancies between immunohistochemical studies and those analyzing mRNA have not been resolved. One explanation is that anti-TrkA antisera could cross-react with other Trk receptors. If this is true, the nodular immunoreactivity we attribute to TrkA expression could represent TrkB or TrkC. A second possibility is that the highly nodular lesions we examine are unique in their Trk receptor expression profile.

The expression of neurotrophins and neurotrophin receptors has been analyzed in normal cerebellar development as well. In situ hybridization analysis indicates that while TrkB is expressed in the outer portion of the EGL, TrkC is present in the better-developed inner portion of the EGL (42). Furthermore, application of the TrkC ligand NT-3 drives cerebellar granule cell maturation (43). Thus the development of neuronal nodules in medulloblastomas is accompanied by localized expression of the same genes found in normal cerebellar differentiation, i.e. Trk receptors, BCL-2, and p53.

Other researchers have also noted nodular or internodular localization of proteins important in neuronal differentiation and tumorigenesis. The low affinity neurotrophin receptor p75 is expressed in only 17% of classic medulloblastomas, but was detected in proliferating internodular regions in all desmoplastic medulloblastomas examined (44). In the same study, Buehran and colleagues documented expression of p75 in the EGL of the developing human cerebellum, but not in the mature neurons of the IGL.

The cell cycle dependent kinase inhibitor p27Kip1 has been detected in nodules but not in internodular areas (45). Kip-1 expression is associated with apoptosis and its nodular expression pattern correlates well with the prominence of intranodular programmed cell death we observed (46). Interestingly, in the maturing cerebellum, Kip-1 is most highly expressed in the internal granule layer (37). Thus Kip-1 also recapitulates its developmental expression pattern in nodular medulloblastomas.

In summary, we have demonstrated immunohistochemical localization of neurotrophin receptors to nodular regions and BCL-2 and p53 to internodular regions of nodular/desmoplastic medulloblastomas. Expression of these and other genes in more differentiated (nodules) and less differentiated (internodular) tumor regions parallels gene expression during normal cerebellar development (Fig. 4). Given the known role of neurotrophin signaling in neuronal maturation and apoptosis, our data suggest that Trk receptor expression plays an important role in the development of nodular medulloblastomas.

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REFERENCES

2. Herpers MJHM, Budka H. Primitive neuroectodermal tumors including the medulloblastoma: Gliad differentiation signaled by immunoreactivity for GFAP is restricted to the pure desmoplastic medulloblastoma (“arachnoidal sarcoma of the cerebellum”). Clin Neuropathol 1985;4:12–18
12. Segal RA, Goumnerova LC, Kwon YK, Stiles CD, Pomeroy SL. Expression of the neurotrophin receptor TrkC is linked to a favorable outcome in medulloblastoma. PNAS 1994;91:12867–71


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