Expression of the ErbB-Neuregulin Signaling Network during Human Cerebellar Development: Implications for the Biology of Medulloblastoma

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ABSTRACT

The four receptor tyrosine kinase I receptors, ErbB-1, ErbB-2, ErbB-3, and ErbB-4, which have been implicated in the development of a variety of normal and malignant tissues, are activated through ligand mediated homo- and heterodimerization. We have previously reported the frequent coexpression, heterodimerization, and prognostic significance of ErbB-2 and ErbB-4 in childhood medulloblastoma, an embryonal tumor of the cerebellar external granule cell layer (EGL). In the present study, we have used immunohistochemistry and Western blotting analysis to analyze the expression of the ErbB receptors and neuregulin (NRG) 1-α and NRG1-β ligands during normal human cerebellar development. We demonstrate that ErbB-1, ErbB-3, ErbB-4, and NRG1-β display specific temporal and topographical distribution in the cerebellum during intrauterine and postnatal life, and that normal ErbB-NRG signaling in the EGL multiplying zone is likely to be mediated by ErbB-4 and NRG1-β. In contrast, ErbB-2, which is expressed in 86% of medulloblastomas, could not be detected at any stage of cerebellar development. Therefore, we propose that positive deregulation of ErbB-2 expression in the cerebellar EGL, leading to the formation of a NRG1-β-driven ErbB-2/ErbB-4 autocrine loop, is an important factor in medulloblastoma tumorigenesis.

In further support of this hypothesis, we provide evidence using reverse transcription-PCR analysis that expression of the ErbB-2 and ErbB-4 receptors, but not ErbB-1 or ErbB-3, is deregulated in medulloblastoma compared with normal developing cerebellum. We also demonstrate NRG1-β expression in 87% (% = 46 of 48) of medulloblastoma primary tumors, with the greatest expression levels occurring in tumors with high ErbB-2 and ErbB-4 receptor coexpression. Furthermore, the expression of all three components of the proposed autocrine loop (i.e., ErbB-2, ErbB-4, and NRG1-β) was significantly related to the presence of metastases at diagnosis (P < 0.05).

INTRODUCTION

There is increasing evidence that cell signaling, involving the activation of membrane-bound receptors by polypeptide growth factors, plays a major role in the development of normal and malignant tissues. In this regard, RTK I has been implicated in regulating the embryogenesis of neuronal and epithelial tissues (1), whereas deregulated RTK I expression is a frequent finding in a variety of human tumors, with the greatest expression levels occurring in tumors with high ErbB-2 and ErbB-4 receptor expression. Although this system affords cells with a great deal of signaling diversity, it is governed by a strict hierarchy, mediated by different receptor ligand binding affinities (22). Despite its apparent lack of a direct ligand, the ErbB-2 receptor appears to play a central role in this signaling network. Evidence suggests that ErbB-2 is the preferred heterodimer partner of the other RTK I receptors, and that this preference leads to competition between receptors to bind ErbB-2 (22, 23). Furthermore, ErbB-2-containing heterodimers have significantly increased signaling potency, which may result from a reduced rate of receptor ligand dissociation and greater efficiency in MAP-kinase activation (7, 24). These properties of the ErbB-2 receptor may explain its potent oncogenicity and prominence in certain human cancers.

It is clear that any influence the ErbB-NRG system exerts in the development of normal and malignant tissues will depend not only on ligand-receptor binding characteristics but also upon their temporal and topographical distribution during normal development and tumorigenesis. There is increasing evidence that ErbB-NRG signaling plays a central role in the development and maintenance of the nervous system (1, 25). For example, knockout mice lacking ErbB-2 (26), ErbB-3 (27), ErbB-4 (28), or NRG1 (29) demonstrate a variety of central and peripheral neurological abnormalities, including defects in cranial ganglia formation, whereas interaction between ErbB-2, ErbB-3, and NRG may play a critical role in the repair and maintenance of peripheral nerves (30).

Within the central nervous system, the cerebellum is emerging as one site in which the ErbB-NRG system appears to have particular significance. Not only do the ErbB receptors demonstrate specific temporal and topographical expression distribution patterns during cerebellar development (12, 31–34), but mice heterozygous for deletion at the NRG1 locus have underdeveloped cerebellums (35). The cerebellum is also the predominant site of NRG2 production (12–14) and the only region to demonstrate an increase in NRG1 expression in adult versus embryonic rodent tissue (36). Recent evidence suggests that at least one important function of the ErbB-NRG system during cerebellar development involves ErbB-4 and NRG-mediated control of immature neurone migration from a superficial germinall layer termed the EGL (Fig. 1) into the cerebellar parenchyma (32).

Medulloblastoma is an embryonal tumor of the cerebellum believed to arise from the EGL (37, 38). We have recently provided evidence that ErbB-NRG signaling may play a significant role in the biology of this disease (39). In this study, ErbB-2 and ErbB-4 coexpression was detected at any stage of cerebellar development. Therefore, we propose that ErbB-NRG signaling may play a significant role in the biology of this disease (39). In this study, ErbB-2 and ErbB-4 coexpression was detected at any stage of cerebellar development. Therefore, we propose that ErbB-NRG signaling may play a significant role in the biology of this disease (39).
related to a poor clinical outcome, and direct evidence of ErbB-2/Erbb-4 heterodimerization was demonstrated in primary tumors by immunoprecipitation. In the present study, we have analyzed the expression of the ErbB receptors and NGFI-a and NGFI-b ligands in normal developing human cerebellum to understand further how deregulation of the ErbB-NGF system may contribute to medulloblastoma tumorigenesis. We have observed that although ErbB-1, ErbB-3, and ErbB-4, and NGFI-b display specific temporal and topographical expression, ErbB-2 cannot be detected within the cerebellum at any developmental stage. We propose that deregulated expression of this receptor may play a central role in this malignancy. In support of this hypothesis, using RT-PCR analysis we have also found higher levels of ErbB-2 and ErbB-4 mRNA expression but not ErbB-1 or ErbB-3 in primary medulloblastoma versus developing cerebellum. Finally, using IHC we show expression of NGFI-b in a large proportion of medulloblastoma primary tumors that coexpress ErbB-2/Erbb-4 receptors, suggesting a role for an ErbB-2/Erbb-4-NGFI-b autocrine loop in the progression of this disease.

MATERIALS AND METHODS

Tissue Samples. The normal tissue used in the study included six, well-preserved, formalin-fixed, paraffin-embedded fetal cerebellar samples, obtained from cases of spontaneous fetal loss. Estimated gestational ages were 15, 20, 22, 25, 25, and 40 weeks, judged by a combination of foot length, crown rump length, and assessment of tissue maturation. Three postmortem, formalin-fixed, paraffin-embedded cerebellum and three fresh-frozen cerebella were also obtained from six cases of nonneurological postnatal death of ages 3, 11, and 18 months, 1 week, and 6 and 20 months, respectively. Tumor tissue analyzed included nine fresh surgical medulloblastoma specimens obtained from patients undergoing surgery for their primary tumors at Newcastle General Hospital. Immediately after resection, samples were treated in Arcton (ICI Chemicals, Middlesbrough, UK) cooled in liquid nitrogen and then stored at −80°C prior to analysis. Patient ages ranged from 1 month to 11 years; six patients were female.

Forty-eight formalin-fixed, paraffin-embedded medulloblastoma primary tumors were also available for study. These patient samples were included in our series published previously of 70 primary tumors diagnosed in Newcastle between 1968 and 1996. Their clinical details and ErbB receptor expression patterns are reported in detail elsewhere (39, 40).
RT-PCR Analysis of mRNA Expression. Total RNA was extracted from the three postnatal cerebellar and nine primary tumor frozen samples using the RNeasy method (41) and used to generate first-strand cDNA by random primer extension reverse transcription. Quantitative RT-PCR analysis of RTK receptor and NRG1 mRNA transcript levels in samples was then performed using a method developed in our laboratory and described in detail elsewhere (42). Briefly, serial cDNA dilutions for each gene of interest were simultaneously and independently amplified over 30 cycles (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min) using otherwise fixed reaction conditions. PCR products were labeled in the reaction by inclusion of [α-32P]dATP (Amersham) in the reaction mix. After amplification, products were separated on 12% polyacrylamide gels, the gels dried under heat and vacuum; and the radioactively labeled PCR products were detected and analyzed using a PhosphorImager (Molecular Dynamics). For each species, the amount of PCR product in the product is then plotted against input cDNA dilution. In the range of amplification showing linear increase of product with increasing template, the ratio of the input total cDNA of each species of interest to that of the standard 18S rRNA is then used to calculate the relative amounts of each cDNA and thus mRNA species in the sample (42). All RT-PCR analyses were performed in triplicate.

All primers used were made on an oligonucleotide synthesizer (Model 392; Applied Biosystems) and, with the exception of ErbB-1 (43), were designed using a computer program computer program by Lowe et al. (44). The NRG1 ligand primers were directed against a conserved sequence and recognize both α and β isoforms. The respective sequences, optimal MgCl2 operating conditions, and the corresponding GDB accession numbers of cDNA sequences used to design the primers were as follows: ErbB-1, 5'-AAT ATT CTT GGA TGC TCT TCT GTA-3' and 5'-TCT GAC TCA CCA GCC CAA AGC AC (MgCl2 concentration, 0.5 mM; accession no. M11730); ErbB-2, 5'-GCT GGT TAT CTC C-3' (MgCl2 concentration, 0.5 mM; accession no. L12261); and the internal standard 18S rRNA, 5'-ATG CTC AAT ATT GTC CT-3' (MgCl2 concentration, 0.75 mM; accession no. M34309); ErbB-4, 5'-CGA TTC TCA GTC AGT GTG TGC-3' and 5'-GTG CTC AAT ATT GTC CT-3' (MgCl2 concentration, 1.25 mM; accession no. M34309). ErbB-1, 5'-GCA TCT TCA GTC AGT GTG TGC-3' and 5'-GCT GGT TAT CTC C-3' (MgCl2 concentration, 0.5 mM; accession no. M11730); ErbB-2, 5'-ATG GAC CTT GAG ATG GGC TGT GCC ATT GTC CT-3' (MgCl2 concentration, 0.75 mM; accession no. M34309); ErbB-4, 5'-GCA TCT TCA GTC AGT GTG TGC-3' and 5'-GCT GGT TAT CTC C-3' (MgCl2 concentration, 0.5 mM; accession no. M11730); NRG1-α, 5'-TCC CCA ATT GAA GAT GAA A-3' and 5'-TGG TAG ATG ATG TAG ATG AAC-3' (MgCl2 concentration, 1.5 mM; accession no. L07869); NRG1-β, 5'-CC AAT GAA GAT GAA A-3' and 5'-TGG TAG ATG ATG TAG ATG AAC-3' (MgCl2 concentration, 1.5 mM; accession no. L12261); and the internal standard 18S rRNA, 5'-ATG CTC TTA GCT GAG TGT CC-3' and 5'-AAC TAC GAC GGT ATC GGA TC-3' (MgCl2 concentration, 1.0 mM; Ref. 40).

RESULTS

IHC and Western Blotting of Normal Cerebellum. IHC was used to analyze expression of the four RTK I receptors and the NRG1-α and NRG1-β ligands during human cerebellar development from 15 weeks of gestation to 18 months of postnatal life. Significant differences in topographical and temporal expression within the different layers of the cerebellum were observed (Fig. 2). ErbB-1 expression was detected in all five layers of the cerebellum during development. From 15 weeks of gestation (the earliest developmental stage analyzed), faint cytoplasmic staining of cells occupying the innermost aspect of the EGL and immediate sub-EGL fibers in the molecular layer was seen (Fig. 2a). This staining showed a marked increase in intensity, which peaked at around 25 weeks of gestation, and then subsequently became negative by term (Fig. 2, a and d). From 20 weeks of gestation, cells in the developing IGL and fibers throughout the molecular layer demonstrated faint to moderate immunoreactivity. Within the IGL, this expression decreased in intensity with subsequent development, becoming negative by term. However, the molecular layer retained faint reactivity (not seen in negative controls), which persisted to 18 months of postnatal life. The white matter demonstrated a moderate staining from 25 weeks of gestation throughout intrauterine development (data not shown). This subsequently decreased postnatally and was undetectable by 11 months. Finally, ErbB-1 expression was observed within the Purkinje cell layer throughout postnatal life. Sustained expression by the Purkinje and molecular layers during postnatal development was confirmed in Western blot analysis (Fig. 3). The three samples analyzed all demonstrated ErbB-1 bands with identical density (IOD) at 1 week and 6 and 20 months of age, respectively (Fig. 3).

In contrast to ErbB-1, all ErbB-3 protein expression was confined to late gestation and postnatal life. The expression by Bergmann glial fibers spanning the molecular layer was most dramatic (Fig. 2e). This staining was first seen at very low levels in the 25-week and term sections. However, by 3 months of postnatal life, intense labeling of these fibers was detected, which persisted in all subsequent sections. Within the molecular layer, expression was also detected in Purkinje cell fibers in addition to glial fibers at 18 months of life. Finally, ErbB-3 expression was also observed in white matter astrocytes (data not shown). This was detected first at term and then at all subsequent stages analyzed. This pattern of increasing ErbB-3 cerebellar expression from term through the first two years of life was observed in the results of Western blotting of whole postnatal cerebellum, which revealed an approximate 2-fold increase in the density of ErbB-3 protein bands from 1 week to 20 months (Fig. 3).

ErbB-4 expression by the EGL and white matter showed the greatest intensity of staining of all of the RTK I receptors. From 15 weeks of gestation, strong immunoreactivity was observed throughout the EGL (Fig. 2a). This then gradually decreased in intensity, becoming negative by 3 months of life. In contrast, white matter expression, which was observed first at term, persisted in the postnatal sections analyzed (data not shown). Staining was also seen in the developing IGL. This increased from 20 to 25 weeks but was reduced to a moderate-to-faint reactivity by 3 months. This then persisted throughout the first 2 years of life. The molecular layer demonstrated a faint reactivity at 25 weeks of gestation and term; however, this site was negative in all subsequent sections. Western blotting of whole normal postnatal cerebellar samples demonstrated a 4-fold decrease in ErbB-4 protein expression from 1 week to 6 months of postnatal life, with a further 2-fold decline from 6 to 20 months of age (Fig. 3). This pattern reflects the declining EGL and IGL ErbB-4 expression during this period.

In contrast to ErbB-1, ErbB-3, and ErbB-4, ErbB-2 expression could not be detected at anytime during intrauterine or postnatal development, either by IHC (Fig. 2, a–d) or Western blotting (Fig. 3). The lack of expression in Western blot analysis was confirmed by the DAOY cell line-positive control (Fig. 3), and the absence of detection by IHC was validated by the use of positive control sections.

The IHC expression pattern of the NRG1-β ligand was similar to that observed for ErbB-4. From 15 weeks of gestation, intense labeling of the EGL was detected. Interestingly, this was confined to a single outer layer of cells in the EGL (Fig. 2, a and b), confirmed by reproducibility in consecutive sections, and an absence of this pattern in negative controls. In keeping with the results of NRG1-β IHC expression in tumors, the distribution of staining within EGL cells appeared both nuclear and cytoplasmic. This expression persisted throughout intrauterine development but had considerably decreased in intensity by term. Staining was also observed from early gestation in the molecular, Purkinje, IGL, and particularly white matter layers, persisting in all postnatal sections. Sufficient material for Western blotting of NRG1-β was only available for the 1-week and 20-month normal cerebellar samples. Both demonstrated significant levels of NRG1-β expression in keeping with the IHC results (Fig. 3). In contrast, expression of the NRG1-α isoform could not be detected by IHC (data not shown) or Western blotting (Fig. 3) at any stage during normal human cerebellar development. The lack of protein expression was confirmed by positive controls in IHC (data not shown) and Western blotting (Fig. 3).
Fig. 2. IHC of ErbB receptor and NRG1-β expression in the EGL and ML of pre- and postnatal developing human cerebellum. Rows a–e correspond to 15, 20, 25, and 40 weeks of gestation and 3 months of postnatal life, respectively. Rows a, c, d, and e, ×400; row b, ×600.
control for Umes BI-B3. by IHC for ErbBl. ErbB-2, ErbB-3, ErbB-4, and NRG1 expression, cerebellar material was available for analysis. Therefore, estimated week and 6 and 20 months of age, respectively. Western blot of actin is shown as loading cell line (HrhB-1 and ErbB-2). mcdulloblastoma primary tumor samples (ErbB-3, ErbB-4, protein expression by postnatal human cerebellum. Ctilulin A, positive controls: DAOY...

Fig. 3. Western blot analysis of ErhB receptor and NRG1-α and NRG1-β ligand protein expression by postnatal human cerebellum. Column A, positive controls: DAOY cell line (ErbB-1 and ErbB-2), medulloblastoma primary tumor samples (ErbB-3, ErbB-4, NRG1-α, and NRG1-β). Column B, Lanes 1–3, normal human postnatal cerebellum at 1 week and 6 and 20 months of age, respectively. Western blot of actin is shown as loading control for Lanes B1-B3.

RT-PCR. To further assess the relative expression of each ErbB receptor in this disease and to examine whether differences in expression were evident at the gene transcript level, we analyzed RTK I and NRG1 mRNA levels in fresh primary tumor samples and compared these with levels in normal cerebellum. Only postnatal fresh normal cerebellar material was available for analysis. Therefore, estimated levels of each mRNA species in the normal cerebellar samples reflect relative expression at postnatal developmental stages only. For each receptor and the NRG ligand, fresh-frozen tumor samples that had previously been shown by IHC to be positive for the respective protein were used to estimate mRNA levels. From an available nine fresh-frozen medulloblastoma samples, 3, 8, 2, 8, and 6 were positive by IHC for ErbB1, ErbB-2, ErbB-3, ErbB-4, and NRG1 expression, respectively. This immunopositivity rate is in keeping with that reported previously by us for the RTK I receptors in medulloblastoma (39). The results of this RT-PCR analysis in normal postnatal cerebellum and tumor material are summarized in Fig. 4.

The limited availability of fresh tumor material and expression of ErbB-1 and ErbB-3 by a minority of medulloblastoma primary tumors precluded formal statistical analysis of the RT-PCR results. However, distinct patterns of mRNA expression were observed (Fig. 4). Two of the three ErbB-1 protein-positive tumors and both ErbB-3-expressing tumors had levels of the respective mRNA species that were 10-fold lower than that seen in normal postnatal cerebellum. The remaining ErbB-1-expressing tumor had an mRNA level equivalent to that measured in cerebellum (Fig. 4, a and c). Interestingly, although we could not detect ErbB-2 protein at any stage during cerebellar development, a low level of ErbB-2 mRNA could be detected (Fig. 4b). ErbB receptor mRNA expression in the absence of protein has been reported previously for a variety of tissues (25). In contrast to the results for ErbB-1 and ErbB-3, five of the ErbB-2 protein-positive tumors had mRNA levels equivalent to those seen in normal cerebellum, whereas three had mRNA levels between 5 and 13 times greater than that of normal cerebellum (Fig. 4b). Similarly, none of the eight samples expressing ErbB-4 protein had ErbB-4 mRNA levels lower than that seen in cerebellum. Most had levels equivalent to the normal tissue, whereas one case had five times the ErbB-4 mRNA level measured in normal cerebellum (Fig. 4d). NRG1 mRNA expression levels demonstrated a mixed picture, with four of the six samples falling in the normal range and two having levels approximately twice that of the normal tissue (Fig. 4e). These results support the hypothesis that deregulation of ErbB-2 and ErbB-4 receptor expression is of greater significance than ErbB-1 or ErbB-3 in medulloblastoma.

NRG1-β IHC of Medulloblastoma Samples. Forty-eight of our previously published series of 70 cases of medulloblastoma (39) had sufficient material available for IHC analysis of NRG1-β expression. Forty-six cases (87.5%) were immunopositive for the ligand (Fig. 5). This is in contrast to the relatively low frequency of NRG1-α expression that we have reported previously in medulloblastoma (39). The percentage of positive tumor cells in each section ranged from <10% to 90%. Tumors with >50% NRG1-β-expressing cells (n = 12) demonstrated an intense, predominantly cytoplasmic immunoreactivity compared with a generally lower level of staining strength in those with <50% positive tumor cells. In addition to cytoplasmic expression, a variable degree of nuclear staining was detected in all positive cases, with two cases having marked nuclear membrane reactivity (Fig. 5). The specificity of nuclear immunostaining was confirmed by the identification by the NRG1-β antibody of a single protein species of the correct size (M, 44,000) on Western blotting (Fig. 3) and the lack of immunopositivity in the IHC-negative controls.

No significant association between patient survival and NRG1-β expression was seen in univariate survival analysis (data not shown). However, NRG1-β is a ligand for the ErbB-2/ErbB-2 heterodimer and may therefore activate these receptors in the context of an autocrine loop in this disease. In our previous study of ErbB expression and clinical outcome, the worst prognosis was seen in patients whose tumors expressed ErbB-2 in >50% of cells and were ErbB-4 positive. Nine of these cases are included in the present study. Interestingly, all nine proved NRG1-β positive, four (44%) of which had intense labeling of >50% of tumor cells. In contrast, only 8 (20%) of the remaining 39 cases in the present study, which included those either ErbB-4 negative or with <50% ErbB-2 tumor cell expression, had >50% NRG1-β tumor cell expression, whereas six of these cases were NRG1-β negative.

Further evidence supporting a role for an ErbB-4/ErbB-2/NRG1-β autocrine loop in the progression of medulloblastoma was seen in the analysis of metastasis at diagnosis and the expression of these three signaling proteins. Twenty-eight of the patients included in the present study had undergone detailed craniospinal imaging at diagnosis and therefore had accurate staging data (39). A significant relationship between tumor expression of all three components of this potential autocrine loop, i.e., ErbB-4, ErbB-2, and NRG1-β, and the presence of spinal metastases at diagnosis was observed (Table 1). Although the numbers are relatively small, Fisher’s Exact test did reach significance (P < 0.03).
ErbB RECEPTORS AND LIGANDS DURING CEREBELLAR DEVELOPMENT

Fig. 4. RT-PCR analysis of ErbB receptor and NRG1 ligand mRNA expression levels in normal postnatal human cerebellar tissue versus medulloblastoma tumor samples. a–e, ErbB-1, ErbB-2, ErbB-3, ErbB-4, and NRG1 ligand, respectively. Bars, SE.

DISCUSSION

The ErbB-2 receptor plays a central role in the ErbB-NRG signaling network (7). Its recruitment into receptor heterodimers significantly increases the potency of ErbB signaling, which results in part from its ability to decrease the rate of ligand-receptor dissociation (24), and potentiates the activity of downstream effector pathways, including mitogen-activated protein kinase and c-jun NH2-terminal kinase/stress-activated protein kinase (23, 24).

These signaling characteristics may provide some explanation for the prominent role the ErbB-2 receptor appears to play in a variety of human malignancies (2). These include breast, ovary, and lung cancer, where ErbB-2 overexpression has been correlated with a poor prognosis (2), chemotherapy resistance (45, 46), and metastatic potential (47, 48). Studies in vitro have also demonstrated the capacity of ErbB-2 to induce malignant transformation when expressed either alone at high levels (49, 50) or together with ErbB-1 (51), ErbB-3, and ErbB-4 (52, 53), when the transforming effect is synergistic. We have previously reported high expression levels of the ErbB-2 and ErbB-4 receptors in childhood medulloblastoma and demonstrated that their coexpression, which occurs in 54% of cases, is associated with heterodimerization and a poor clinical outcome (39).

Medulloblastoma is an embryonal tumor of the cerebellum believed to arise from the EGL (37, 38). This layer of proliferating precursor cells covers the surface of the cerebellum from early gestation up to the first year of postnatal life and may be further divided into two zones (54). The outer “multiplying zone” consists of rapidly dividing cells and gives rise to an inner “premigratory zone,” from which cells migrate into the cerebellar parenchyma during development, eventually forming the IGL (Fig. 1). There are few published studies in the literature of ErbB receptor and ligand expression during EGL and cerebellar development; almost all have focused on rodent development (12, 31–34). Therefore, to increase our understanding of how deregulated ErbB receptor expression may contribute to the initiation and/or progression of medulloblastoma, we have investigated the expression of these receptors, and their ligands NRG1-α and NRG1-β, in developing human fetal and postnatal cerebellum.

Of the six components of the ErbB-NRG network analyzed (the four RTK I receptors and NRG1-α and NRG1-β ligands), only three, ErbB-1, ErbB-4, and NRG1-β, displayed significant expression levels in the developing EGL (Fig. 2, a–d), whereas ErbB-3 was expressed predominantly in glial cells of the postnatal cerebellum (Fig. 2e). In contrast, despite its high expression frequency in medulloblastoma (39), we did not detect the ErbB-2 receptor in any part of the EGL or cerebellum at any time during development. Expression of the NRG1-α isoforms was also not detected.

With regard to the ErbB receptors, ErbB-4 demonstrated the great-
ErbB receptors and ligands during cerebellar development

Fig. 5. NRG1-β IHC immunostaining of primary medulloblastoma sample demonstrating cytoplasmic and nuclear membrane expression. ×400.

Table 1. Coexpression of ErbB-2, ErbB-4, and NRG1-β in metastatic versus nonmetastatic medulloblastoma primary tumors (Fisher's Exact test, p < 0.05)

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<th>ErbB-2, ErbB-4, and NRG1-β positive</th>
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<td>Metastatic</td>
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Table 1 Coexpression of ErbB-2, ErbB-4, and NRG1-β in metastatic versus nonmetastatic medulloblastoma primary tumors (Fisher's Exact test, p < 0.05)

In this study, we investigate the expression of ErbB receptors in the developing cerebellum. The ErbB-2 receptor was not detected at any postnatal age, whereas the ErbB-1 receptor demonstrated a significant increase and decrease respectively in detectable protein (Fig. 3).

These data are in keeping with a number of recently published studies of ErbB receptor expression in rodent tissue. Using in situ hybridization, Pinkas-Kramarski et al. (34) have demonstrated that although expression of ErbB-3 and ErbB-4 mRNA can be detected in adult cerebellum, only the latter is expressed in E14.5 embryonic cerebellum (34). Furthermore, in Northern blot analysis of mRNA extracted from whole, postnatal rat cerebella aged 0 to 22 days, they also identified a steady increase in ErbB-3 expression with a concomitant decrease in ErbB-4 levels. The cellular distribution of these two receptors in rat cerebellum also appears to be similar to that identified by us in human tissue. Again using in situ hybridization, Ozaki et al. (33) identified ErbB-3 expression predominantly in glial cells, whereas ErbB-4 transcripts were enriched in the EGL and IGL of early postnatal (P14) rat cerebellum. Finally, one study has recently identified two novel juxtamembrane domain isoforms of the ErbB-4 receptor and demonstrated expression of both in adult human and mouse cerebellum (55).

Our data from the present study of human cerebellum and those from investigations in rodent tissue support the hypothesis that the ErbB-1 and ErbB-4 receptors play important roles in early cerebellar development. In contrast, the confinement of ErbB-3 receptor expression to late gestation and postnatal tissue suggest that this member of the RTK I family predominates in the later stages of development and cerebellum. The role of the ErbB-2 receptor in the developing cerebellum is less clear. Although there is some evidence that this receptor is expressed in postnatal rat cerebellum (33), we did not identify any detectable ErbB-2 protein in either pre- or postnatal human cerebellum. Similarly, in their study of human tissues, Press et al. (56) could not detect expression of ErbB-2 protein or mRNA transcripts in fetal or adult cerebellum. Therefore, we propose that the ErbB-2 receptor is not involved in normal human cerebellar development.

Because ErbB receptor signaling is activated through their recruitment into hetero- and homodimers by cognate ligands, we have also analyzed expression of NRG1-α and NRG1-β, which are ligands for ErbB-3 and ErbB-4. NRG1-β expression displayed a similar temporal expression pattern to that of ErbB-4, but within the EGL, was confined to a single outer layer of cells in the “multiplying zone” (Fig. 2, a and b). In contrast, NRG1-α could not be detected in either pre- or postnatal cerebellum by IHC (data not shown) or Western blotting (Fig. 3). These data are in keeping with the previously reported
distribution pattern of the NRG1 ligands. The NRG1-β isoforms are predominantly expressed in neuronal tissue, whereas the α isoforms are principally produced by mesenchymal tissue (16). Furthermore, recent evidence from work in rodent tissue indicates that the ErbB-4 receptor is the major transducer of NRG1 signaling in developing cerebellum (34), with the full-length NRG1-β protein but not the α isoform having a significant effect on the phenotype of cultured cerebellar cells in vitro (33).

During development, ErbB-NRG signaling within tissues appears to operate through a system of receptor-ligand, short-range paracrine interactions (1). With regard to interplay between the ErbB-4 and NRGs during cerebellar development, one recent study has reported a critical role for these proteins in coordinating the migration of granule cell precursors from the EGL to the IGL during rat postnatal cerebellar development (32). In the present study, we have demonstrated expression of NRG1-β by a single outer layer of germinal cells in the multiplying zone of the EGL. This NRG1-β expression occurred in parallel with ErbB-4 receptor expression by cells of the underlying EGL. In the context of the paracrine model in which ErbB-NRG signaling appears to operate, this pattern of expression suggests that the outer layer of cells in the EGL multiplying zone may act as a source of NRG1-β for the stimulation of the underlying, primitive, ErbB-4-expressing cells.

As outlined above, we have previously reported the frequent expression of ErbB-2 expression in medulloblastoma primary tumors. However, in contrast, we and others have not detected expression of this receptor in the cerebellum at any time during pre- and postnatal development (Fig. 2, a–d, and Fig. 3; Ref. 56). Although the present study cannot rule out a short period of ErbB-2 expression by very immature cerebellum (<15 weeks gestation), there is another, more likely explanation for the difference between the levels of this receptor in tumor and normal cerebellar tissue. We propose that abnormal positive deregulation of ErbB-2 receptor expression within the EGL during development is a major contributing factor in the initiation and or progression of medulloblastoma. This would result in the expression of ErbB-2 receptor by EGL cells already expressing ErbB-4, adjacent to a source of NRG1-β ligand. Under these conditions, the formation of low-affinity ErbB-4 homodimers with low signaling potency (which our IHC results suggest are likely to mediate normal EGL ErbB receptor signaling), would be superseded by formation of the high-affinity, high-potency ErbB2/ErbB-4 heterodimer (22, 23, 53). In support of this hypothesis, work in vitro has demonstrated that although NIH 3T3 cells expressing ErbB-4 only can respond to NRG stimulation by increasing cell proliferation (a potential role for ErbB-4/NRG interplay in the normal multiplying zone of the EGL), it does not result in malignant transformation (53). However, the response to NRG by NIH 3T3 cells coexpressing both ErbB-2 and ErbB-4 is dramatically different, leading to increased receptor phosphorylation and malignant transformation as measured by focus formation (53).

The possibility that deregulated ErbB-2 receptor expression by ErbB-4-expressing EGL cells may result in an oncogenic ErbB-NRG autocrine loop in medulloblastoma was investigated further in the present study by: (a) comparing ErbB receptor mRNA levels in normal cerebellum and medulloblastoma samples; and (b) investigating the expression of the NRG1-β in medulloblastoma tumor samples whose ErbB receptor expression status was known.

Although limited in number, our RT-PCR analyses do support the hypothesis that ErbB-2 and ErbB-4 overexpression is an important feature of this malignancy. In tumor samples, we found mRNA levels of these two receptors to be equivalent to or greater (5–15 times greater in the case ErbB-2) than that of normal postnatal cerebellum (Fig. 4 b and d). In contrast, estimated ErbB-1 and ErbB-3 mRNA levels in primary tumors were equal to or less than that of normal tissue (Fig. 4, a and c). The likelihood that these latter two receptors are of less biological significance in this disease is suggested by this result and their much lower frequency of expression in medulloblastoma and lack of influence on patient prognosis (39). Moreover, although expression of ErbB-1 and ErbB-3 was observed by us in developing cerebellum, in contrast to ErbB-4 their distribution was limited to sites outside the multiplying zone of the EGL, where malignant transformation is most likely to occur (Fig. 2).

In addition to the high expression levels of ErbB-2 and ErbB-4 in medulloblastoma, the present study identified NRG1-β ligand expression in 87.5% (46/48) of medulloblastoma tumor samples analyzed. This confirms the coexpression of all three members of the proposed autocrine loop, i.e., ErbB-2, ErbB-4, and NRG1-β in a high proportion of primary tumors. Furthermore, we observed the greatest NRG expression levels in patients with the highest levels of ErbB-2 and ErbB-4 expression. All cases whose tumors had >50% ErbB-2-immunopositive cells and expressed ErbB-4, and hence particularly aggressive disease (39), expressed the NRG1-β ligand, with almost one-half displaying intense expression in >50% of tumor cells. In addition, tumor coexpression of ErbB-2, ErbB-4, and NRG1-β at diagnosis was significantly related to the presence of central nervous system metastases at diagnosis (P < 0.05).

Finally, a number of observations from our IHC results suggest that in addition to the activation of the ErbB receptors, the NRG1-β ligand may have receptor-independent functions in medulloblastoma. In particular, a number of tumors were identified that expressed high levels of ligand in the absence of either the ErbB-3 or ErbB-4 receptors, and in many tumors the unexpected pattern of NRG1-β nuclear immunoreactivity was observed (Fig. 5). Although these results may reflect cross-reactivity of the antibody and false-positive results, the consistency observed in our IHC controls and the identification of a single correctly sized protein in Western blotting (Fig. 3) do not support this. Rather, we believe that our results are in keeping with the increasing recognition that these growth factors are multifunctional. Nuclear targeting-like sequences have been identified at the NH2 terminus of both α and β NRG1 isoforms (10), and nuclear accumulation after internalization has also been reported for some ErbB-1-specific ligands, e.g., amphiregulin (57). Furthermore, using immunofluorescence microscopy, Li et al. (58) recently demonstrated the uptake and accumulation of NRG1-β in the nucleus of cultured SK-Br-3 breast cancer cells. This was shown to be associated with the up-regulation of expression and nuclear translocation of c-myc (58). The amplification and/or overexpression of this oncogene has been reported previously in medulloblastoma (59). Therefore, in addition to activating the RTK I receptors, the NRGs may contribute to the malignant phenotype in medulloblastoma by inducing the synthesis and nuclear transport of c-myc. We are presently analyzing the relationship between the expression of this oncogene and the NRG1-β ligand in the present study population.

In summary, these data, in concert with our previous observations of ErbB receptor expression in medulloblastoma (39), support the hypothesis that deregulation in the expression of ErbB-2 within the EGL during development may significantly contribute to the initiation and or malignant progression of medulloblastoma and that this is mediated through receptor heterodimerization between ErbB-2 and ErbB-4 under the stimulatory effect of NRG1-β. In addition, the NRG1-β ligand may contribute to the malignant phenotype in medulloblastoma through receptor-independent mechanisms.
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ErbB receptors and ligands during cerebellar development


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