Differential Expression of Pleiotrophin and Midkine in Advanced Neuroblastomas

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INTRODUCTION

Neuroblastoma is one of the most common pediatric neoplasms and originates from the sympathoadrenal lineage of the neural crest. Tumors in infants and young children have a tendency to spontaneously regress or to differentiate (1). However, tumors that occur in older children are frequently metastatic at diagnosis, and these children have a poor prognosis.

Recently, neurotrophic factors and their receptors were found to play an important role in regulating growth, differentiation, and cell death in neuroblastoma (2-8). The members of the TRK family of protein-tyrosine kinases have been identified as the genes encoding the functional receptors that mediate the trophic properties of the neurotrophic factors whose expression is developmentally regulated. PTN and MK are members of a new family of neurotrophic factors, the acidic fibroblast growth factors (12-16). MK is a 13-kDa molecule whose expression is developmentally regulated (12-16). These hep-

neurite outgrowth in an autocrine or paracrine manner (7, 8). To date, there are no published reports on TRK-C or NT-3 expression in neuroblastomas.

MK and PTN are members of a new family of neurotrophic factors, whose expression is developmentally regulated (12-16). These heparin-binding proteins have about 50% homology and no sequence homology with other heparin-binding proteins, such as basic and acidic fibroblast growth factors (12-16). MK is a 13-kDa molecule and is rich in basic amino acids and cysteine (17, 18). The gene was originally cloned by a differential hybridization procedure from the embryonal carcinoma cells which were induced to neuronal differentiation by treatment with retinoic acid (12). PTN (also known as HB-GAM) has a molecular mass of 17 kDa and was purified independently from developing rat brain (14) and bovine uterus (15). MK is mitogenic to some cell lines (19-21) and PTN transforms NIH 3T3 fibroblasts (22). However, both MK and PTN act on embryonic brain cells and spinal ganglia as a neurotrophic factor to promote survival and induce neurite outgrowth (16, 20, 21).

Here we report that MK and PTN are expressed in many primary neuroblastomas, but their patterns of expression are quite different. MK mRNA is expressed in essentially all primary neuroblastomas and cell lines, independent of disease stage. In contrast, PTN mRNA is preferentially expressed in favorable stage neuroblastomas and those lacking N-myc amplification. This correlation between PTN and favorable outcome suggests that it may be playing a biological role in the behavior of these tumors.

MATERIALS AND METHODS

Patients. Of 72 neuroblastomas and 5 ganglioneuromas, 63 were obtained from Japanese patients and 14 from patients at Pediatric Oncology Group institutions or others in the United States. Twenty-five Japanese patients were identified by a mass screening program that began in 1985. Neuroblastoma tissues were obtained from the primary tumors of 55 untreated and 17 pre-
treated patients (one or two courses of high-dose cyclophosphamide and/or cisplatin; Ref. 23). All diagnoses of neuroblastoma were confirmed by histo-

logical assessment of a surgically resected tumor specimen. Patients were treated according to previously described protocols (3). The tumors were staged according to the system of Evans et al. (24). The median follow-up period after diagnosis was 32 (range, 8-116) months. Among the 17 treated patients, 15 had stage III or IV disease and two had stage IV-S. Thus, there was a high level of confounding between treatment and stage. There were 55 neuroblastomas, 17 ganglioneuroblastomas, and 5 ganglioneuromas. Neuro-
blastomas and ganglioneuroblastomas were combined and analyzed as neuro-

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1 The abbreviations used are: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; MK, midkine; PTN, pleiotrophin; du, density units.
Southern Blot and Northern Blot Analyses. The cDNA probes used were a 487-base pair human MK cDNA fragment (from T. M.; Ref. 18) and a 2.4-kilobase human PTN cDNA (from T. F. D.; Ref. 25). There was no cross-reactivity between the probes under the conditions used in this study. The human N-myc probe was a gift from J. Michael Bishop (26). N-myc amplification was measured by Southern blot analysis (26). Total RNA was prepared and the mRNA level was determined by Northern blot analysis, as described previously (2, 3).

All bands of N-myc DNA, as well as MK, PTN, and β-actin RNA, were analyzed by scanning the autoradiographs on an Apple Scanner. One RNA sample was analyzed for PTN expression but then degraded and could not be analyzed for MK expression. The intensity of mRNA expression was measured by a computer-based densitometry program (Densitometer-on-a-Disc; compliments of IMAGENETICS and AMOCO Technology Co., Naperville, IL). mRNA expression of MK and PTN was normalized to β-actin and expressed as arbitrary du. Thus, the results for expression of the two neurotrophins, and between the primary tumors, cell lines, and tissues, can be directly compared. A level of <100 du indicates that a weak band was barely visible, as opposed to between the primary tumors, cell lines, and tissues, can be directly compared. Therefore all analyses were done either on the natural logarithm of the density unit scale (t tests, ANOVAs) or using nonparametric statistics. The distinction between high and low levels of expression of PTN and MK was based on the value on the histograms that gave the best natural separation (data not shown), regardless of stage, N-myc amplification, or survival. Kaplan-Meier survival curves were calculated, and survival distributions were compared using the log rank test. Cox regression models were used to explore associations between PTN and MK expression and survival, as well as to explore the prognostic value of each in the presence of other prognostic variables such as N-myc amplification.

RESULTS

Expression of PTN and MK in Neuroblastomas and Other Tissues. Examples of the mRNA expression of PTN and MK are shown in Fig. 1, and the distribution of expression by stage and N-myc amplification is shown in Table 1. Because they were skewed, Table 1 gives both summary nonparametric statistics and transformed after normalization values. For one patient, there were no MK values available. The distribution based on the histogram of all of the values of both PTN and MK expression was bimodal, and a cutoff value of 150 du completely separates the high and low values (Fig. 2). An overall ANOVA test shows that PTN levels were different in the different stages (P = 0.0125). In particular, there was a significant difference between the PTN expressions in the early stages (median, 103; IQR 16-579) and advanced stages (median, 14; IQR 7-29; 95% CI 27-1814). Between high and low levels of expression of PTN and MK was based on the value on the histograms that gave the best natural separation (data not shown), regardless of stage, N-myc amplification, or survival. Kaplan-Meier survival curves were calculated, and survival distributions were compared using the log rank test. Cox regression models were used to explore associations between PTN and MK expression and survival, as well as to explore the prognostic value of each in the presence of other prognostic variables such as N-myc amplification.

Table 1 Distribution of PTN and MK expression by stage and N-myc amplification

<table>
<thead>
<tr>
<th>Category</th>
<th>PTN mRNA expression</th>
<th>MK mRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR) du</td>
<td>Mean (95% CI) du</td>
</tr>
<tr>
<td>Stage I</td>
<td>19</td>
<td>189 (15-730) du</td>
</tr>
<tr>
<td>Stage II</td>
<td>13</td>
<td>67 (24-422) du</td>
</tr>
<tr>
<td>Stage IV-S</td>
<td>12</td>
<td>103 (19-640) du</td>
</tr>
<tr>
<td>Stage III</td>
<td>14</td>
<td>15 (9-199) du</td>
</tr>
<tr>
<td>Stage IV</td>
<td>14</td>
<td>13 (6-25) du</td>
</tr>
<tr>
<td>Stage IV-S</td>
<td>44</td>
<td>103 (16-579) du</td>
</tr>
<tr>
<td>Stage III + IV</td>
<td>28</td>
<td>14 (7-29) du</td>
</tr>
<tr>
<td>N-myc amplification (-)</td>
<td>63</td>
<td>65 (14-468) du</td>
</tr>
<tr>
<td>N-myc amplification (+)</td>
<td>9</td>
<td>9 (6-12) du</td>
</tr>
</tbody>
</table>

a IQR, interquartile ranges, i.e., 25th and 75th percentiles; CI, confidence interval.

b Mean and 95% CI based on log scale values transformed back to du.

c One patient’s MK data were not available.
MK was expressed at a very high level in almost all neuroblastomas. An overall ANOVA showed no significant differences between the stages, both overall and grouped as early versus advanced stages. The tumors with N-myc amplification appeared to have higher MK expression; however, the difference did not achieve significance \( (P = 0.10) \). Ganglioneuromas had relatively low MK expression. All 11 neuroblastoma cell lines expressed very high levels of MK mRNA compared to primary tumors.

For comparison, we analyzed other cell lines and tissues (Table 2). The rat pheochromocytoma cell line PC12 is known to express the TRK-A receptor and is responsive to NGF. It expressed a low level of PTN, but it did not express MK at all. The melanoma cell line A875 expressed neither PTN nor MK. The infant adrenal tissue showed a high level of PTN mRNA, but not MK. Two Wilms’ tumors expressed high levels of both PTN and MK transcripts. In three hepatoblastomas and three primitive neuroectodermal tumors, the expression pattern of PTN and MK was variable (Table 2).

**Survival and Expression of PTN and MK.** Higher PTN was associated with prolonged survival, as expected, based on the difference in PTN among the stages of disease (Fig. 3A). The group with PTN expression of more than 150 du \((n = 26)\) had a 5-year survival rate of 93\%, while the group with PTN expression of less than 150 du \((n = 46)\) had a 5-year survival rate of 43\% \((P = 0.0023, \text{log rank test})\). PTN expression was associated with survival in a Cox regression model \((P < 0.0005)\). MK expression was also associated with a difference in survival (Fig. 3B). The group with MK expression higher than 150 du \((n = 45)\) had a 55\% survival rate at 5 years, while the group with MK expression lower than 150 du \((n = 26)\) had a 92\% survival rate at 5 years \((P = 0.031)\). MK expression was also associated with survival in a Cox regression model \((P = 0.029)\).

The most significant prognostic factor for survival was N-myc amplification \((P < 0.0005)\). All nine patients with more than one copy died, as opposed to 8 of 68 patients whose tumors had one copy per haploid genome. TRK-A expression which had been analyzed previously \((3)\) was strongly prognostic \((P < 0.0005)\). Also, patient age was prognostic, with younger patients faring substantially better \((P = 0.011)\). Finally, patients with favorable stages (I, II, and IV-S)
Differential Expression of PTN and MK in Neuroblastomas

Fig. 3. Cumulative survival curves of patients with neuroblastoma, according to expression of PTN (A, n = 72) and MK (B, n = 71). The Kaplan-Meier curves show the probability of survival in terms of the level of expression of PTN and MK. High levels of PTN expression were defined as values >150 du and low levels as values <150 du. High levels of MK expression were defined as values >150 du and low levels as values <150 du.

had a better prognosis than those with advanced stages (P < 0.0005), but histology was not prognostic, and prior treatment was not either, once stage was accounted for.

PTN expression was prognostic even in the presence of other prognostic variables. Table 3 gives several Cox regression models with PTN and other prognostic variables as well as MK expression and other prognostic variables. Table 3 shows that PTN expression remained prognostic even in the presence of N-myc amplification, and that the best-fitting model included N-myc amplification, stage at diagnosis, and PTN expression. Indeed, this model fit better than including TRK-A. MK expression, while prognostic when considered separately, was not a significant predictor once any strong predictor was in the model.

In Situ Hybridization of PTN. A neuroblastoma tissue sample was obtained from the adrenal primary of a 1-day-old girl with stage II neuroblastoma. The tumor sample was frozen and later processed to analyze the in vivo expression pattern of PTN. As shown in Fig. 4A, the antisense PTN probe hybridized to almost all tumor cells in the cluster. A control sense probe did not hybridize at all (data not shown). Fig. 4B shows the result of PTN in situ hybridization in an

Table 3 Cox models with stage, N-myc amplification, and expression of MK, PTN, and TRK-A

<table>
<thead>
<tr>
<th>Model</th>
<th>Variable</th>
<th>P</th>
<th>Variable</th>
<th>P</th>
<th>Variable</th>
<th>P</th>
<th>P</th>
<th>P</th>
<th>Log likelihood</th>
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<tr>
<td>A</td>
<td>N-myc amplification</td>
<td>&lt;0.0005</td>
<td>PTN (log)</td>
<td>&lt;0.0005</td>
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<td></td>
<td>-43.589</td>
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<tr>
<td>B</td>
<td>N-myc amplification</td>
<td>&lt;0.0005</td>
<td>MK (log)</td>
<td>0.17</td>
<td></td>
<td></td>
<td>-45.801</td>
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<tr>
<td>C</td>
<td>TRK-A (log)</td>
<td>&lt;0.0005</td>
<td>PTN (log)</td>
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<td></td>
<td>-46.477</td>
<td></td>
<td></td>
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<tr>
<td>D</td>
<td>TRK-A (log)</td>
<td>&lt;0.0005</td>
<td>MK (log)</td>
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<td></td>
<td>-46.605</td>
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<tr>
<td>E</td>
<td>Stage (2 cat.)</td>
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<td>PTN (log)</td>
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<td>-52.314</td>
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<td></td>
<td>-49.859</td>
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<td>N-myc amplification</td>
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<td>TRK-A (log)</td>
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<td>PTN (log)</td>
<td>0.001</td>
<td>-43.129</td>
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<td>N-myc amplification</td>
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<td>PTN (log)</td>
<td>0.004</td>
<td>-39.289</td>
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<tr>
<td>I</td>
<td>N-myc amplification</td>
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<td>Stage (2 cat.)</td>
<td>0.013</td>
<td>TRK-A (log)</td>
<td>0.23</td>
<td>PTN (log)</td>
<td>0.003</td>
<td>-38.541</td>
</tr>
</tbody>
</table>

* cat., categories.
aggressive stage IV neuroblastoma with N-myc amplification. Hybridization of antisense PTN was not seen in any area of the tumor. These and other in situ hybridization results (data not shown) correlated well with the overall PTN mRNA expression level in each tumor.

DISCUSSION

PTN was highly expressed in favorable neuroblastomas, which often show spontaneous regression and differentiation. Mature ganglioneuromas also expressed high levels of PTN transcripts. In contrast, PTN expression was extremely low in stage III and IV tumors, especially those with N-myc amplification. Also, there was a tendency for neuroblastoma cell lines without amplification to have trace amounts of PTN expression compared to no detectable expression in N-myc-amplified cell lines. MK was expressed at high levels in almost all neuroblastoma primary tumors and cell lines, and aggressive neuroblastomas seem to have a relatively increased amount of MK transcripts. Thus, neuroblastomas clearly show quite different patterns of expression for these two closely related genes.

PTN and MK are members of the same family of neurotrophic factors and they share about 50% homology (16). Both are cysteine-rich, basic proteins that are secreted and have heparin-binding activity (14, 15, 19). The expression of PTN and MK genes is developmentally regulated (12, 16, 28), and they are both expressed in a variety of tissues in the mid-gestation mouse embryo. Their expression decreases in later embryogenesis, but then increases again postnatally in restricted organs or tissues. In mouse brain, MK is expressed more intensely than PTN during mid-gestation, but postnatally PTN expression is more intense than MK. Both proteins have neurotrophic activity on embryonic brain cells and dorsal root ganglion cells (16, 20, 21, 29, 30). MK is inducible by the treatment with retinoic acid (12), whereas PTN is not (25, 30). Furthermore, PTN, but not MK, has recently been known to be induced by platelet-derived growth factor (25). These findings suggest that, although PTN and MK are homologous, they have rather different biological functions.

It is interesting that PTN is expressed in ganglioneuromas as well as in neuroblastomas with the potential to differentiate. It is possible that PTN acts on neuroblastoma cells to potentiate the neuronal and/or Schwannian differentiation. On the other hand, PTN has been found to be secreted from human breast carcinomas and implicated as a tumor analogous, they have rather different biological functions.

The pattern of expression of PTN in favorable neuroblastomas is similar to that of TRK-A (2–6) which encodes the high-affinity NGF receptor. Neuroblastoma cells expressing a high level of TRK-A differentiate in the presence of NGF, but die in the absence of NGF in the primary culture (3). Furthermore, both PTN and TRK-A expression are very low or absent in neuroblastomas with N-myc amplification, which have a poor prognosis. Thus, expression of PTN and/or TRK-A may be useful as prognostic markers for neuroblastoma, and they may play an important role in the biological and clinical behavior of these tumors.

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REFERENCES

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