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Pancreastatin Immunoreactivity in Favourable Childhood Neuroblastoma and Ganglioneuroma

P. Kogner, P. Bjellerup, T. Svensson and E. Theodorsson

Neuroblastoma and its benign counterpart, ganglioneuroma, are tumours of the sympathetic nervous system, and known to produce and release various regulatory peptides. In this study, pancreastatin, a 52 amino acid regulatory peptide derived from chromogranin A, was analysed in plasma and tumour tissue from 15 children with neuroblastoma and one with ganglioneuroma. Detectable pancreastatin immunoreactivity (>1.9 pmol/l) was found in plasma in 13 of 15 children with highest concentrations in samples from children with favourable outcome ($P < 0.05$). In tumour tissue, non-metastatic tumours showed higher concentrations of pancreastatin immunoreactivity ($P < 0.05$). However, the highest concentrations were detected in tumours from children with favourable prognosis, regardless of clinical stage at presentation ($P < 0.01$). Serial plasma samples from one child with neuroblastoma and one with ganglioneuroma were investigated and showed significant systemic release of pancreastatin immunoreactivity during surgical manipulation of tumours with high pancreastatin concentrations. It is concluded that pancreastatin immunoreactivity may be detected in plasma samples and tumour extracts from children with neuroblastoma and ganglioneuroma. Systemic release during surgery implied tumour origin of elevated plasma pancreastatin. Furthermore, higher pancreastatin concentrations correlate with tumour differentiation, localised clinical stage and a favourable outcome for children with these tumours. It is suggested that pancreastatin in plasma and tumour tissue may be utilised as a marker indicating favourable tumour behaviour.

Key words: chromogranin A, pancreastatin, ganglioneuroma, neuroblastoma, plasma, differentiation, prognosis, tumour marker, radioimmunoassay

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INTRODUCTION

NEUROBLASTOMA, an embryonal malignant tumour of the sympathetic nervous system, is the most common paediatric extracranial tumour mainly diagnosed during infancy or early childhood. Neuroblastoma shows a remarkable heterogeneity both in biological and clinical behaviour, ranging from spontaneous regression or complete remission after minimal therapy in one subset, to unfavourable outcome due to aggressive tumour growth in spite of intensive multimodal therapy in another subset [1]. The benign differentiated counterpart, ganglioneuroma, usually has an excellent clinical prognosis.

Neuroblastomas and ganglioneuromas frequently produce various regulatory peptides, including neuropeptide Y (NPY), somatostatin and vasoactive intestinal peptide (VIP) [2]. These peptides may cause symptoms when released, but also influence tumour growth and differentiation in an autocrine or paracrine fashion. Analysing neuropeptides in tumours and plasma may be useful for diagnosis, prognostic assessment, and clinical follow-up of children with these tumours, and further indicate novel therapeutic modalities.

Pancreastatin is a 52 amino acid regulatory peptide derived from a 439 amino acid precursor, chromogranin A, the major soluble protein of catecholamine storage vesicles in adrenomedullary cells [3–5]. In the present study, we investigated plasma and tumour samples from children with neuroblastoma and ganglioneuroma for pancreastatin immunoreactivity.

MATERIALS AND METHODS

Patient material and sample handling

15 children with neuroblastoma and one with ganglioneuroma were included in the study (Table 1). All children were diagnosed and staged according to INSS criteria [6]. All 16 children were monitored at a single institution from diagnosis to death (0–19 months, $n = 6$) or last follow-up, 12–47 months from diagnosis. Blood samples were obtained at diagnosis in 15 children and during surgery in two of these (Nos 1 and 6, Table 1). Venous blood was collected in prechilled heparinised tubes, transported in an ice-bath and centrifuged within 30 min at +4°C for 10 min. The plasma was decanted and stored at −20°C until analysis. Plasma samples were extracted using C_{18} cartridges (SepPak, Millipore, U.S.A.).

Fresh tumour tissue was obtained at surgery, quick frozen on dry ice, and stored at −70°C until analysis. Tumour tissue was

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Table 1. Patient material and pancreastatin immunoreactivity in plasma and tumours

Patient number	Diagnosis	Stage*	Age (months)	Sex	P-PST-LI pmol/l	T-PST-LI pmol/g	Outcome	FU (months)
1	GN		60	M	19	0.7	NED	30+
2	NB	1	1	M	13	0.7	NED	47+
3	NB	1	12	F	11	0.3	NED	30+
4	NB	1	18	F	4.6	0.56	NED	19+
5	NB	1	21	M	<1.9	0.54	NED	25+
6	NB	2A	33	F	11.3	2.41	NED	12+
7	NB	2B	32	M	7.8	0.95	NED	31+
8	NB	3	0	F	6	nd	NED	12+
9	NB	3	0	M	nd	0.65	Dead	0−
10	NB	4	9	M	7	0.05	DOD	8−
11	NB	4	19	F	4	0.41	DOD	4−
12	NB	4	32	M	4	0.13	DOD	12−
13	NB	4	41	F	7.2	5.1	NED	47+
14	NB	4	52	F	<1.9	0.26	DOD	19−
15	NB	4	139	M	7	0.41	DOD	9−
16	NB	4S	0	M	10	0.27	NED	18+

* Clinical stage according to INSS [6]; P-PST-LI, plasma pancreastatin-like immunoreactivity at diagnosis; T-PST-LI, tumour tissue pancreastatin-like immunoreactivity; FU, follow-up; GN, ganglioneuroma; NB, neuroblastoma; nd, not done; NED, no evidence of disease; Dead, dead from postsurgical cardiovascular complications; DOD, dead of disease. *MYCN* amplification was only detected in tumours from patient Nos 10, 11 and 15.

cut while frozen, boiled in 10 volumes of 1 mol/l acetic acid, homogenised and centrifuged. Plasma eluates and tumour tissue supernatants were lyophilised and stored at −70°C until analysis.

Radioimmunoassay for pancreastatin

Pancreastatin-like immunoreactivity (PST-LI) was analysed using a competitive radioimmunoassay with an rabbit anti-pancreastatin (porcine) antiserum (RIK-7158, Peninsula, Belmont, California, U.S.A.). This antiserum shows a 91.4% crossreactivity with human pancreastatin (Chromogranin A [250-301]-amide). The intra- and inter-assay coefficients of variation were 6 and 9%, respectively. The lower detection limit was 1.9 pmol/l.

Statistical analysis

Statistical significance was calculated using Wilcoxon and Mann-Whitney test for two independent samples with two sided probability. The median and interquartile range (median: lower quartile-upper quartile) were used as measures of central tendency and variation, respectively.

Ethical approval

The study was approved by the ethics committee of the Karolinska Institute, Stockholm, Sweden.

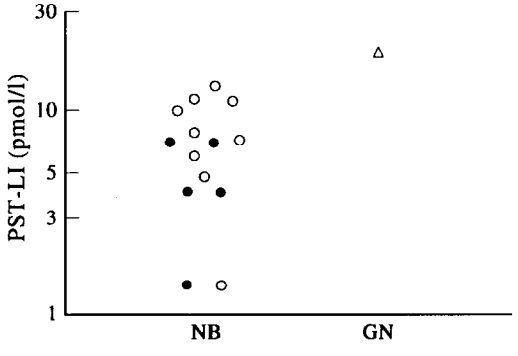

Figure 1. Pancreastatin-like immunoreactivity (PST-LI) in plasma samples obtained at diagnosis from 14 children with neuroblastoma (NB) and one child with ganglioneuroma (GN) (see Table 1 for details). Solid symbols indicate those who died during follow-up, remaining children have been followed for >12 months from diagnosis and are without evidence of disease after concluding therapy. Children with unfavourable outcome had lower plasma PST-LI at diagnosis ($P < 0.05$).

RESULTS

Pancreastatin in plasma at diagnosis

All but 2 of the 15 children under investigation (patient No. 8 not tested) showed detectable plasma concentrations of PST-LI at diagnosis (>1.9 pmol/l), with the highest concentration found in a child with a benign differentiated ganglioneuroma (19 pmol/l) (Table 1 and Figure 1). 8 children with ganglioneuroma or neuroblastoma of favourable stage (INSS stage 1, 2 and 4S) had significantly higher concentrations of plasma PST-LI (median: lower-upper quartile, 10: 5.4–10.8 pmol/l) compared with the 7 with neuroblastoma of advanced stage (3 or 4), (6: 3–7 pmol/l, $P < 0.05$), (Table 1). The 10 children who are alive without evidence of disease had significantly higher plasma PST-LI concentrations at diagnosis (9: 5.3–11.3 pmol/l) compared with the 5 patients who died from tumour progression during follow-up (4: 3.6–7 pmol/l, $P < 0.05$) (Figure 1).

Pancreastatin in tumour tissue

Tumour concentrations of PST-LI were significantly lower in primary tumours from children with unfavourable metastatic neuroblastoma stage 4 compared with those with localised or regional neuroblastoma or ganglioneuroma (0.34: 0.13–0.41 pmol/g wet weight versus 0.68: 0.54–0.70 pmol/g, $P < 0.05$), (Table 1). However, the highest PST-LI concentration measured in any single tumour was detected in a stage 4 primary neuroblastoma from a child (No. 13, Table 1) with bone metastases at presentation but an ultimate favourable outcome, alive with no evidence of disease 47 months from diagnosis.

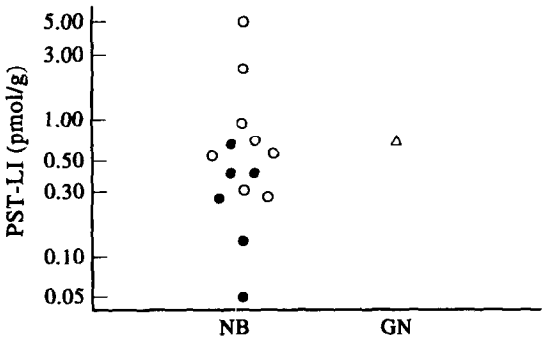

Figure 2. Pancreastatin-like immunoreactivity (PST-LI) in tumour tissue extracts of 14 neuroblastomas (NB, circles) and one ganglioneuroma (GN, triangle). Solid symbols indicate those who died during follow-up, open symbols those alive for >12 months from diagnosis without evidence of disease after concluded therapy (see Table 1 for details). Tumours from children with favourable outcome had higher PST-LI concentrations than those from children who died ($P < 0.05$).

Children with favourable outcome had significantly higher tumour concentrations of PST-LI (0.70: 0.42–1.68 pmol/g) compared to those dying from tumour progression (0.26: 0.11–0.41 pmol/g, $P < 0.01$) (Figure 2).

Pancreastatin release during surgery

Serial plasma samples were available for analysis from 2 children with relatively high tumour concentrations of pancreastatin immunoreactivity (0.7 and 2.41 pmol/g, respectively). The plasma samples were obtained before surgery, during surgical tumour manipulation and 1 and 24 h post operatively in both children (Figure 3). Both children (No. 1 with ganglioneuroma and No. 6 with a neuroblastoma stage 2A, Table 1) showed detectable plasma PST-LI concentrations at diagnosis (19 and 11.3 pmol/l, respectively), and none had pre-operative chemotherapy. There was a significant increase in PST-LI concentrations during surgical tumour manipulation (0.40–1.83-fold), with the greatest increase in the child undergoing surgery for a asymptomatic thoracic ganglioneuroma (Figure 3). In both children, there was a subsequent return at 24 h after surgery to plasma PST-LI concentrations lower than those detected pre-operatively (11–33% lower) (Figure 3).

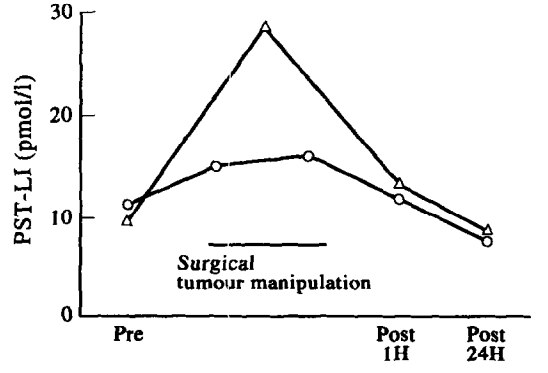

Figure 3. Serial plasma PST-LI in one child with ganglioneuroma (triangles) and one with neuroblastoma (circles), (Nos 1 and 6, respectively, Table 1). Samples were obtained immediately pre-operatively (Pre), during surgical tumour manipulation, and post operatively after 1 h (Post 1H) and after 24 h (Post 24H).

DISCUSSION

Several regulatory peptides are produced and released by neuroblastoma and ganglioneuroma tumours, and may bear significance for symptoms, and growth or differentiation of tumour cells [2]. Elevated concentrations of neuropeptide Y (NPY) in plasma and tumour tissue correlate with tumour growth and poor outcome [2, 7, 8, 9], while high concentrations of somatostatin and vasoactive intestinal peptide correlate with differentiated tumours and favourable clinical outcome [2, 10]. Pancreastatin was recently shown to be produced by human small cell lung carcinoma cells, and elevated concentrations of PST-LI were found in plasma of most patients with this neuroendocrine tumour [11, 12]. The present study is the first to present data on pancreastatin immunoreactivity in plasma and tumour tissue from children with neuroblastoma and ganglioneuroma. Although derived from only 16 children, the results showed a significant association of pancreastatin with favourable clinical stage and clinical outcome.

Pancreastatin is a regulatory peptide consisting of 52 (human) or 49 amino acid residues (porcine) that inhibits glucose-induced insulin secretion [3]. In the present study, we detected a significant release of pancreastatin during surgical tumour manipulation of neuroblastoma and ganglioneuroma (Figure 3). However, it remains to be further studied whether pancreastatin may exert any pathophysiological effects in children with neuroblastoma or ganglioneuroma. Plasma concentrations of the pancreastatin precursor, chromogranin A (CGA), have been reported to be elevated in children with neuroblastoma, with the highest levels recorded in those with widespread tumours (Evans stage III, IV and IV-S) [13]. High concentrations of CGA-like immunoreactivity (>190 ng/ml) were associated with poor outcome in children with regional or metastatic tumours (stage III and IV) [13]. The majority of neuroblastoma cell lines analysed showed expression of CGA as reported by Cooper and coworkers [14]. Using immunohistochemistry, CGA can be detected in most childhood tumours of neural crest origin, predominantly those with more differentiated morphology (ganglioneuroma and ganglioneuroblastoma) [15, 16].

From the present study, we conclude that pancreastatin immunoreactivity may be detected in plasma and tumour tissue from children with neuroblastoma and ganglioneuroma. Furthermore, we have provided evidence that increased circulating plasma pancreastatin is of tumour origin. From this limited series, it appears that clinically more favourable tumours show higher concentrations of pancreastatin immunoreactivity. These results may imply the usefulness of analysing pancreastatin in plasma and tumours as a marker of differentiation and an indicator of favourable clinical outcome.

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Genetic Alterations Associated with Metastatic Dissemination and Chemoresistance in Neuroblastoma

J. Bénard

Knowledge about genetic alterations specific to the metastatic process and chemoresistance in neuroblastoma is progressing steadily. Low or no *CD44* expression, increased *NM23* expression and specific mutations of the 5′ coding regions of *NM23* are distinct features of aggressive, metastatic neuroblastoma. *MYCN* down-regulates Class I HLA antigen expression in many neuroblastoma cell lines and, in turn, may be regulated by a suppressor gene. The *MYCN* amplified human neuroblastoma cell line, IGR-N-91, established *in vitro*, metastasises in the nude mouse and has exhibited co-activation of *MYCN* and *PGY1*, resulting from direct activation of the oncoprotein on the *PGY1* promoter. In this model, the *MYCN* product activates angiogenesis, the dissemination process and chemoresistance via specific genes (*PGY1* and *GST3*). *MYCN*, like the *BCL-2* and *TP53* products, may also play a key role in apoptosis. The implication of these genes in the potential for metastasis and chemoresistance in neuroblastoma is discussed.

Key words: neuroblastoma, metastatic dissemination, genetic alterations, *MYCN* activation, chemoresistance, *in vivo* models

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INTRODUCTION

THE GENETIC analysis of tumour tissues has offered considerable insights into the tumour heterogeneity of neuroblastoma. Indeed, cytogenetic and molecular biology studies, currently in progress, have identified recurrent genetic alterations which, when combined, appear to denote the existence of neuroblastoma subtypes [1, 2]. In the clinic, knowledge of such genetic anomalies has provided immediate applications for the treatment and outcome of patients with localised forms and stage IV-S [1, 3, 4]. So far, however, there has been no impact on the management of clinically unfavourable forms (disseminated stage IV) of neuroblastoma at diagnosis, in children older than a year. It is possible that information on genetic alterations specific to metastatic cells could help to improve the prognosis and to tailor therapy to these aggressive neuroblastomas.

Cancer invasion and dissemination is characterised by a long