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Somatostatin and Vasoactive Intestinal Peptide (VIP) in Neuroblastoma and Ganglioneuroma: Chromatographic Characterisation and Release During Surgery

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Neuroblastomas and ganglioneuromas frequently produce somatostatin (SOM) and vasoactive intestinal peptide (VIP), and elevated concentrations in tumour tissue are associated with favourable outcome. Both somatostatin and VIP have been shown to have an autocrine effect on tumour growth and differentiation *in vitro*, and VIP may cause clinical symptoms when released systemically. Using gel-permeation chromatography and specific radioimmunoassays, we further characterised somatostatin-like immunoreactivity (SOM-LI) and VIP-like immunoreactivity (VIP-LI) in neuroblastoma and ganglioneuroma tumour tissue. The major part of SOM-LI and VIP-LI in both neuroblastoma and ganglioneuroma represents the biologically active forms SOM-28, SOM-14 and VIP-28, respectively. 21 children with neuroblastoma and ganglioneuroma were monitored with serial plasma samples during surgery. In 8 children with measurable concentrations of SOM-LI, all showed increased concentrations during tumour manipulation ($P = 0.004$) that subsequently decreased below preoperative levels in all but one case ($P = 0.06$). The only child presenting with diarrhoea showed the highest preoperative plasma VIP-LI in the study (54 pmol/l). 2 children with increased concentrations of VIP-LI preoperatively showed a rapid decrease after surgical tumour removal. These findings indicate a systemic release from the tumours. It is concluded that plasma and tumour tissue from children with neuroblastoma and ganglioneuroma contain biologically active molecular forms of somatostatin and vasoactive intestinal peptide. These peptides may bear significance both for specific symptoms in certain patients as well as influencing tumour growth and differentiation *in vivo*.

Key words: neuroblastoma, ganglioneuroma, vasoactive intestinal peptide, somatostatin, neuropeptide Y, tumour markers, gel-permeation chromatography, radioimmunoassay
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INTRODUCTION

NEUROBLASTOMA, one of the most common tumours of childhood, is of neural crest origin and may arise wherever sympathetic tissue is present including the adrenal medulla, the most common site of primary tumours. In contrast to many other malignancies of childhood, there has been only little improvement in the long-term survival of patients with advanced neuroblastomas [1].

Neuroblastomas, as do other tumours of neuroendocrine origin, produce and secrete various neuropeptides. Neuropeptides are biologically active regulatory peptides, produced by neuronal cells, that act as neuromodulators or neurotransmitters. Neuropeptides have recently also been shown to have both potent trophic and differentiational effects on a cellular level with potential autocrine and paracrine functions [2–4]. Measuring some of these peptides may be of value for diagnosing and monitoring of tumour growth as recently reviewed [5]. Serial measurements of neuropeptide Y (NPY) in plasma have been shown to correlate well with the clinical course of neuroblastoma [6, 7]. Plasma NPY concentrations have been found to increase considerably during surgical tumour manipulation indicating systemic release, and both plasma and tumour tissue contain different NPY immunoreactive molecular forms [8].

Somatostatin (SOM) is a neuropeptide derived from a 92 amino acid precursor, prosomatostatin. Somatostatin exists in two different biologically active forms, a 14 amino acid peptide (SOM-14) and an N-terminally extended form with 28 amino acids (SOM-28). It is widely distributed throughout the body, including the central and peripheral nervous systems, the gastrointestinal tract and different glands [9]. Somatostatin has a wide range of different physiological effects including inhibition of growth hormone and thyroid stimulating hormone release from the pituitary gland. Somatostatin has growth inhibitory effects on malignant neuroendocrine cells *in vitro*, and somatostatin analogues are used for treatment of neuroendocrine tumours [10, 11]. Ganglioneuromas, benign childhood tumours of neural crest origin, contain higher concentrations of somatostatin-like immunoreactivity (SOM-LI) compared to neuroblastomas, healthy adrenals and other tumours of neuroendocrine origin [7, 12].

Vasoactive intestinal peptide (VIP), a 28 amino acid neuropeptide distributed throughout the central and peripheral nervous systems, is involved in a wide variety of biological processes acting as a neurotransmitter, neuromodulator or a hormone [13, 14]. VIP may play a significant role in the development and differentiation of the sympathetic nervous system [15]. Intact VIP induces growth inhibition and differentiation of malignant neuroblastoma cell lines [16, 17]. VIP secreting tumours usually have a favourable prognosis [18]. Recently, ganglioneuromas were shown to have higher concentrations of VIP-like immunoreactivity (VIP-LI) when compared to neuroblastomas, healthy adrenals and other tumours of neuroendocrine origin [7].

The aim of the present study was to further characterise somatostatin and VIP immunoreactivities in neuroblastoma and ganglioneuroma tumour tissue. Using gel-permeation chromatography and radioimmunoassays, we investigated if this immunoreactive material may represent biologically active homologues. Furthermore, serial plasma samples obtained during surgery were analysed to monitor systemic release of these neuropeptides.

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MATERIALS AND METHODS

Patient material and sample handling

Plasma and tumour samples were obtained from children with neuroblastoma and ganglioneuroma. The clinical diagnosis and staging were performed according to the International Neuroblastoma Staging System (INSS) [19]. We have previously investigated concentrations of SOM-LI and VIP-LI in a series of tumours of neural crest origin [7]. From this extended series (in total 96 neuroblastomas and 10 ganglioneuromas), tumour tissue with high concentrations of SOM-LI and VIP-LI from one child with neuroblastoma (patient No. 1) and one child with ganglioneuroma (patient No. 2) were selected for chromatographic characterisation (Table 1).

For monitoring surgical release, 21 consecutive children with neuroblastoma (n = 20) and ganglioneuroma (n = 1) were followed with blood samples before, during and after surgery. Fresh tumour tissue was obtained at surgery and quick frozen to −70°C on solid CO_2. Plasma samples were obtained in prechilled heparinised tubes and stored in ice-water for a maximum of 30 min until centrifuged at +4°C for 10 min, decanted and subsequently frozen to −20°C. Plasma samples were extracted using C_{18}-cartridges (SepPak) (Millipore, U.S.A.). Tumour samples were cut in small pieces without previous thawing, and extracted and homogenised in 10 volumes of boiling acetic acid (1 mol/l) for 10 min. The plasma eluates and the tumour tissue supernatants were lyophilised and stored at −70°C until analysis.

Gel-permeation chromatography

A 100 cm long column with an inner diameter of 26 mm, packed with Sephadex G-50 superfine (Pharmacia, Sweden) was used for gel-permeation chromatography. The column was eluted (0.5 ml/min) with a formic acid solution (0.1 mol/l) containing 0.2% bovine serum albumin (Sigma, U.S.A.) and 0.02% sodium azide (Merck, Germany). The fractions (2 ml) were collected, lyophilised and stored at −20°C until radioimmunoassays were performed. The column was calibrated in separate runs using low concentrations of synthetic SOM-14, SOM-28 (= $SOM\text{-}28_{[1-28]}$), $SOM\text{-}28_{[1-14]}$ and VIP-28. The void column (V_o) was determined using dextran blue 2000 (Pharmacia, Sweden) and the total volume (V_t) using ^{22}Na (Amersham, U.K.).

Radioimmunoassays for somatostatin, VIP and NPY

SOM-LI was analysed using an antiserum raised against conjugated SOM-14 as described earlier [20]. This antiserum reacts with both SOM-14 and SOM-28. However, the antiserum

Table 1. Clinical data for the three patients whose tumours were selected for chromatography

Patient No.	Gender	Age (months)	Diagnosis stage
1	F	56	NB 4
2	M	24	GN
3	M	8	NB 3

NB, neuroblastoma; GN, ganglioneuroma.

shows no crossreactivity with the N-terminal peptide fragment SOM-28$_{[1-14]}$. Intra- and interassay coefficients of variation were 7 and 11%, respectively.

VIP-LI was analysed using antiserum VIP2 raised against conjugated natural intact porcine VIP. The antiserum did not crossreact with gastrin, pancreatic polypeptide, glucagon, NPY or neurotensin (data not shown). The limit of detection was 3 pmol/l. Intra- and interassay coefficients of variation were 9 and 13%, respectively. NPY-LI was analysed using antiserum N1 raised against natural intact porcine NPY as previously described [21]. Intra- and interassay coefficients of variation were 7 and 11%, respectively.

Statistical methods

The sign test was used for significance calculations.

Ethical approval

The present study was approved by the ethics committee of Karolinska Institute, Stockholm, Sweden (KI: 1987: 198–201).

RESULTS

Characterisation of somatostatin-like immunoreactivity

Chromatographic characterisation, using gel-permeation chromatography, in neuroblastoma tumour tissue from patient No. 1 (Table 1) revealed three different components of SOM-LI (Figure 1a). The two major components eluted at the positions of SOM-28 and SOM-14, respectively. The third component, a minor fraction, eluted at an earlier position. This fraction, representing molecular forms with larger Stokes radius, was not further characterised. In ganglioneuroma tumour tissue, a similar pattern was detected (Figure 1b), with two immunoreactive components corresponding to SOM-28 and SOM-14, and a third component corresponding to a larger molecular form. In addition to these three peaks, immunoreactive material eluted between SOM-28 and SOM-14 in the ganglioneuroma tumour extract (Figure 1b).

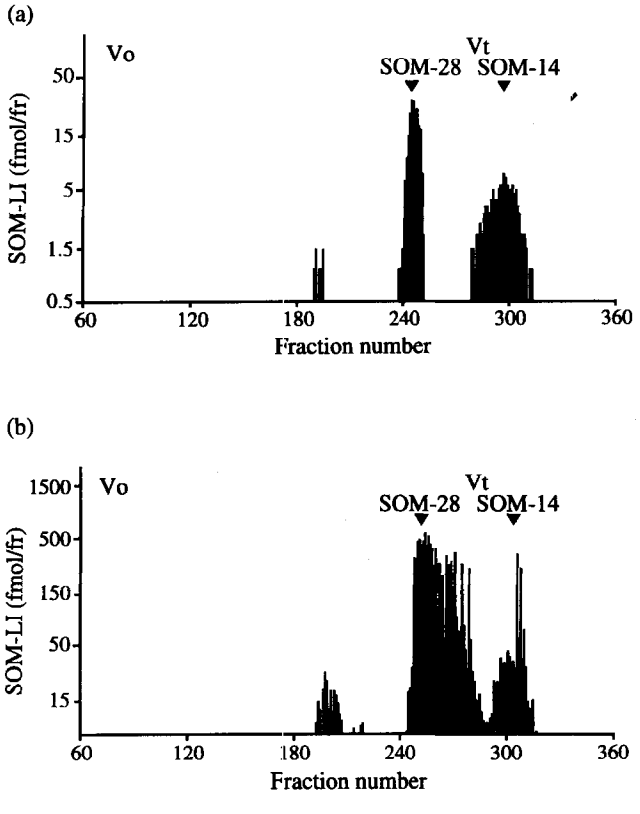

Figure 1. Gel-permeation chromatogram of SOM-LI obtained from (a) the primary tumour from patient No. 1 with neuroblastoma stage 4; and (b) tumour tissue from patient No. 2 with ganglioneuroma. In both tumours, two major peaks were identified eluting at a similar position as SOM-28 and SOM-14. A minor fraction of SOM-LI eluted as a molecular form with a larger Stokes radius. In addition to these three peaks, immunoreactive material eluted between SOM-28 and SOM-14 in the ganglioneuroma tumour extract. Elution positions of SOM-28, SOM-14, void volume (V_o) and total volume (V_t) are indicated.

Characterisation of VIP-like immunoreactivity

Chromatographic characterisation of VIP-LI in tumour tissue from the same patients as above revealed one major component of VIP-LI in both patients (Figure 2). This peak eluted at the same position as VIP-28. As for SOM-LI, a minor fraction eluted at an earlier position in both neuroblastoma and ganglioneuroma tumour tissue, representing a molecular form with a larger Stokes radius.

Plasma SOM-LI and VIP-LI during surgery

Of the 21 children monitored during surgery, 10 children had no detectable plasma concentrations of SOM-LI or VIP-LI. Of the children with measurable concentrations of SOM-LI, all 8 showed increasing concentrations during surgery ($P = 0.004$), and 24 h postoperatively, 6 out of 7 had a lower concentration compared to preoperatively ($P = 0.06$) (Figure 3a).

5 of the 8 children that showed measurable concentrations of

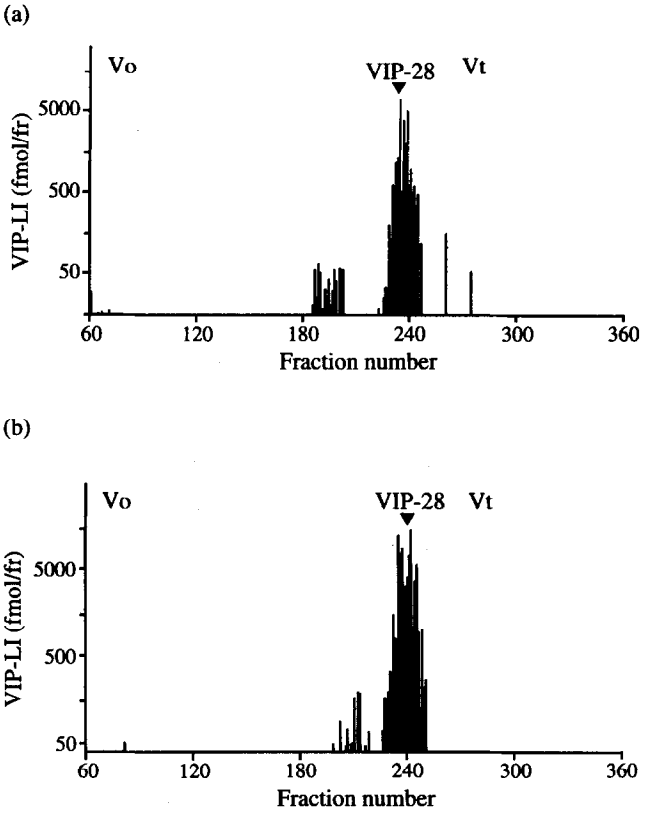

Figure 2. Gel-permeation chromatogram of VIP-LI obtained from (a) the primary tumour from patient No. 1 with neuroblastoma stage 4; and (b) tumour tissue from patient No. 2 with ganglioneuroma. In both tumours, only one major component was identified eluting at a similar position as intact VIP. A minor fraction of VIP-LI eluted as a molecular form with a larger Stokes radius. Elution position of VIP-28 indicated.

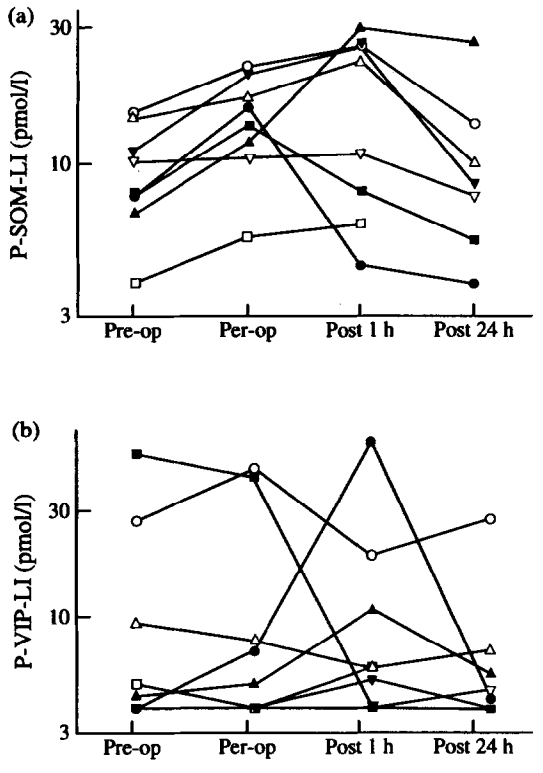

Figure 3. Serial plasma immunoreactivity preoperatively (pre-op), during surgery (per-op), postoperatively 1 h (post 1h) and 24 h (post 24h) for those children with (a) at any time detectable concentrations of SOM-LI (n = 8); and (b) at any time detectable concentrations of VIP-LI (n = 8). The total number of children was 21. Each line represents measurements for one child.

VIP-LI (Figure 3b) had low concentrations (<10 pmol/l) at all times. One child had increased concentration during surgery, one had increased concentrations after surgery and one had a high concentration preoperatively that decreased during surgery and was unmeasurable postoperatively (Figure 3b). This child (patient No. 3, Table 1), the only one presenting with diarrhoea, is described in more detail in Figure 4.

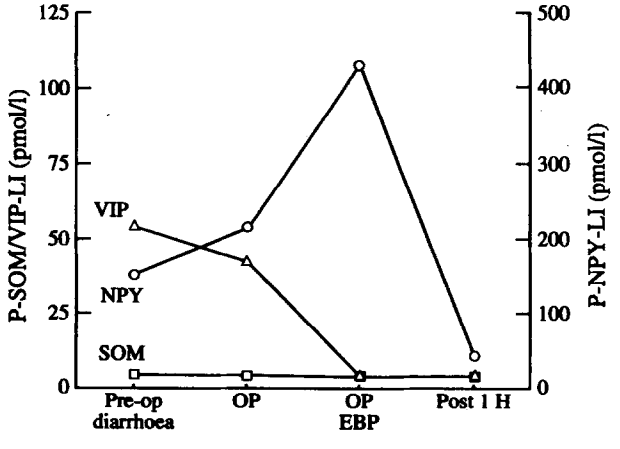

Figure 4. Serial plasma immunoreactivity for SOM, VIP and NPY during surgery for patient No. 3 (Table 1). Pre-op, preoperatively; OP, during surgery; OP EBP, elevated blood pressure during surgery; Post 1 H, 1 h postoperatively.

DISCUSSION

Monitoring different neuropeptides in plasma has been shown to be a useful tool for diagnosis, prediction of outcome and follow-up in children with neuroblastoma [5]. One of the most promising neuropeptides in this context, NPY, is present in different molecular forms, possibly with different biological significances [7]. Recent studies have indicated an autocrine function of both somatostatin and VIP in neuroblastoma tumour growth and differentiation *in vitro* [16, 17, 22, 23, 24]. Benign ganglioneuromas and favourable neuroblastomas contain high concentrations of somatostatin and VIP immunoreactivities [7, 12]. Recently, methods have been made available for the *in vivo* detection of tumour tissue expressing somatostatin and VIP receptors [11, 25]. In the present study, we characterised SOM-LI and VIP-LI in tumour tissue using gel-permeation chromatography, demonstrating molecular forms with putative biological activity *in vivo*. We also investigated the systemic release from tumour tissue during surgical manipulation.

SOM-LI in neuroblastoma and ganglioneuroma tumour tissue was separated into two major components representing SOM-14 and SOM-28 (Figure 1). Both SOM-14 and SOM-28 are produced from the same gene product, prepro-somatostatin. The majority of SOM-14 is processed directly from the precursor, but a small proportion originates from further processing of SOM-28 [9]. Both SOM-14 and SOM-28 are biologically active, but have individual affinity for different somatostatin receptors indicating targeted actions in tumour growth and differentiation [23, 24]. The molecular form of SOM-LI with a larger Stokes radius (Figure 1) was not further characterised. It may represent an intermediate product in the processing of pro-somatostatin, presumably smaller than the deduced 92 amino acid precursor. In the ganglioneuroma tumour tissue, a fourth component between SOM-28 and SOM-14 was seen but not further characterised.

VIP induces neuroblastoma differentiation *in vitro* [16] and children with VIP secreting tumours and diarrhoea have an excellent prognosis [18]. Differentiated ganglioneuromas, also from children without gastrointestinal symptoms, show higher VIP concentrations than unfavourable neuroblastomas indicating a role for VIP in neuroblastoma differentiation *in vivo* [7, 12]. Recent data imply that VIP acts in an autoregulatory manner and stimulates neuroblastoma cells both to VIP synthesis and to expression of VIP receptors [17, 22]. In tumour tissue from both the child with neuroblastoma and the child with ganglioneuroma VIP-LI was detected as one major component representing VIP-28 (Figure 2). VIP-28 is the biologically active form of VIP, and the only molecular form of the glucagon-secretin family that has been shown to have differentiating activity [26]. The molecular form of VIP-LI with larger Stokes radius (Figure 2) was not further characterised. It may represent some intermediate product in the processing of prepro-VIP [27]. Both neuroblastoma and ganglioneuroma have the same chromatographic pattern indicating that there is no major qualitative difference in the biosynthetic processing of VIP.

The preoperative increase in plasma SOM-LI during tumour manipulation indicates a systemic release from the tumour itself (Figure 3a). The postoperative decrease to levels below preoperative concentrations indicates that spontaneous systemic release is the source of detectable plasma SOM-LI in children with neuroblastoma and ganglioneuroma.

Fluctuations in plasma VIP-LI during surgery can be subdivided into three different categories: (a) no measurable concentrations; (b) low concentrations with no significant variation;

and (c) preoperatively high concentrations probably correlating with clinical symptoms, with decreasing concentrations postoperatively (Figure 3b). The only exception was an infant, only 2 weeks of age with a neuroblastoma stage 3, that had an increase 1 h postoperatively that was reversed to undetectable concentrations 24 h later.

The infant with high concentration of plasma VIP-LI preoperatively (patient No. 3; Table 1) had voluminous diarrhoea for 2 weeks before admission. The plasma concentration of VIP-LI (54 pmol/l) preoperatively was well above the concentrations known to cause persistent diarrhoea [18, 28]. After recovery, the diarrhoea ended and the VIP-LI concentration in plasma was normalised. During surgery this child had an episode of elevated blood pressure simultaneously with a decrease in VIP-LI concentration and an increase in NPY-LI to 428 pmol/l (Figure 4). Although increased concentrations of NPY are not necessarily correlated to increased arterial blood pressure [29], the combination with a decrease in VIP-LI concentration possibly had a causal relationship to the elevation in blood pressure [14].

In summary, we conclude that neuroblastomas and ganglioneuromas may contain SOM-LI and VIP-LI. Chromatographic characterisation showed that these immunoreactivities represent molecular forms with putative biological activities. Serial plasma determinations showed SOM-LI release during surgical tumour manipulation. The decrease of SOM and VIP immunoreactivity postoperatively indicated tumoral origin of systemic VIP and SOM. Furthermore, these circulating regulatory peptides seemed to have significance for pathophysiology, e.g. diarrhoea and blood pressure. SOM and VIP may also play a role in neuroblastoma differentiation and/or regression, although further studies are warranted. The influence of SOM and VIP in endogenous tumour regression and differentiation may indicate novel therapeutic possibilities in children with neuroblastoma using synthetic SOM and/or VIP analogues.

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