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Original Paper

Somatostatin in Neuroblastoma and Ganglioneuroma

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Neuroblastoma, a childhood tumour of the sympathetic nervous system, may in some cases differentiate to a benign ganglioneuroma or regress due to apoptosis. Somatostatin may inhibit neuroblastoma growth and induce apoptosis *in vitro* and was therefore investigated. Using a radioimmunoassay, we found that all ganglioneuromas contained high somatostatin concentrations (> 16 pmol/g), significantly higher than neuroblastomas ($n = 117$, median 2.8 pmol/g), healthy adrenals, Wilms' tumours, phaeochromocytomas and other neuroendocrine tumours ($P < 0.001$). Neuroblastomas contained more somatostatin than control tumours ($P < 0.001$–0.05). Neuroblastomas amplified for the *MYCN* oncogene contained less somatostatin than non-amplified tumours (1.2 pmol/g versus 4.0 pmol/g, respectively; $P = 0.026$). In a clinically unfavourable neuroblastoma subset (age > 12 months, stage 3 or 4) 16 children with high concentrations of somatostatin in primary tumours had a better prognosis than 23 with low somatostatin (46.7% versus 0% survival at 5 years, $P < 0.005$). Scintigraphy using ^{111}In-pentetreotide identified tumours expressing high-affinity somatostatin receptors *in vivo*. However, no significant correlation was found between somatostatin receptor expression and peptide content in 15 tumours. Similarly, human SH-SY5Y neuroblastoma xenografts grown in nude rats showed low somatostatin concentrations, but were positive for somatostatin receptor scintigraphy. Treatment of these rats with the somatostatin analogue octreotide seemed to upregulate *in vivo* receptor expression of somatostatin and vasoactive intestinal peptide more effectively than 13-*cis* retinoic acid. In conclusion, somatostatin in neuroblastoma is associated with differentiation to benign ganglioneuromas *in vivo* and favourable outcome in advanced tumours. Furthermore, somatostatin receptor scintigraphy may identify tumours with high-affinity receptors in children that might benefit from targeted therapy using synthetic somatostatin analogues. © **1997 Published by Elsevier Science Ltd.**

Key words: somatostatin, neuroblastoma, ganglioneuroma, ganglioneuroblastoma, octreotide, vasoactive intestinal peptide, neuropeptide Y, retinoic acid, prognosis, xenograft, autocrine
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INTRODUCTION

NEUROBLASTOMA IS an embryonal tumour of neural crest origin with an extraordinary clinical and biological heterogeneity. The clinical outcome, ranges from differentiation to ganglioneuroma, or regression due to apoptosis after no or minimal therapy in the most favourable subset, to tumour progression and poor clinical outcome despite intensive therapy in the most unfavourable subset. Neuropeptides, a class of regulatory peptides produced by neural cells and acting as neurotransmitters,

Correpondence to P. Kogner.

may have clinical and biological significance in neuroblastoma and ganglioneuroma tumours. Neuropeptides may both cause specific symptoms as well as regulate cellular growth and differentiation [1].

Somatostatin (SOM), or somatotropin-release inhibiting factor (SRIF), is extensively distributed in the human body including the central and peripheral nervous systems, the gastrointestinal tract and various exocrine and endocrine glands [2, 3]. SOM is a cyclic neuropeptide with two different biological active forms (SOM-14 and SOM-28) derived from a 92 amino acid precursor, presomatostatin. Both SOM-14 and SOM-28 bind with high affinity to five known G protein-coupled SOM-receptor subtypes displaying a widespread, overlapping but characteristic pattern of expression with evidence that several subtypes may be co-expressed in certain cells [4]. SOM was shown to have growth inhibitory effects on malignant neuroendocrine cells *in vitro* and long-acting analogues have been developed and used for the treatment of neuroendocrine tumours *in vivo* [5, 6]. Recent studies have shown that neuroblastoma cells may express SOM receptors with high-affinity binding, allowing biological activities *in vitro* [7–9]. Somatostatin receptors have also been identified in neuroblastoma tumour tissue, predominantly in samples from tumours of localised clinical stages without *MYCN* amplification [7, 10]. It has also been demonstrated that children with tumours expressing these receptors have a much better chance of survival [10]. Recently, techniques for the detection of somatostatin receptor-expressing tumour cells *in vivo* have been established [11]. Preliminary results indicate that somatostatin receptor scintigraphy (SRS) is positive at diagnosis or relapse in a majority of children with neuroblastoma, with a tendency that SRS detects tumours in children with a more favourable outcome [12, 13].

We have previously reported that high concentrations of SOM immunoreactivity (SOM-LI) may be detected in ganglioneuromas and a subset of neuroblastomas [14, 15]. Furthermore, we have shown that SOM-LI represents molecular forms (SOM-14 and SOM-28) with putative biological activity, indicating that SOM may play a functional role in neuroblastoma differentiation *in vivo* [16]. In the present study SOM-LI concentrations were analysed in a larger number of tumours and the results were compared to clinical and biological features. In a subset of children with neuroblastoma and ganglioneuroma, we analysed both SOM-LI in tumour tissue and SOM receptor expression *in vivo*, using SRS. Finally, SOM-LI concentrations and SOM receptors were analysed in human neuroblastoma SH-SY5Y xenografts in nude rats, and the therapeutic effects of an SOM analogue (octreotide), vasoactive intestinal peptide (VIP) and 13-*cis* retinoic acid were analysed with respect to the *in vivo* expression of SOM and VIP receptors.

MATERIALS AND METHODS

Patients and sample handling

Children with neuroblastoma ($n = 117$) and ganglioneuroma ($n = 13$) diagnosed and staged according to the International Neuroblastoma Staging System (INSS) [17], were included in the study. Seventy-nine of these children are alive and disease-free with a follow-up of 12–96 months from diagnosis (66 with neuroblastoma and all 13 with ganglioneuroma). Thirty-nine children with neuroblastoma died during follow-up (33%), whereas 12 are still under therapy (10%). As control tissues, 3 healthy adrenals from children over 1 year of age, 6 adult phaeochromocytomas, 5 other neuroendocrine tumours and 16 paediatric Wilms' tumours were analysed.

Primary tumour tissue was obtained at surgery, fresh frozen on solid CO_2 or liquid N_2 and kept frozen until analysis. Tumour samples were cut while still frozen, extracted and homogenised in 10 volumes of boiling acetic acid (1 mol/l) for 10 min.

Radioimmunoassay for somatostatin immunoreactivity (SOM-LI)

SOM-LI in acid tumour extracts was analysed using an antiserum raised against conjugated SOM-14 as described earlier [18]. This antiserum reacts with both SOM-14 and SOM-28 [16, 18]. The intra- and interassay coefficients of variation were 7% and 11%, respectively.

Animals and SH-SY5Y xenografts

Nude rats (WAG rnu/rnu) were injected with 20×10^6 cells (passage 28) of the adrenergic neuroblastoma cell line SH-SY5Y (kind gift from J Biedler) [19] s.c. on the lateral side of each hind leg. After establishment of significant tumour growth, animals were randomly selected for treatment with octreotide (1.5 or 7.5 μg, s.c. twice daily), VIP (10 or 40 μg, s.c., twice daily), 13-*cis*-retinoic acid (2 or 4 mg, oral, twice daily) or controls without therapy. Animals were anaesthetised with pentobarbital (50 mg/kg) during SRS and VIP receptor scintigraphy (VIP-RS). Tumour tissue from control animals was analysed for SOM-LI.

Somatostatin receptor scintigraphy

The somatostatin analogue pentetreotide (DTPA-D-Phe^1-octreotide) was labelled with ^{111}In and a mean dose of 34 MBq was injected at diagnosis into 15 children from whom tumour tissue was available for analysis of SOM-LI. Images were acquired at 4 and/or 24 h as previously described [12]. Rats were placed on the gamma-camera and injected with 20 MBq in the tail vein, and planar images were obtained as previously described after the injection and at 1, 4, 24 and 48 h [19].

Vasoactive intestinal peptide receptor scintigraphy (VIP-RS)

Iodination and purification of ^{131}I-VIP was performed using porcine VIP (similar amino acid sequence as rat and human) as previously described [19]. The rats were placed on the gamma-camera, 20 MBq ^{131}I-VIP was injected i.v. and images were obtained after the injection and at 1, 4, 24 and 48 h [19].

Statistical analysis

Statistical analysis was performed using Fisher's exact test for 2×2 tables, the Wilcoxon, Mann–Whitney test for 2 independent samples and the Kruskal–Wallis test with multiple comparisons for more than two groups. The median and interquartile range (median: lower quartile–upper quartile) were used as measures of central tendency and variation, respectively. Survival probability was calculated using the product limit method of Kaplan and Meier and compared using the Mantel–Haenszel log-rank test.

RESULTS

Somatostatin in tumour tissue

All 13 ganglioneuromas had very high SOM-LI concentrations (>16 pmol/g wet weight), significantly higher than

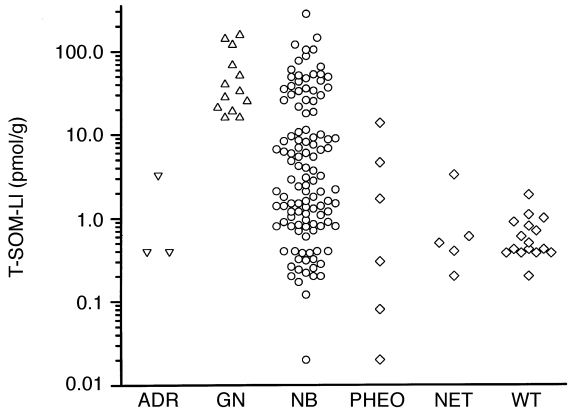

Figure 1. Somatostatin in tumour tissue extracts (SOM-LI pmol/g wet weight) of ganglioneuromas (GN, $n = 13$) and neuroblastomas (NB, $n = 117$) compared to healthy adrenals (ADR, $n = 3$) and control tumours (6 phaeochromocytomas, PHEO, 5 other neuroectodermal tumours, NET and 16 Wilms' tumours, WT). GN showed higher SOM-LI concentrations than all other tumours ($P < 0.001$) whereas NB had higher concentrations compared to control tumours ($P < 0.05$– 0.001).

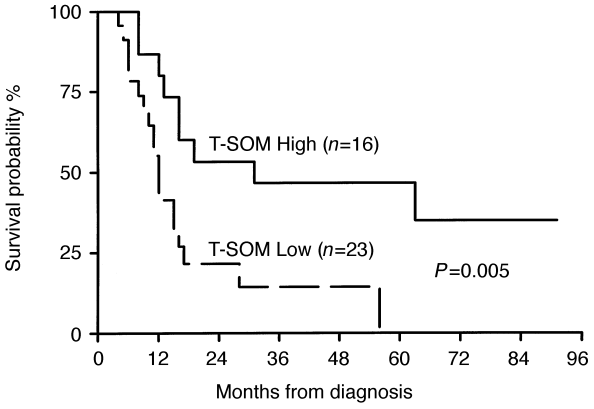

Figure 2. Survival from diagnosis for 39 children with advanced clinical stage (INSS 3 or 4) and > 12 months of age at diagnosis according to high (SOM-LI > 8 pmol/g, $n = 16$) or low somatostatin concentration in primary tumour tissue, respectively. Survival probability according to Kaplan–Meier was better for children with high SOM-LI (46.7 ± 12.9% and 35 ± 14% versus 14.3 ± 8.4% and 0% survival probability at 3 and 6 years, respectively) according to the Mantel–Haenszel log-rank test ($P = 0.005$).

neuroblastomas, healthy adrenals or control tumours ($P < 0.001$, Figure 1). Neuroblastomas contained higher SOM-LI concentrations (2.8: 0.84–20.05 pmol/g, median: lower quartile–upper quartile) than Wilms' tumours (0.45: 0.4–0.6 pmol/g, $P < 0.001$), phaeochromocytomas (0.75: 0.19–3.15 pmol/g, $P = 0.03$) and other neuroendocrine tumours (0.5: 0.3–1.95 pmol/g, $P = 0.018$). There was no significant difference between neuroblastomas of different stages, although there was a non-significant trend towards higher concentrations in stage 1, 2 and 4S tumours compared to tumours at unfavourable clinical stages 3 and 4. 18 neuroblastomas amplified for the *MYCN* oncogene had lower SOM-LI (1.2: 0.8–2.4 pmol/g) compared to 99 non-amplified tumours (4.0: 0.9–25.35 pmol/g, $P = 0.026$) *MYCN* amplified neuroblastomas from 10 children over 24 months at diagnosis had the lowest SOM-LI concentrations, significantly lower than those in 8 *MYCN* amplified tumours from younger children ($P < 0.05$).

In the whole neuroblastoma series, there was a non-significant tendency to higher SOM-LI in tumours from children with favourable clinical outcome ($P = 0.11$). This difference was significant in the most unfavourable subset of children, aged over 1 year with widespread tumours of stage 3 and 4 ($P = 0.03$). In this subset of 39 children, the 16 children with high SOM-LI content (> 8 pmol/g wet weight) had better survival probability than the 23 with low SOM-LI (46.7 ± 12.9% and 35 ± 14% versus 14.3 ± 8.4% and 0% survival probability at 3 and 6 years, respectively, $P = 0.005$, Figure 2).

Somatostatin receptor scintigraphy and somatostatin in tumour tissue

SRS was performed in 15 children and visualised significant tumour tissue in 10 of these, 9/14 neuroblastomas and 1/1 ganglioneuroma. Interestingly, there was no significant correlation between SOM-LI concentrations in tumour tissue and receptor expression as analysed with SRS (Table 1).

Somatostatin and somatostatin receptors in neuroblastoma xenografts

The SH-SY5Y xenografts grown in nude rats showed only a low level of SOM-LI concentration (0.08 pmol/g). However, SRS showed tumour tissue expressing somatostatin receptors (Figure 3).

Treatment with octreotide, VIP and 13-cis *retinoic acid* in vivo

Expression of somatostatin receptors but low levels of somatostatin peptide concentrations in the xenografts prompted us to try treatment with the somatostatin analogue octreotide, compared with VIP and 13-*cis*-retinoic acid. After four days of treatment, SRS and VIP-RS was performed as described. All treated rats showed positive scans for ^{111}In-pentetreotide, indicating the presence of high-affinity somatostatin receptors (Figure 4). Rats treated with octreotide or VIP showed higher uptake in the tumour tissue at ^{131}I-VIP scans compared to rats receiving treatment with retinoic acid or no treatment at all (Figure 5). From the scans, it was observed that rats receiving octreotide in the higher dose

Table 1. *Tumour somatostatin peptide content and receptor scintigraphy*

	Tumour concentration of somatostatin immunoreactivity				
	>16 pmol/g	8–16 pmol/g	4–8 pmol/g	1–4 pmol/g	<1 pmol/g
SRS					
Positive	1 + 1*	1	1	4	2
Negative	2		1		2

Somatostatin immunoreactivity in 14 neuroblastoma tumours and one ganglioneuroma (*), from children analysed at diagnosis for specific receptor expression using somatostatin receptor scintigraphy (SRS).

Figure 3. Somatostatin receptor scintigraphy in a nude rat bearing human SHSY-5Y neuroblastoma xenografts positively labelled for ^{111}In-pentetreotide (Octreoscan, Mallinckrodt). Scanning was performed at 25 min, 2 h and 4 h postinjection. The highest uptake is seen in the kidneys, bladder and at the injection site in the tail vein.

range showed more intense *in vivo* labelling of tumour tissue for both ^{111}In-pentetreotide and ^{131}I-VIP compared to control animals and rats receiving 13-*cis*-retinoic acid (Figures 4 and 5).

DISCUSSION

Somatostatin is known to inhibit growth and induce apoptosis in certain neuroblastoma cell lines *in vitro*. In the current study, we present data supporting a role of somatostatin in neuroblastoma biology *in vivo*. We found a higher somatostatin content in fully differentiated benign ganglioneuromas. In an unfavourable subset of advanced neuroblastomas, high somatostatin concentration was associated with favourable prognosis. Somatostatin receptor scintigraphy detected tumours expressing high-affinity receptors *in vivo*. This was found in tumours both with low and high concentrations of somatostatin immunoreactivity, respectively. Furthermore, SH-SY5Y xenografts with somatostatin binding but only very low peptide content were treated with a synthetic somatostatin analogue and showed an increase in scintigraphic detection of somatostatin and VIP receptors *in vivo*.

Using a specific radioimmunoassay for somatostatin, we found higher concentrations of immunoreactivity in ganglioneuroma and favourable neuroblastoma tumours in agreement with our previous findings [14, 15]. Furthermore, the analysed immunoreactivity represents the molecular forms SOM-14 and SOM-28 with biological activity *in vivo* [16]. In another study, higher tissue concentrations of SOM-LI were found in tumours with morphological differentiation and favourable clinical stage [21]. However, in that study comprising 16 neuroblastomas, no correlation with clinical outcome could be found for SOM-LI.

Plasma SOM-LI has only been studied in a limited series of children with neuroblastoma. Among 21 children, only 8 showed detectable plasma SOM-LI [16]. However, all these children showed increasing concentrations during surgical tumour manipulation. In all but one, postoperative concentrations were lower than those measured before surgical tumour removal, indicating tumour origin of increased systemic SOM-LI.

Somatostatin receptors have been shown both in cell lines and neuroblastoma tumour samples using binding studies and autoradiography, and *in vivo* in neuroblastoma patients using somatostatin receptor scintigraphy [7, 8, 10, 12, 13]. The presence of specific receptors is necessary for biological effects on neuroblastoma cells [7, 8]. In agreement with *in vitro* data, clinical effects of tumour treatment with somatostatin analogues in adult patients with carcinoid tumours are confined to those with tumours positive for SRS [22]. Hence, the described neuroblastoma xenograft model seemed to be a

Figure 4. ^{111}In-pentetreotide scintigraphy (Octreoscan) at 24 h postinjection in nude rats with SH5Y-5Y neuroblastoma xenografts treated for four days with octreotide (left, 7.5 μg×2, s.c.), VIP (middle, 40 μg×2, s.c.) and 13-*cis*-retinoic acid (right, 4 mg×2, p.o.) as described in *Materials and Methods*. Tumour sites are indicated by arrows.

Figure 5. ^{131}I-VIP scintigraphy at 4 h postinjection in nude rats with SHSY-5Y neuroblastoma xenografts treated for 4 days with octreotide (upper left, 7.5 μg×2, s.c.), VIP (upper right, 40 μg×2, s.c.) and 13-*cis*-retinoic acid (lower left, 4 mg×2, p.o.) and an untreated rat (lower right). Tumour sites are indicated by arrows.

useful model to investigate the *in vivo* effects of a somatostatin analogue in neuroblastoma treatment. However, the detection of high-affinity somatostatin binding sites in the xenografts (Figure 3) was somewhat unexpected since Maggi and associates [8] reported SH-SY5Y cells to only bind SOM-14 with low affinity. We selected to analyse VIP receptors and compare the effects of octreotide to VIP and retinoic acid since the SH-SY5Y cell line has been reported to have an autocrine expression of VIP and VIP-R and also to respond to retinoic acid with upregulation of VIP-R [23, 24]. From our results in a limited series of *in vivo* experiments, it seems that octreotide was more effective than 13-*cis*-retinoic acid in upregulating the somatostatin and VIP binding sites (Figures 4 and 5). Previous data have indicated that VIP receptor expression in neuroblastoma is regulated by VIP in an autocrine manner [25, 26]. However, we are not aware of studies showing this effect from somatostatin on SOM or VIP receptors *in vitro* or *in vivo*. Downregulation of SOM binding in neuroblastoma tumour samples was observed in 5 children during disease progression [7]. VIP synthesis is increased in neuroblastoma cells by differentiation induced by retinoic acid as well as by VIP [26, 27]. We only found low concentrations of VIP immunoreactivity in the untreated xenografts (data not shown), and the effect of treatment has not yet been analysed in this regard. In conclusion, results obtained from clinical tumour samples support a correlation of somatostatin with neuroblastoma tumour differentiation and favourable prognosis. Using a xenograft model, treatment with the somatostatin analogue octreotide indicated the usefulness of this therapeutic option in human neuroblastoma. Somatostatin receptor scintigraphy seems to be useful not only to detect tumour tissue for diagnosis and staging, but also for the future selection of patients for specific targeted therapy using the growth inhibitory effects of somatostatin in neuroblastoma.

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