Neurokinin-immunoreactivity in human neuroblastomas

Evidence for selective expression of the preprotachykinin (PPT) II gene

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Factors regulating differentiation of the peripheral nervous system (PNS) have been widely studied in neuroblastomas which are tumors of the PNS. Five neuroblastomas were investigated for their content of tachykinin neuropeptides, which arise from two distinct genes which appear differentially expressed in the PNS. Radioimmunoassay and column chromatography revealed large amounts of neurokinin B in three of these tumors and the absence of substance P, neurokinin A, neuropeptide K and neuropeptide γ from all five tumors. This suggests that neuroblastomas can selectively express the preprotachykinin (PPT) II gene and that they may be valuable for investigating the factors involved in the regulation of these two structurally-related neuropeptide genes.

Neuroblastoma; Tachykinin; Neurokinin B; Human

1. INTRODUCTION

The mammalian tachykinin family of neuropeptides are derived from two distinct genes. Four tachykinin peptides, substance P, neurokinin A and the neurokinin A-derived, neuropeptide K and neuropeptide γ, arise from the preprotachykinin (PPT) I gene [1-3] whereas neurokinin B is the only tachykinin deriving from the PPT II gene [4,5]. Substance P has been the most extensively studied of these peptides and has a widespread distribution in the central and peripheral nervous systems (CNS and PNS) [6]. This undecapeptide is an invariable product of PPT I gene expression. However, PPT I gene expression also involves cell-specific differential post-transcriptional (alternative mRNA splicing) and post-translational (alternative endoproteolysis) processing [7], which affect the tissue concentrations of neurokinin A and its amino-terminally extended derivatives neuropeptides K and γ, relative to those of substance P [7-9]. Similar heterogeneity in the expression of the PPT II gene is not evident. Neurokinin B appears to have a distinctly different distribution to that of substance P and neurokinin A. In the CNS in situ hybridisation studies of PPT I and PPT II mRNAs indicate that, besides at least one example of colocalisation of these mRNAs [10], they are mostly expressed in quite separate sets of neurons [11]. Also this differential expression of PPT I and II genes would seem to occur in the PNS. Thus substance P and neurokinin A are found in many peripheral organs, predominantly within nerve terminals of C-fibre afferent neurons [12,13] whose cell bodies are in the cranial and dorsal root sensory ganglia and contain large amounts of PPT I mRNA [11,14] but no PPT II mRNA [11].

Peripheral sympathetic neurons of the rat have also been shown to express the PPT I gene and have been the focus of studies by Black and coworkers, investigating the regulatory factors which influence PPT I gene expression [15,16]. There is no evidence that the neurons express the PPT II gene. Neuroblastomas are neoplasms of the peripheral nervous system which appear to be derived from progenitive neuroblasts of sympathetic neurons. They are of potential value in investigations of the mechanisms of peripheral neuronal development and differentiation [17]. The aim of this study was to characterise which tachykinins are present in these tumors. Radioimmunoassays for the tachykinins have been combined with analytical column chromatographic methods including reverse-phase (rp) HPLC capable of resolving the different mammalian tachykinins.

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Abbreviations: PNS, peripheral nervous system; CNS, central nervous system; PPT, preprotachykinin; NKA-IR, neurokinin A-immunoreactivity; SP-IR, substance P-IR; rpHPLC, reverse-phase high-pressure liquid chromatography; K_a, affinity constant; ID_{50} concentration giving 50% inhibition

2. MATERIALS AND METHODS

2.1. *Materials*

Peptides were obtained from either Peninsula Labs, St. Helens, UK

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or Bachem Biochemica GmbH, Heidelberg, Germany. 'Iodogen' reagent (1,3,4,6-tetrachloro-3α,6α-diphenyl glycouril) was obtained from Pierce, Heidelberg, Germany. rpHPLC cartridges and solvents were obtained from Merck, Darmstadt, Germany. Sepak C_{18} cartridges were obtained from Waters Associates, Milford, USA. Sephadex G_{50} (fine) for gel permeation chromatography was obtained from Pharmacia Uppsala, Sweden.

2.2. *Tissues and extraction method*

Specimens from 5 freshly diagnosed abdominal neuroblastomas were obtained during surgery and kept frozen at −80°C until investigation. Diagnosis was based on clinical signs, elevated circulating catecholamines and their metabolites as well as on histopathology. For comparison with normal peripheral tissues, human specimens of duodenum and colon removed at surgery for different reasons and taken for the purpose of routine histology and found to be normal, were used. The peptide contents of the tumor and intestinal tissues were extracted in boiling 0.5 M acetic acid [18]. 20 ml of each of the extract solutions were partially purified by adsorption onto a Sepak cartridge of octadecylsilica as previously described in detail [19]. The eluted fractions were evaporated in a vacuum centrifuge (Speed Vac) to between 100 and 200 μl. Complete drying of samples was avoided to maximise recovery of peptide. Samples were then diluted to 2 ml with 0.1% TFA and then stored at −80°C until assay and chromatographic analysis.

2.3. *Radioimmunoassays (RIAs)*

The substance P RIA was that previously described [18] except that the radiolabelled tracer was prepared similarly as described below for the neurokinin RIA. The RIA employs the rabbit antiserum SP3 which exhibits less than 0.1% crossreaction with neurokinins A and B and neuropeptide K and neuropeptide γ. The neurokinin RIA employed a rabbit antiserum raised to neurokinin A coupled by glutaraldehyde to bovine serum albumin (BSA). The antiserum $\alpha\beta$4 was selected because of its high affinity ($K_a = 2.4 \pm 0.3 \times 10^{11}$ 1/mol) and its significant crossreaction with neurokinin B, neuropeptides K and γ and not with substance P (see Results). The tracer was radiolabelled ^{125}I-tyr-0-NKA prepared using the Iodogen reagent, followed by purification on rpHPLC.

2.4. *rpHPLC*

For rpHPLC analysis, 1 ml of the partially-purified tissue extracts was diluted in 1 ml 50 mM potassium phosphate buffer, pH 2.5 (solvent A), and centrifuged (10 000 × *g*) to remove insoluble debris. Each sample was injected onto a 125 × 4 mm LiChro CART column of C_{18} LiChrospher (100-RP-18,5 μm), preequilibrated with solvent A. After sample injection the column was eluted with a linear gradient of solvent B (acetonitrile/water (70:30) containing potassium phosphate buffer, final concentration 37 mM, pH 2.5). The exact gradient conditions used are indicated in Fig. 2. Each of the fractions was evaporated to 50–100 μl in a vacuum centrifuge, followed by addition of 1 ml RIA buffer and then assayed at dilution for tachykinin-immunoreactivity. The column was calibrated with picomole quantities of standard synthetic tachykinins and elution times were determined by RIA. In this way possible contamination of the injector, which was also thoroughly washed between runs, and column was minimised.

2.5. *Gel permeation chromatography*

2 ml of each of the partially purified tissue extracts were loaded onto a 90 × 1.6 cm column of G_{50} Sephadex (fine), eluted with 40 mM potassium phosphate buffer, pH 7.4, containing 150 mM sodium chloride, 0.2% BSA and 0.25% sodium azide. The flow rate was 30 ml/h and 4 min (2 ml) fractions were collected and assayed at dilution. The void and total volumes of the column bed were determined from the elution time of Dextran blue (Pharmacia) and NaI (containing a trace amount of $Na^{125}I$), respectively. The elution time of the different tachykinins was determined under identical conditions and was detected by RIA.

3. RESULTS AND DISCUSSION

Table I shows the sequence homology shared by neurokinin A, neurokinin B, neuropeptide K and neuropeptide γ. The latter two contain the complete neurokinin A sequence at the carboxy-terminus and are derived, respectively, from preprotachykinins β and γ which both arise from the PPT I gene by way of alternative mRNA splicing [7]. Neurokinin B shares with neurokinin A the complete carboxy-terminal amidated pentapeptide sequence. The polyclonal antibody used in this study, $\alpha\beta$4, is clearly directed to this sequence since it exhibits significant crossreaction with all these homologous peptides (Fig. 1). Taking the ID_{50} values for inhibition of binding of ^{125}I-Tyr-0-neurokinin A to polyclonal antibody, $\alpha\beta$4, for each of these four peptides and dividing them into the value obtained for neurokinin A, allows a quantitative estimate of crossreactivity. The assay shows full crossreaction with neuropeptide K (100%), reduced crossreaction with neurokinin B (30%) and, unexpectedly, the polyclonal antibody appears to have greater affinity for neuropeptide γ (180%) than for neurokinin A. The differing amino-terminal sequences of neurokinin B and neuropeptide γ must confer some conformational change in and/or steric influence on the carboxy-terminal sequence in order to affect the immunoreactivity in opposing ways.

Table II shows that high concentrations of neurokinin A-immunoreactivity (NKA-IR) were measured in 3 of the 5 neuroblastomas but that there was no detectable substance P-immunoreactivity (SP-IR). This is a novel observation in view of the widely reported parallel distribution of neurokinin A and substance P in peripheral organs [7–9]. In Table III this is illustrated by the similar concentrations of NKA-IR and SP-IR measured in extracts of normal human intestine;

Table I

Primary sequences of neurokinin A and related peptides with extent of carboxy-terminal sequence homology outlined

neurokinin A	HKTDSFVGLM-NH_2
neuropeptide K	DADSSIEKQVALLKALYGHGQISHKR HKTDSFVGLM-NH_2
neuropeptide γ	DAGHGQISHKR HKTDSFVGLM-NH_2
neurokinin B	DMHDF FVGLM-NH_2

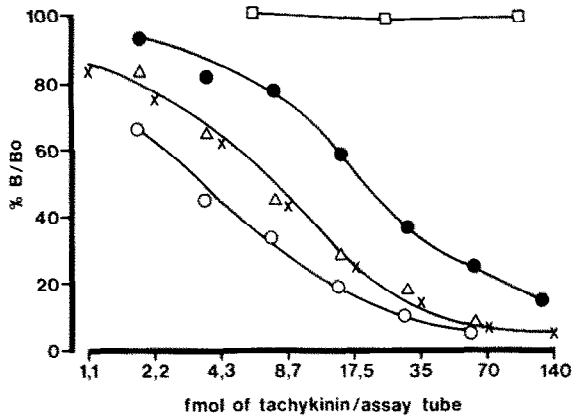

Fig. 1. Displacement of binding (% B/B_0) of ^{125}I-Tyr-0-neurokinin A to polyclonal antibody $\alpha\beta 4$ by increasing concentrations of neurokinin A (×), neurokinin B (●), neuropeptide K (△), neuropeptide γ (○) and substance P (□).

rpHPLC, under optimised conditions, could resolve the various tachykinins (Fig. 2). The NKA-IR of each of the three NKA-IR positive tumor extracts elutes as one major peak coeluting with the neurokinin B standard. The profile for two of the tumors is shown in Fig. 2. Treatment of synthetic neurokinin B, which contains two methionine residues, with hydrogen peroxide under conditions known to convert methionine-containing peptides to their sulphoxide form [20] was found to generate two earlier eluting peaks (arrows 4 and 5 in Fig. 2). The neuroblastoma NKA-IR presented on rpHPLC a significant peak of immunoreactivity with the elution time of one of these presumed oxidised forms of neurokinin B. None of the neurobolastoma NKA-IR eluted in the position of neurokinin A, neuropeptide K or neuropeptide γ. In contrast, the NKA-IR of an extract of human colon appears more heterogeneous on rpHPLC and peaks of immunoreactivity were resolved with elution times corresponding to the various tachykinin standards and some of their oxidised forms. A similar profile was obtained for an extract of human duodenum (data not shown). In these human intestinal extracts the major peak of immunoreactivity corresponds to neurokinin A.

Table II

Concentrations (pmol/g wet weight) of neurokinin A (NKA)- and substance P (SP)-like immunoreactivities in individual samples of human neuroblastoma and intestine

	NKA	SP
Neuroblastoma		
1	32	<0.5
2	400	<0.5
3	<0.5	<0.5
4	160	<0.5
5	<0.5	<0.5
Duodenum	43	39
Colon	38	41

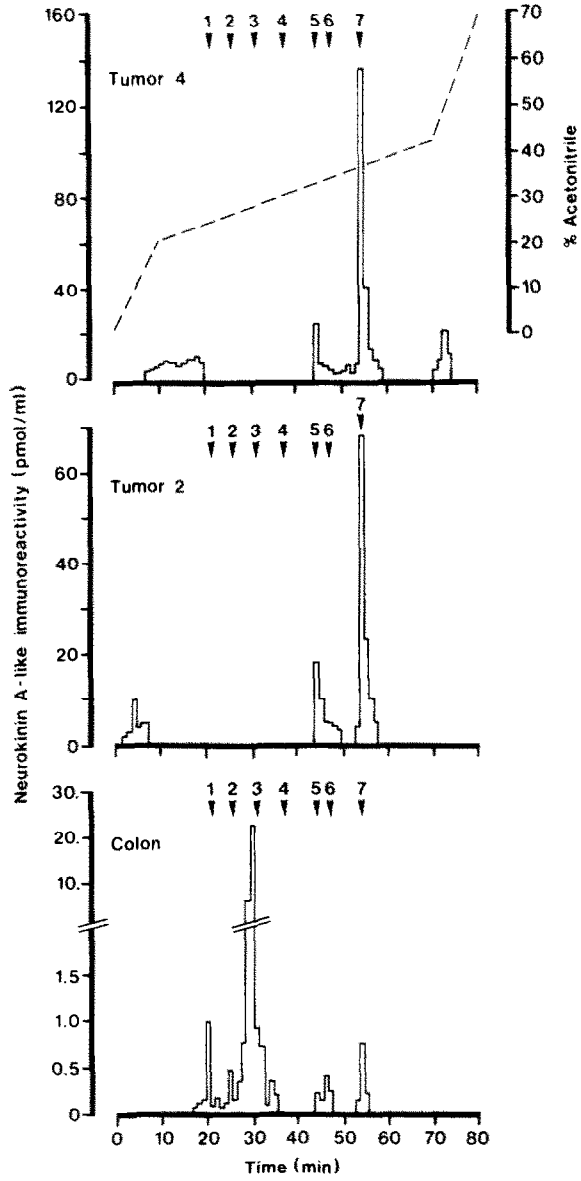

Fig. 2. Profiles of NKA-IR of extracts of two neuroblastoma tumors (2 and 4) and one human colon, fractionated on an rpHPLC column. The column was eluted with a linear gradient of increasing concentration of acetonitrile in the eluting solvent. Arrows indicate elution-times of oxidised neurokinin A (1), neuropeptide γ (2), neurokinin A (3), oxidised neurokinin B (4 + 5), neuropeptide K (6) and neurokinin B (7).

The identity of the neuroblastoma NKA-IR as neurokinin B was further supported by the gel permeation analysis. Neurokinin B, despite its very similar molecular weight to neurokinin A, elutes from the G_{50} Sephadex column with a peak elution volume significantly greater than that of neurokinin A (Fig. 3). This probably results from the peptide's particularly hydrophobic properties, resulting in some adsorption to the Sephadex which retards elution. The neuroblastoma NKA-IR elutes with a peak elution volume corresponding to that of the neurokinin B standard. For com-

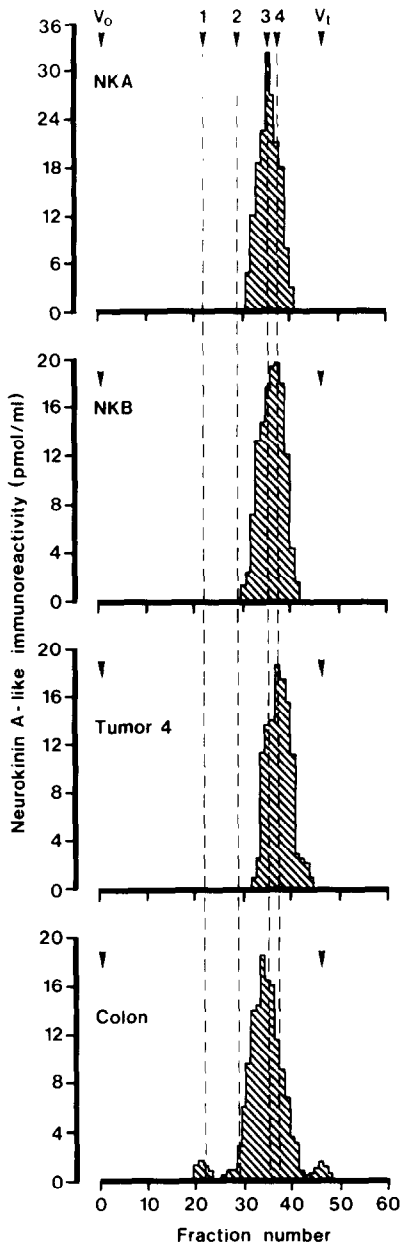

Fig. 3. Profile of NKA-IR fractionated on a G_{50} Sephadex of synthetic neurokinin A and B standards and of extracts of one neuroblastoma tumor (number 4) and a human colon. Arrows indicate calibrated elution positions of void (V_o) and total (V_t) volumes, synthetic neuropeptide K (1), neuropeptide γ (2), neurokinin A (3) and neurokinin B (4).

parison, in Fig. 3 is shown the elution profile of NKA-IR of a human colonic extract, which reveals one major peak of immunoreactivity with a peak elution volume corresponding to that of NKA. A discrete peak of NKA-IR was also found in the elution position corresponding to neuropeptide K. The relatively low quantities of neuropeptide γ and neurokinin B compared to neurokinin A which were resolved on rpHPLC of the identical extract are probably obscured on elution from the G_{50} Sephadex column by the large neurokinin A component.

Taking into account the reduced crossreaction of neurokinin B in the RIA employed here, significant amounts of the peptide appear to be present in the human intestine, albeit at about 10% of the concentration of neurokinin A. This is an important observation in light of a recent report, using a neurokinin B-specific RIA demonstrating the absence of the neuropeptide from rodent intestine [21]. Neurokinin B has been postulated as the endogenous ligand of the pharmacologically-defined neurokinin-3 receptor which appears to mediate tachykinin effects on mammalian enteric neurons [22].

Whether neurokinin B in humans is produced by mature sympathetic neurons needs to be determined. Perhaps neurokinin B in neuroblastomas represents a transient expression of the PPT II gene during differentiation of sympathetic neurons or it is being produced ectopically. In the ontogenetically related adrenal medulla, PPT II mRNA has been detected in the ox [4]. Also in one human phaeochromocytoma, a tumor of the adrenal medulla, neurokinin B was detected using a neurokinin B-specific RIA, but at concentrations much lower than the neuroblastoma concentrations reported here [23]. In this same study, Kage and Conlon detected neurokinin B in only one of a series of 10 phaeochromocytomas and, interestingly, it was also the only one of the series in which products of the PPT I gene were detected. However, there was no histochemical assessment of whether cellular co-localisation of PPT I and II gene products occurs in this tumor.

Evidence has been obtained here for the production by some neuroblastomas of neurokinin B but neither neurokinin A nor substance P which strongly suggests that the PPT II gene and not the PPT I gene is being selectively expressed. In normal tissues these genes appear often to be seperately expressed which indicates that they are regulated by different control mechanisms. Similarly in carcinoid tumors, which are also neuroendocrine neoplasms, substance P and neurokinin A and its derivatives are frequently produced but not neurokinin B [20]. Perhaps further studies comparing neuroblastoma and carcinoid tumor cells may reveal the factors which are involved in differential expression of the tachykinin genes. Furthermore neuroblastoma tissue could be a valuable source of PPT II mRNA and so facilitate cloning of cDNA and elucidation of the structure of the human neurokinin B precursor protein and also of the gene. So far only the rat and bovine neurokinin B precursor cDNAs and the corresponding bovine gene have been cloned and sequenced. The neuroblastomas investigated here did not differ with respect to their histological grading. The high expression of neurokinin B in 3 of the 5, however, suggests that differences at the molecular level, which may become important for diagnosis and subclassification of the disease, do in fact exist.

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