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Tachykinin Production by Carcinoid Tumours in Culture

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Abstract—*Tissue specimens from 5 patients with metastatic midgut carcinoid tumours were kept in organ culture for up to 6 months. The tumour cells were confined to the suspension in the form of condensed cell clusters and appeared to retain their endocrine characteristics. Radioimmunoassay for tachykinin immunoreactivity showed high concentrations in 4 out of 5 culture media. The concentrations were highest in the beginning of the experiment, but subsequently decreased. The 4 patients from which these tumours were taken had all elevated tachykinin concentrations in extracted plasma. The fifth culture medium had low tachykinin concentration, and the concentration in extracted plasma from this patient was within the normal range. Reversed-phase high-performance liquid chromatography of the culture media with elevated tachykinin concentrations revealed immunoreactive components with the characteristics of synthetic neuropeptide K, neurokinin A and eledoisin, components also found in plasma and tumour tissues of carcinoid patients. Our findings indicate that carcinoid tumour cells produce tachykinins. These peptides are biologically very active, resulting in flush and hypotension when infused intravenously into normals, and might contribute to the clinical symptoms of the carcinoid syndrome.*

INTRODUCTION

SMALL intestinal carcinoids (mid-gut carcinoids) are the most frequently occurring malignant neuroendocrine tumours of the gastrointestinal tract. Carcinoid tumours may contain and secrete several biologically active substances into the circulation, i.e. amines such as serotonin and peptides such as substance P [1, 2]. The pathophysiological effects of the tumour secretory products are particularly evident in the presence of liver metastases, where the patients present symptoms of flush, diarrhoea, asthma and tricuspidal valve insufficiency.

Substance P (SP) belongs to the tachykinin family of peptides. The tachykinins share a common C-terminal amino acid sequence and have similar biological effects. Thus both SP and the tachykinin eledoisin have been found to induce flushing by i.v. infusion in normals [3]. Until recently, substance P was regarded as the only tachykinin present in vertebrates. However, it has now been shown that vertebrate tissues may contain an entire family of tachykinins including neurokinin A (NKA), neurokinin B (NKB) and neuropeptide K (NPK) [4, 5]. In earlier studies we have found elevated concentrations of tachykinin-like immunoreactivity (including NKA, NKB, NPK and an eledoisin-like peptide) in plasma and tumour extracts from patients with carcinoid tumours [6, 7]. Thus, it seemed of interest to study the secretion of tachykinins from carcinoid tumour cells in organ culture.

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MATERIAL AND METHODS

The study was approved by the local ethical committee on human experimentation at the University Hospital in Uppsala. Tissue specimens from 5 patients with metastatic carcinoid tumours were studied. Four of the patients had the primary tumour located to the ileum, but in 1 patient the origin of the primary tumour was unknown. All patients suffered from cutaneous flushing, 3 of them had diarrhoea and all had elevated levels of urinary 5-hydroxyindole acetic acid (5-HIAA) [8] (Table 1).

Fresh tissue specimens were obtained at operation in 1 patient, and in the other 4 by ultrasonically-guided needle (dia. 2.1 mm) biopsies by a previously described technique [9]. The tumour

Table 1. Primary tumour localization, concentrations of tumour markers (TKLI(K12) in plasma and 5-HIAA in urine), and clinical symptoms in 5 patients with classical carcinoid tumours

Patient No.	Primary tumour	Flush	Diarrhoea	Urinary 5 HIAA	Plasma TKLI(K12)	Culture length
1	ileum	+	+	126	< 6	1.5 months
2	ileum	+	−	585	727	5 months
3	ileum	+	+	800	322	6 months
4	ileum	+	+	1675	290	4.5 months
5	unknown	+	−	290	48	5 months

The culture period is the length of time the tumour cells have been kept alive in RPMI-1640 culture media. Ref. values: 5-HIAA < 80 μmol/24 hr, TKLI(K12) < 12 pmol/l.

material was used for tissue culture and histological and immunocytochemical analyses.

Histopathology

The small carcinoid tumour pieces were fixed in 10% neutral formalin, dehydrated through a series of graded alcohols and paraffin embedded. About 4 μm thin sections were cut, deparaffinized and stained with haematoxylin–eosin, the argentaffin reaction of Masson [10], the argyrophil reaction of Sevier–Munger [11] and the argyrophil reaction of Grimelius [12]. The tumour sections were also used for immunocytochemistry according to the peroxidase–antiperoxidase (PAP) method of Sternberger [13], with a monoclonal antibody against serotonin (Mas 055 clone YC5/45 HLK, Seralab Limited, Crowley Down, Sussex, U.K.) [14] and with antiserum against neuronspecific enolase (NSE) [15].

Tissue culture

The fresh tumour material was immediately transferred into RPMI-1640 medium [16]. The specimens were mechanically disintegrated into small fragments and if necessary, trypsinated (0.25%) for 0.5–1 hr. The minced tumour was then put into Ehrlenmeyer (E) flasks containing a bottom layer of contact inhibited glial feeder cells [17] and RPMI-1640 medium with 10% foetal calf serum supplemented with penicillin (100 IU/ml) and streptomycin (50 μg/ml). The primary cultures were incubated at normal atmospheric pressure in humidified air, 37°C, containing 5% CO_2. The cultures were observed routinely and half of the medium was renewed twice a week. Supernatants from the media were stored at −70°C until assayed for tachykinin immunoreactivities.

Electron microscopy

Small cell clusters in organ culture were fixed in 2% glutaraldehyde in 0.1 M sucrose and 0.1 M Na-cacodylate buffer for 1 hr, embedded in agar 100 and cut into ultrathin sections on an LKB 5 ultratome. The sections were contrasted with uranyl acetate and lead citrate and examined in Philips 205 electron microscope operated at 60 kV.

Radioimmunoassay

The radioimmunoassay for tachykinin immunoreactivity was based on antiserum K12 which was raised against a kassinin conjugate but has higher affinity for neurokinin A (NKA) than for kassinin (KAS) [5]. NKA was labelled to a specific activity of 69 Bq/fmol according to Bolton and Hunter [18] and used as radioligand. NKA was used as standard since kassinin does not seem to be present in mammals [5, 6]. The IC_{50} value of antiserum K12 was 21.8 fmol of NKA, and the detection limit for NKA was 0.6 fmol/tube. Using the crossreactivity to NKA as the 100% reference, the crossreactivity of antiserum K12 to other tachykinins was: KAS 84%, eledoisin (ELE) 30%, NKB 26% and NPK 61%. The crossreactivity for SP and bombesin was less than 0.01%. Immunoreactivity as measured with antiserum K12 is called TKLI(K12).

Preparation of the supernatants from culture medium

Before radioimmunoassay, the supernatants from the culture media and the plasma samples from the patients, were extracted with acid ethanol. One ml of acid ethanol (0.15% hydrocloric acid in absolute ethanol) was added to 0.5 ml plasma or supernatant. After vortexing and centrifugation, the supernatants in these tubes were evaporated to dryness under nitrogen gas at 45° C and dissolved in the assay buffer before assay.

Before reversed phase high performance liquid chromatography (rpHPLC), 16.4 ml of culture supernatants were extracted using a Sep Pac® cartridge as described by Ping Wu *et al.* [19].

High performance liquid chromatography

A nucleosil®, C18, 5 μm 4.6 × 300 mm column (Merck) was used for rpHPLC. It was eluted (1 ml/min) with a 40 min linear gradient of 20% eluent B to 40% eluent B. Eluent A consisted of 0.1% trifluoroacetic acid (TFA) in double distilled, ultrafiltrated water, and eluent B of 0.1% TFA in

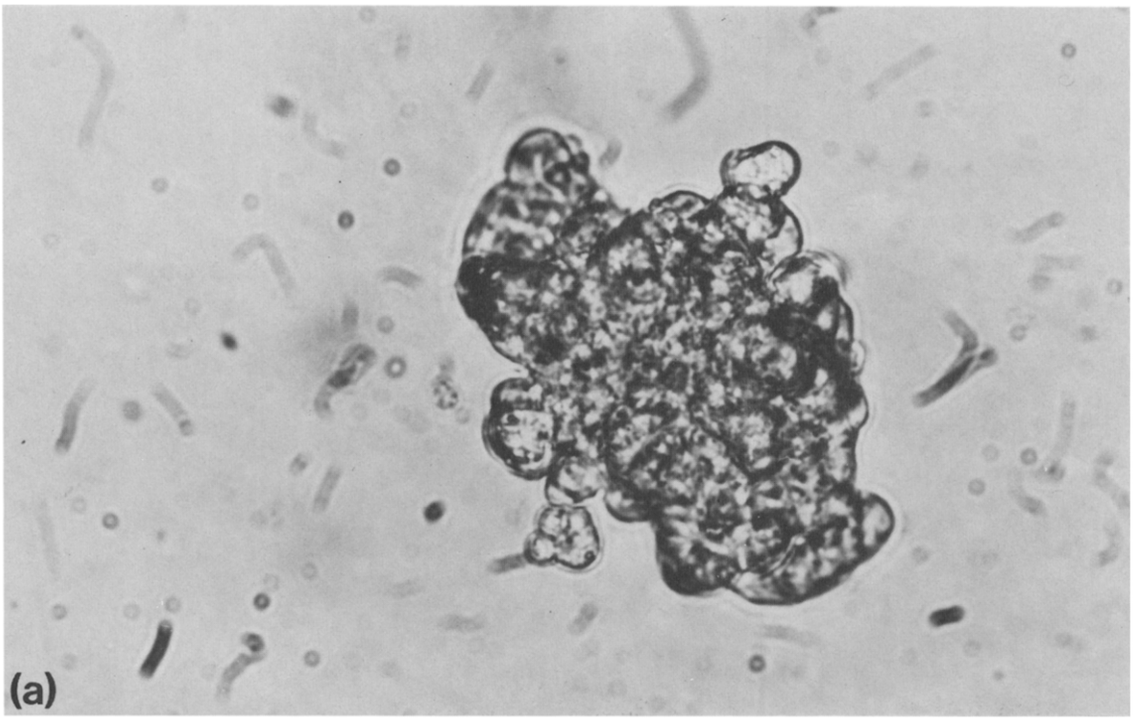

Fig. 1(*a*). *Possible proliferating clusters of carcinoid tumour cells in suspension.* × 637.

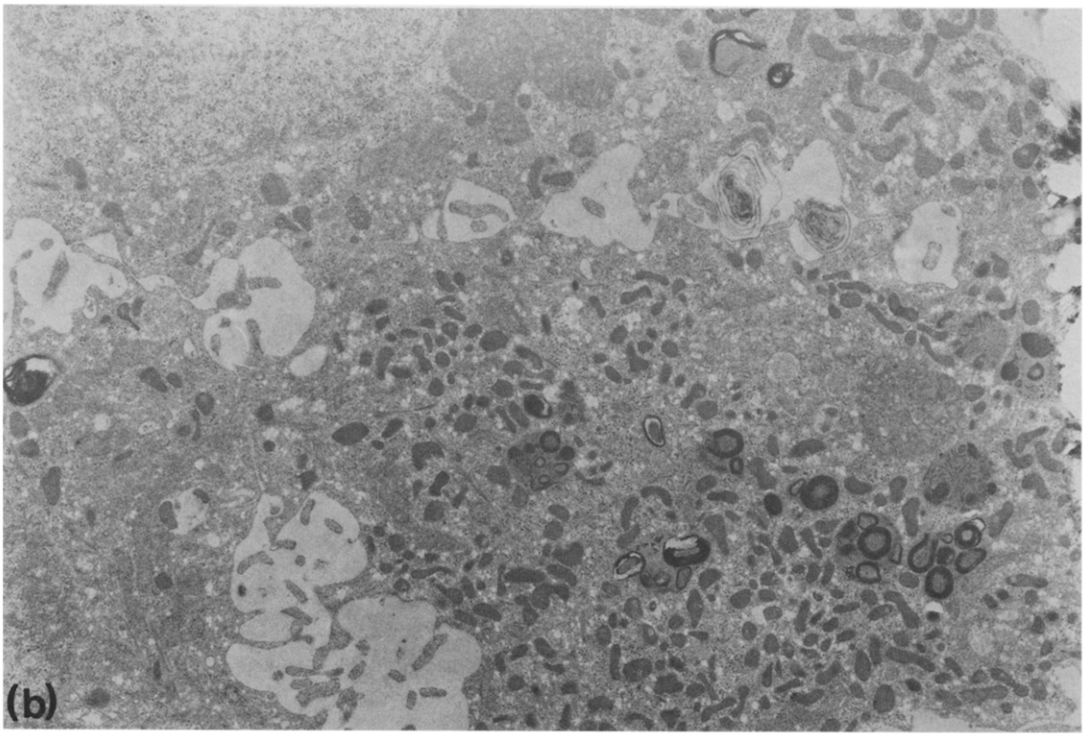

Fig. 1(*b*). *Electron micrograph of tumour cells from case No.* 4 *after* 2 *months in culture. The tumour cells display an abundance of heteromorphic granules of neurohormonal type.* × 5700.

acetonitrile (Rathburn Chemicals, HPLC grade S). Two Milton Roy Constamatic III HPLC pumps were used, controlled by Sinclair Spectrum/Bercol I microcomputer. Extracted supernatants from organ cultures were passed through Millipore GS filters (0.22 μm) to remove particulate matter before chromatography. Samples of 100 μl were injected using a Rheodyne injector (715S). Fractions (0.5 ml) were collected, lyophilized, redissolved in 100 μl of distilled water and analysed in the tubes used for collection of fractions. The HPLC column was calibrated in separate runs with small amounts (500–800 fmol) of synthetic tachykinins.

RESULTS

Histopathology

All the tumour specimens displayed light-microscopically the morphology of carcinoid tumours, with regular tumour cells growing in an insular pattern. The argyrophil reaction and/or NSE immunohistochemistry disclosed a positive reaction in all cases, further emphasizing the neuro-endocrine origin of the tumours. The carcinoid tumours seemed all to be of intestinal origin, all containing serotonin as identified by an argentaffin reaction and by serotonin immunoreactivity.

Tissue culture

All tumours were kept as primary tissue cultures. The tumour cells were confined to the suspension in the form of condensed cell clusters from which small bats of possibly proliferating tumour cells emerged (Fig. 1a). However, any obvious increase in the number or size of the tumour cell aggregate was not seen during the culture period. Proliferation in monolayer or established cell lines was not obtained. After 2 months in culture, some tumour aggregates were collected for light and electron microscopic examination (Case No. 4). At the light microscopical level, the tumour clusters were argentaffin and argyrophil. Well-preserved tumour cells, with an abundance of cytoplasmic pleomorphic granula of neurohormonal type, were seen, using the electron microscope. Several myelin bodies were also present (Fig. 1b). After up to 6 months in culture, the tumour clumps deteriorated and disappeared.

Tachykinin-like immunoreactivity in culture supernatants

The 5 carcinoid patients had TKLI(K12) levels in extracted plasma as follows: < 6, 48, 290, 322 and 727 pmol/l (ref. value < 12 pmol/l) (Table 1). The TKLI(K12) concentration in the RPMI-1640 culture medium was 20 pmol/l. The mean initial concentration of the extracted supernatants was

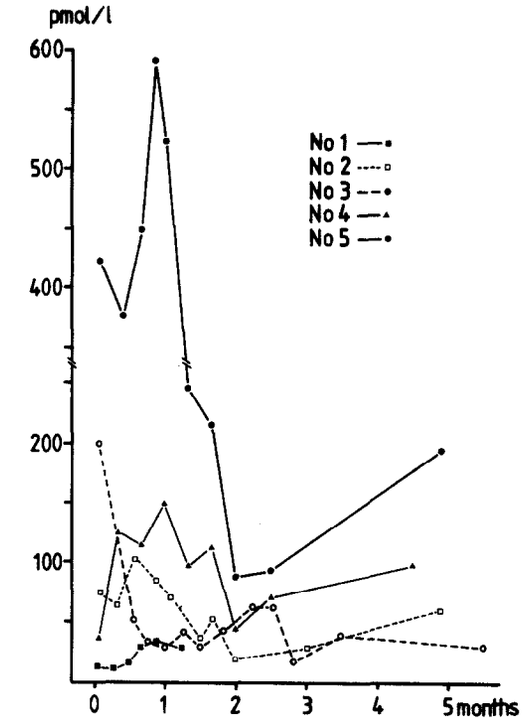

Fig. 2. TKLI(K12) concentration (pmol/l) *in extracted supernatants from culture media containing carcinoid cells, as a function of time.*

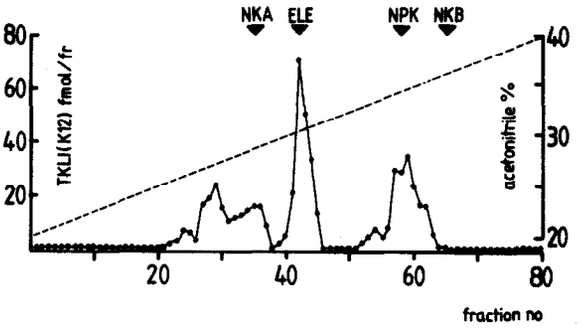

Fig. 3. Reversed phase high performance liquid chromatography of supernatants from culture media of carcinoid cells. The antiserum used was K12. Elution conditions are given in the text.

165 pmol/l (range 14–411 pmol/l) (Fig. 2). With time the TKLI(K12) concentrations decreased, and in 2 cases (after 1 and 1.5 months) the levels reached the RPMI-1640 culture medium reference level.

RpHPLC of supernatants from carcinoid tumour cell cultures are shown in Fig. 3. A peak of immunoreactivity eluted in the position of synthetic ELE, and peaks were also seen in the position of synthetic NPK and NKA.

DISCUSSION

Carcinoid cells in culture have been found to produce multiple hormones such as serotonin, histamine, insulin, gastrin and thyrocalcitonin [20, 21]. In the present investigation, evidence is presented for the productions of tachykinins by carcinoid tumour cells in culture. Tumour cells from

5 midgut carcinoid tumours were held in culture in the form of condensed cell clusters for up to 6 months. Tumour aggregates from one of the patients were collected for light and electronic microscopic examination after 2 months in culture. The cells were argentaffin and argyrophil, and cytoplasmic granula of neurohormonal type were seen on electron microscopy, indicating still viable cells with the capacity of hormone production. Radioimmunoassay of supernatants from the culture media showed increased concentrations of tachykinin-like material in 4 out of 5 tumour cultures. The tumour cells in the culture medium with undetectable TKLI(K12) concentrations came from a patient presenting normal TKLI(K12) levels in extracted plasma in spite of large liver metastases. This might indicate a relationship between production of tachykinins in the tumour cells and the tachykinin concentrations found in plasma. The TKLI(K12) levels in all the media decreased over time which may be due to a gradual degradation of the tumour cells as none of the cultures became established cell lines. The increasing peptide levels seen in 3 cultures just before exit might be due to a final cell lysis and release of stored peptides to the medium.

RpHPLC of the supernatants showed immunoreactive components corresponding to NPK, NKA and ELE. This supports our earlier findings of multiple immunoreactive tachykinins in tumour extracts and plasma from carcinoid patients [6, 7]. Tachykinins which are biologically very active substances increase in plasma from carcinoid patients during flush [22] and might be a result of tachykinin secretion from the carcinoid tumour cells. However, *in vitro* stimulatory tests have to be performed to elucidate the precise mechanisms of tachykinin release.

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