Expression of Gastrointestinal Endocrine Tumours in Culture Systems*

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Summary. Human endocrine tumours were studied in in vitro systems (cell suspensions and tissue cultures) and in in vivo systems (tumour transplants to the anterior eye-chamber of immunosuppressed rats). In the experimental systems the tumour cells were demonstrated to synthesize and secrete the same hormonal products as in the patient. Intraocular transplants of a gastrinoma secreted gastrin-17 into the chamber fluid. This molecule, normally not secreted in the rat, was also detected in the peripheral plasma of tumour-bearing rats. Intraocular transplants of midgut carcinoid tumours released serotonin (5-HT) at adrenoceptor stimulation, of a similar type as demonstrated in acute tumour cell suspensions. However, in tissue cultures genuine β-adrenoceptors seemed to be modified, since pretreatment with β-adrenoceptor antagonists or calcium deprivation did not prevent stimulated 5-HT release. Tachykinins were not liberated by adrenoceptor stimulation.

In certain cultures of midgut carcinoid tumour cells, two different phenotypes developed: small rounded endocrine tumour cells with positive immunoreactions against 5-HT and tachykinins (TK), and large elongated neuron-like cells, which gradually lost 5-HT immunoreactivity, while TK immunoreactivity remained unchanged. These cultured tumour cells may produce an endogenous factor inducing transformation into a neuron-like phenotype. One candidate factor is nerve growth factor (NGF), since NGF-like immunoreactivity was demonstrated in cells of the endocrine phenotype.

The present paper describes some results obtained in experiments on human endocrine tumours, using material from patients undergoing curative or palliative surgery. Most studies have been carried out on carcinoid tumours of midgut origin, but one gastrinoma has also been investigated. The results indicate that endocrine tumours can be studied either in in vitro systems, i.e. in cell suspensions (NILSSON et al., 1985, 1986a; GRÖNSTAD et al., 1987), and in tissue culture (AHLMAN et al., 1988) or in in vivo systems, i.e., tumour transplants to the anterior eye-chamber of immunosuppressed rats (NILSSON et al., 1986a, b). In these experimental situations, we have been able to demonstrate that the tumour retains most of its endocrine characteristics and can be made to synthesize and secrete the same hormonal products as in the patient. This implies that endocrine tumours may be investigated ex corpore using these three experimental systems, thus supplying additional information of clinical importance. We have also noticed, in certain carcinoid tumours in culture, that the endocrine cells may be transformed into a neuron-like phenotype, a most intriguing observation.

METHODOLOGY

Tumour material was obtained from lymph node metastases at surgery. The tumour pieces were immediately placed in sterile, ice-cold, oxygenated Krebs’ solution and were rapidly transferred to the laboratory, where the tissue was further processed and material was prepared for various experiments.

Immunocytochemistry

Small pieces of tumour tissue were fixed in 4% paraformaldehyde (pH 7.4) for 4 h, rinsed in PBS/
sucrose (5–10%), frozen and cryostat sectioned at 10–12 μm. The sections were placed on gelatin-coated glass slides and processed for immuno-cytochemistry using the indirect technique as described earlier (cf. NILSSON et al., 1986b). Specific antisera against serotonin (5-HT, from HWM Steinbusch, code SER 7-7, Amsterdam), gastrin/cholecystokinin (CCK, code RPN 1592, Amersham Internat. UK), various tachykinins [including neuropeptide K (NPK) and substance P (SP) code SK12], and against the neuronal markers neurofilament (NF, i.e., the 3 subunits) and neuron specific enolase (NSE), (from K. HAGLID, Göteborg, Sweden) and the transmembrane synaptic vesicle component SV2 (mouse monoclonal 10H, from R. KELLY, San Francisco, USA) were employed as indicated below. For fluorescence microscopy, a Nikon Optiphot with incident light was used. As control for the specificity of observed immunofluorescence, sections were incubated with normal serum instead of the primary antiserum, or the primary antiserum was omitted. Such control sections were negative.

**Tumour cell suspensions**

The tumour tissue was carefully cleaned from non-tumour tissue, minced and treated with a 2% collagenase solution with 0.04% DNA-ase per 10 ml solution added. After stirring for 60 min at room temperature under continuous oxygenation, the cells were filtered through sterile gauze, centrifuged and washed three times in Krebs' solution to remove collagenase. Cell viability in the final solution was between 85 and 90%, estimated by the dye exclusion test (cf. GRONSTAD et al., 1987). Tumour cell suspensions (1×10⁶ cells per ml) were incubated in Krebs' solution with various receptor agonists and antagonists added in an attempt to trigger (or block) the release of cell products (cf. GRÖNSTAD et al., 1987). Following incubation with the pharmacological agents, 200 μl samples were removed from the incubated suspensions and rapidly centrifuged (Airfuge®, Beckman Instruments, USA) for 20 sec to remove cells. The supernatants were kept frozen (−20°C) until assay of 5-HT and peptides.

**In oculo transplants**

Under ether anaesthesia, small pieces of tumour tissue were transplanted into the anterior eye-chamber (cf. OLSON, 1970; NILSSON et al., 1984, 1985) of Sprague-Dawley rats (180–200 g), immunosuppressed during the period of study by daily injections of cyclosporin A (20 mg/kg s.c., Sandoz AG, Switzerland). Generally bilateral transplantations were carried out. Control animals were either sham-operated without inoculation of the tissue, or transplanted with normal tissue of similar size as the tumour tissue. Animals were allowed free access to food and water and kept in rooms with a 12 h dark/light schedule. The transplants were inspected daily. Samples from the anterior chamber fluid were removed, under ether anaesthesia, for assay of the products released from the tumour transplants with at least 24 h intervals.

**Tissue cultures**

Sterile pieces of tumour metastases removed at surgery were minced into 1–2 mm pieces and treated with collagenase and DNA-ase as described above. The tumour cells in the final suspension were seeded onto a matrix of either collagen or Matrigel™ (Collaborative Res. Inc., USA) in plastic tissue culture dishes with 1 ml wells. The culture medium was RPMI 1640 medium (pH 7.4) supplemented with 5% or 10% fetal calf serum, 1% glutamine and penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cultures were incubated at 37°C in a 90% humidified atmosphere of 20% O₂ and 5% CO₂. The medium was changed every 3 days. After 2–3 weeks in the primary culture, cells were harvested using Dispase (Collaborative Res. Inc, USA) for reseeding on fresh matrix. The tissue cultures were inspected daily in an inverted phase contrast microscope (Nikon Optiphot). The cultured cells were fixed in paraformaldehyde (see above) and characterized immunocytochemically using appropriate antisera.

**Stimulation tests and assay systems**

Stimulations using the adrenoceptor agonists isoprorenaline (IP, 10⁻⁶–10⁻⁴ M) or noradrenaline (NA, 10⁻⁶–10⁻⁴ M) were carried out in culture under sterile conditions without change of media prior to stimulation. Samples of 20 μl were drawn from stimulated and control wells 0, 2, 5, and 10 min after the addition of agonists (blocking agents were added 5 min prior to the agonists), 5-HT was assayed using HPLC with electrochemical detection (cf. NILSSON et al., 1986b), and tachykinin (TK)-like immunoreactivity was measured in radio-immunoassay (RIA) according to TEODORSSON-NORHEIM et al. (1985), using rabbit antiserum code SK 12. Using crossreactivity to NK A (NKA) as the 100% reference, this antiserum shows crossreactivity to the following peptides: kassinin
(84%), eleidosin (30%), NK B (26%), and NP K (61%). Since the major immunoreactive component measured in patients with carcinoid tumours is NPK, the activity observed with the antiserum SK 12 will be referred to as NPK-like immunoreactivity.

STUDIES ON A GASTRINOMA TUMOUR

Tumour specimens from a patient (female, age 17, no MEN I syndrome) were obtained at surgery. Both the pancreatic gastrinoma and its metastases were investigated.

*Immunocytochemical* studies using a rabbit anti-synthetic CCK-8, crossreacting with CCK-8, gastrin (G)-17 and G-5 (code RPN 1592 Amersham International, Plc, UK) revealed G-like immunoreactivity in the original tumour. In a transplant of the tumour to

![Fig. 1 a and b. Chromatograms showing molecular forms of gastrin in the patient and a tumour-bearing rat.](image)

![c and d. Intraocular tumour transplants and iris of a tumour bearing eye demonstrate a positive reaction with the gastrin antiserum.](image)
the rat anterior eye-chamber, small cords and nests of immunoreactive tumour cells were seen. In addition, the iris epithelium exhibited a medium to strong fluorescence of G like immunoreactivity (Fig. 1). Control sections were negative.

Gelpermeation chromatography combined with RIA of eluted fractions was employed on the tumour tissue, patient serum and urine, and on serum and chamber fluid from rats with intraocular transplants. The concentration of G like immunoreactivity in this patient was 21,400 pmol/l in the serum (reference value < 50 pmol/l) and 1,400 pmol/24 h in the urine (reference value < 9.3 pmol/24 h). In extracts of the tumour 2,447 pmol/g of G was assayed. G-34 was discovered in all compartments studied. In both serum and tumours G-17 was observed as well as a larger molecule named Component 1 (REHFELD and STADIL, 1973). In rat plasma, G-34 as well as G-17 were present in addition to some smaller molecular species. The chamber fluid from the transplant-carrying eyes contained G-17 as the major immunoreactive molecule, but traces of both smaller species and of G-34 and Component 1 were present. In control rats G-34 was the only detectable component in the plasma (Fig. 1).

The above results indicate that the tumour transplanted in oculo to the rats was hormonally active, based on immunohistochemical and gelfiltration chromatographic studies. The intraocular tumour seemed to have preferentially released G-17 into its local

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Fig. 2 a. Midgut carcinoid tumour cells after 3 w culture. b. Levels of tachykinins and 5-HT in med during a period of culture.
environment and this molecule had escaped into the systemic circulation (Fig. 1).

MODIFIED ADRENORECEPTOR RESPONSES OF CARCINOID CELLS

Carcinoid tumour cells have been shown immuno-cytochemically to contain not only 5-HT but also co-existing peptides, e.g., NPK and SP. In cell suspension experiments 5-HT has earlier been shown (NILSSON et al., 1986a; GRÖNSTAD et al., 1987) to be released upon stimulation with either α- or β-adrenergic agonists. Our impression is that tumours responding to α-adrenergic stimulation appear to have a more aggressive clinical course than tumours responding to β-adrenoceptor stimulation (unpublished observation). Cell suspensions from carcinoid tumours generally respond to adrenoceptor agonists in concentrations of $10^{-6}$ to $10^{-4}$ M in a dose-dependent pattern (NILSSON et al., 1985; AHLMAN et al., 1986; GRÖNSTAD et al., 1987; ÅHLUND et al., 1989a, b).

Adrenoceptor responses in carcinoid tumour cells may be modified in different ways:

1) When transplanted into the anterior eye-chamber of immunosuppressed rats, midgut carcinoid tumours have been demonstrated to be hormonally active in releasing not only 5-HT, but also the precursor 5-HP and the metabolite 5-HIAA, upon stimulation with conjunctivally applied adrenoceptor agonists (NILSSON et al., 1986a; ÅHLUND et al., 1989b). In most cases there was a good concordance between the receptor responses in cell suspensions and intraocular tumour transplants. However, in a few cases we have noticed that β-adrenergic responses can be altered into α-adrenoceptor responses after intraocular growth and vice versa (GRÖNSTAD et al., 1987).

2) After the introduction of treatment with a somatostatin analogue, SMS 201-995, we investigated the effect of this compound (4 µg/kg s.c. daily) on carcinoid tumour transplants. Four d after pretreatment of the rats with SMS 201-995, conjunctival IP stimulation was carried out. The release of 5-HT from the tumour transplants was decreased to about 35% of the value induced in the same transplant before treatment with SMS 201-995 (ÅHLUND et al., 1989a).

3) Carcinoid tumour cells have been kept in tissue cultures for up to 9 months with maintained immuno-cytochemical characteristics concerning contents of 5-HT-, SP- and NPK-like immunoreactivities (ÅHLUND et al., 1988; ÅHLUND et al., 1989b). The release of 5-HT and peptides from these cultured cells has been studied, both as spontaneous release into the tissue culture medium and as adrenoceptor stimulated release (ÅHLMAN et al., 1988). The results indicate that carcinoid tumour cells in long-term cultures maintain the spontaneous release of 5-HT, assayed by HPLC, into the culture media in a manner essentially unchanged over the first 2 months (ÅHLMAN et al., 1988; ÅHLUND et al., 1989b). The spontaneous release of NPK and SP, assayed by RIA, decreased to very low levels in about 2 months (Fig. 2). The release was high during the first two weeks in culture and gradually declined thereafter, despite the good viability of the cells as observed in phase contrast microscopy and as indicated by the maintained spontane-

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| Well no 15 | '' | 5575 | - | - | - |
| Well no 16 | '' | 5256 | - | - | 34550 |
Fig. 3. Transformation of endocrine tumour cells (t.c.) into neuronlike cells (a, b). During transformation t.c. loose 5-HT-like immunoreactivity (c), while TK-like immunoreactivity remained unchanged (d) NGF-like immunoreactivity present in rounded t.c., frequently attached to elongated t.c. (e, f). Elongated t.c. also express NF-like immunoreactivity (g).
ous 5-HT secretion (Fig. 2). These findings indicate different turnover rates for the two secretory products and/or different storage sites for peptides and amine in the cultured cells.

In stimulation experiments a dose dependent release of 5-HT, but not of tachykinins, was induced by the β-adrenoceptor agonist IP at 10^{-6}, 10^{-5} and 10^{-4} M, dose dependently (Table 1). Stimulation of α-adrenoceptors with NA was not effective in releasing any of the substances. Pretreatment of cultures with the β-adrenoceptor antagonist propranolol did not influence the IP-induced release of 5-HT, not even when given in equimolar concentrations. Nor did calcium deprivation of media, using EDTA, influence the IP induced 5-HT release. These observations indicate a modification of the genuine β-adrenoceptors during culture, or suggest that IP-induced 5-HT secretion under these conditions may rather be due to a receptor independent mechanism (cf. REPKE and LIEBMANN, 1987).

**EXPRESSION OF NEURON-LIKE PHENOTYPES IN CARCINOID TUMOUR CELLS**

During long-term culturing of carcinoid tumour cells, we have often noticed that typical carcinoid tumour cells gradually lose their characteristic rounded appearance, become elongated and extend processes (Fig. 3). They finally became neuron-like, extending long processes with varicose-like enlargements and growth-cones. These cells have been characterized immunohistochemically to contain immunoreactivities for TK and 5-HT-like immunoreactivities. In addition, they were clearly positive with antibodies against SV2, a synaptic vesicle membrane component. Electron-microscopically they were found to contain typical electron-dense granules and in addition neurofilaments.

In order to investigate whether the cultured cells may produce an endogenous factor, which could induce transformation into neuron-like phenotypes, we incubated carcinoid cell cultures with a commercial antibody to NGF (AB927, Chemicon Int. Inc., USA). Using this antiserum we could demonstrate that some of the small rounded tumour cells contained a strong immunoreactivity (referred to as NGF-like immunoreactivity). Such NGF-like immunoreactive cells were frequently seen to adhere to the elongated neuron-like cells, which gradually lost their expression of NGF-immunoreactivity during the extension of processes (Fig. 3). To test if the culture media contained NGF activity, such media were pooled and assayed for biological activity of NGF using the chick embryo sympathetic ganglion bioassay (carried out by Prof. T. EBENDAL, Uppsala, Sweden). In this assay, no biological activity of NGF could be detected. Cultures of carcinoid tumour cells have also been extracted for NGF and tested in a similar mode, but no activity has so far been detected despite the occurrence of many cells with NGF-like immunoreactivity. Possible explanations may be either that: 1) the amounts of NGF-immunoreactive material are too small to induce a biological effect in the assay system used; or that 2) the NGF antiserum used may cross-react with some other factor which could be responsible for the transformation of the carcinoid tumour cells into neuron-like cells. In situ-hybridization techniques will be employed to further investigate this interesting observation.

The endogenous production of growth factors by endocrine tumours has been described earlier, e.g., the production of “transforming growth factor” by cultured melanoma cells (TODARO et al., 1980; MARQUARDT and TODARO, 1982). Also, melanoma cell lines can express the NGF-receptor, when stimulated by endogenously produced NGF-like molecules (SHERWIN et al., 1979). It is of interest to recall that the earliest discovery of NGF was due to an unusually large outgrowth of adrenergic nerves induced by a chick tumour (LEVI-MONTALCINI and ANGELETTI, 1968). Considering these observations, it would not seem surprising if also carcinoid tumours expressed growth factors promoting neuronal phenotypes.

These observations of the transformation of carcinoid tumour cells into neuron-like cells are most interesting, both from the theoretical and clinical point of view. Carcinoid tumour cells originate from gut enterochromaffin cells, which may be derived from the neural crest (cf. FUJITA, 1976). They are thus closely related embryologically to nerve cells. It is well known that other cell types among the paraneurons, e.g., adrenal medullary cells, can express neuronal phenotypes when stimulated with NGF (THOENEN and BARDE, 1980). Such a transformation of carcinoid tumour cells has not been demonstrated previously. However, it is reasonable that also these tumour cells, when stimulated with an appropriate trophic factor, e.g., NGF, may undergo a similar modification as adrenal medullary cells. From the clinical point of view, it is of interest to find out if carcinoid tumours, producing neuronotrophic factors, may induce an aberrant innervation pattern in gut regions reached by the secreted factor. In fact, such a
hyperinnervated gut mucosa has been noticed in one patient (The cell culture material illustrated in Figure 3 was derived from this patient) with a large midgut carcinoid tumour mass without any other regional pathological-morphological lesions. In the future, it may be of interest to study carcinoid tumour patients also with respect to the possible co-secretion of growth factors and peptide hormones.

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REFERENCES


