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Inhibition of the Growth of Transplanted Rat Pancreatic Acinar Carcinoma with Octreotide

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The effects of octreotide on transplanted azaserine-induced pancreatic acinar tumours were investigated in the rat. When tumours became palpable, rats were treated either with octreotide (40 μg/kg per day, by infusion) or NaCl 0.9% (controls) for 14 days. Tumours were then analysed for their size, composition and somatostatin receptors. Octreotide induced a 80% reduction in tumour growth rate during the first 2 days of treatment. This rate was less marked from day 4 to day 15. The tumour weight, protein, DNA, RNA and enzyme content were reduced in parallel by 50 to 60%. A homogeneous distribution density and a high affinity of somatostatin receptors were found by receptor autoradiography and *in vitro* binding assays in tumours of both groups. These findings indicate that octreotide reduces the growth rate of the transplanted pancreatic acinar tumour and may exert its inhibitory effect directly via specific somatostatin receptors on tumour cells.
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INTRODUCTION

IT IS well recognised that pancreatic growth is affected by a number of gastrointestinal hormones and neuropeptides. Some, including the cholecystokinin family (cholecystokinin, cholecystokinin-8, caerulein), the gastrin family (pentagastrin, tetragastrin) and the bombesin family (bombesin, gastrin-releasing peptide), induce pancreatic growth [1–4]. Others, especially somatostatin and its structural analogues exert antitropic effects on the exocrine pancreas [5, 6]. The role of these factors in the development and growth of pancreatic cancer is not clearly understood, but it is likely that they may influence the growth of malignant cells of the pancreas [7–10]. As reported by Lhoste and Longnecker [11], bombesin and caerulein are able to stimulate growth of preneoplastic acinar cell lesions induced in the rat by azaserine. In addition, it was shown by Howatson *et al.* [8] that cholecystokinin enhances pancreatic ductal carcino-

genesis in the hamster nitrosamine model. Somatostatin and some of its analogues have been shown to exert various effects on exocrine pancreatic tumour growth in animals [12, 13].

Octreotide is a metabolically stable somatostatin analogue with a longer half-life in the circulation than native somatostatin *in vivo* [14]. It is being used more and more frequently in the treatment of acromegaly [15], pancreatic endocrine tumours [16, 17] and other non-pituitary disease states [18]. Octreotide inhibits the growth of human pancreatic ductal carcinoma transplanted into nude mice [13].

The aim of this work was to study the effect of octreotide on the growth of a chemically-induced acinar cell adenocarcinoma transplanted into Lewis rats and to investigate the distribution and affinity of somatostatin receptors in tumour tissue.

MATERIALS AND METHODS

Materials

The original tumour was induced by azaserine [19]. Octreotide and SDZ 204-090, its stable Tyr^3 analogue, were from Sandoz Pharma (Basel). Mini-osmotic pumps (Alzet, model 2002) were supplied by Scientific Marketing Associates (London). Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml), Fungizone 0.25 μg/ml and 10% fetal calf serum were obtained from Gibco/BRL. Other chemicals were standard reagent grade.

Animals, tumour dissociation and transplantation procedures

The initial pancreatic tumour of acinar origin was maintained in our laboratory as a transplantable tumour in Lewis rats (CNRS, Orléans, France). The tumours were removed from the rats, washed in ice-cold DMEM supplemented with antibiotics and chopped into small fragments which were passed through an 18 gauge needle. The resulting slurry was incubated for 10 min in Hanks' solution containing 2.5 mmol/l EDTA (lacking Ca^{2+} and Mg^{2+}), centrifuged at 500 *g* for 5 min and washed twice with cold medium. The pellet was resuspended in DMEM supplemented with antibiotics and 10% fetal calf serum. 2–3 mg aliquots (500 μl) of tumour tissue were injected subcutaneously in the scapular region of male adult Lewis rats weighing 150–180 g.

Experimental procedure

When the tumours became palpable (~ 10 days after injection), the animals were divided into two groups of 13 animals each. The treatment group received octreotide (40 μg/kg per day) delivered by means of osmotic minipumps for 14 days. The control group received saline solution according to the same procedure. The dose of octreotide used was chosen for its optimal effect on pancreatic growth induced by caerulein as reported previously [6]. Tumours were measured three times per week with calipers and tumour volume was calculated by the formula $\pi LD^2/6$ where L is the longest diameter and D the diameter at right angles to it [20]. At the end of the experiment, animals were killed. Blood was collected in order to measure plasma octreotide levels by radioimmunoassay [21]. Tumours were excised, cleaned of any adherent tissue and coagulated blood, weighed and analysed biochemically, ultrastructurally and by receptor autoradiography.

Biochemical studies

Tumours were homogenised in ice-cold, twice distilled water (100 mg/ml) in a Polytron (medium speed). Protein was determined according to Lowry *et al.* [22]. After extraction, DNA was determined by the diphenylamine method using calf thymus DNA as standard [23] and RNA by the orcinol method using yeast RNA as a standard [24]. Amylase was measured according to Danielsson [25] using maltose as standard. Chymotrypsin was assayed as indicated by Nagel *et al.* [26] after activation of chymotrypsinogen by trypsin. Lipase was measured according to Verduin *et al.* [27].

Ultrastructural studies

Small portions of tumours were excised and immediately fixed in 2% glutaraldehyde buffered with 0.2 mol/l sodium cacodylate, pH 7.4 and postfixed in 1% buffered osmium tetroxide. After dehydration in a graded sequence of alcohol, the samples were embedded in araldite. Semithin sections (0.5 μm) were stained with toluidine blue and examined in a light microscope. Ultrathin sections (≈ 0.07 μm) were contrasted with uranyl acetate and lead citrate, then examined in a Philips EM 300 electron microscope.

Somatostatin receptor autoradiography

Visualisation of somatostatin receptors in pancreatic tumours was performed using ^{125}I-SDZ 204-090, as specific radioligand. SDZ 204 090 was iodinated by chloramine-T and purified using high performance liquid chromatography (HPLC) (Lichrosorb RP-18, Merck, Darmstadt, Germany) with a linear gradient of 10–30% acetonitrile in 0,02 mol/l tetramethyl ammonium phosphate (pH 2.5). Somatostatin receptor autoradiography was performed as previously described [28]. Briefly, frozen tissue blocks were mounted on microtome chucks and sectioned at −20°C using a microtome cryostat (Leitz 1720, Leitz, Wetzlar, Germany). 10 μm sections were mounted on gelatin-coated glass slides, preincubated in Tris/HCl buffer (15 mmol/l, pH 7.4) containing $CaCl_2$ (2 mmol/l) and KCl (5 mmol/l) for 10 min at room temperature and washed twice for 2 min in the same buffer without salts. Incubation was carried out at room temperature for 2 h in Tris-HCl buffer (0.17 mol/l, pH 7.4) containing 1% bovine serum albumin, bacitracin (40 μg/ml) and $MgCl_2$ (5 mmol/l). The ligand concentration was 60 pmol/l. Non-specific binding was determined by using an excess of octreotide (1 μmol/l). At the end of the incubation, the sections were washed twice for 5 min each in cold incubation buffer containing 0.25% bovine serum albumin and then dipped in cold double-distilled water before being dried under a stream of cold dry air.

Autoradiograms were generated by exposing the labelled sections to [^{3}H]-sensitive Ultrofilm (LKB, Broma, Sweden). The exposure time was 10 days.

SRIF receptor assay

Pancreatic tumours in which somatostatin receptors were visualised with autoradiographic methods were also characterised biochemically in homogenate binding assay. One part of the tumoral tissue (the other one being used for histological and autoradiographic examinations) was quickly placed on ice and processed to obtain membrane homogenates for *in vitro* somatostatin binding assays. The membranes were prepared as described

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previously by Reubi *et al.* [29]. The tumours were homogenised in 5–10 volumes of 70 mmol/l Hepes buffer, pH 7.6, on ice. The homogenate was centrifuged once for 20 min at 48 000 *g* at 4°C and the resulting pellet was resuspended in 5–10 volumes of 10 mmol/l Hepes buffer, pH 7.6.

Binding studies were performed as described previously [30]. Briefly, tumour membranes (corresponding to ≈ 30–50 μg protein) were incubated in triplicate in a total volume of 300 μl at 22°C for 30 min with increasing concentrations of radioligand in 10 mmol/l Hepes buffer (pH 7.6) containing 0.5% BSA. The incubation was terminated by rapid filtration through Whatman GF/B glass fiber filters, which were then washed four times each with 5 ml ice cold 10 mmol/l Tris/150 mmol/l NaCl. The filters were counted in a LKB counter at 78% counting efficiency. Specific binding was total binding minus non-specific binding defined in the presence of 1 μmol/l somatostatin-14 or octreotide.

Experiments were carried out in triplicate. Protein was determined with the Bio-Rad protein assay kit. The dissociation constant (K_d) and number of binding sites (B_{max}) were calculated from Scatchard plots of the data.

Statistical analysis

For tumour volume, weight and biochemical parameters, the mean and standard error of the mean were calculated; for group comparisons, a one-way analysis of variance followed by a Newman–Keuls test was applied.

RESULTS

Survival

During the 14-day treatment with octreotide, a marked difference in survival rates was noted between the tumour-bearing groups: in the control group it was 77% vs. 92% in the octreotide treated group.

Body weights

At the end of the 14-day treatment, there were no significant differences in body weights between the treated and the control groups.

General morphology

When the tumours became palpable they were firm and highly vascularised; later they became soft, haemorrhagic and

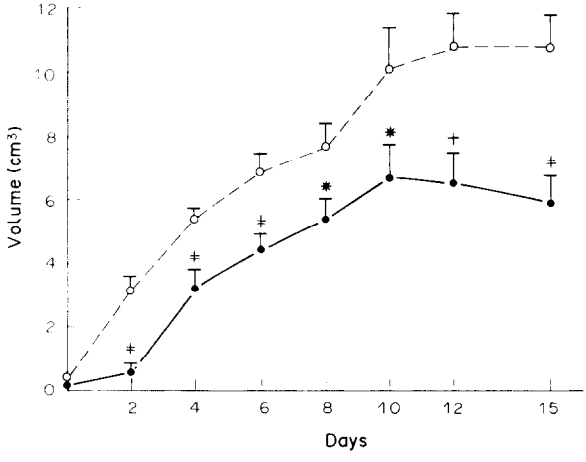

Fig. 1. Absolute tumour size as a function of time after implantation of acinar tumour cells. Results are expressed as mean (S.E.) (bars). Comparison between control group (– – –) and octreotide treated group (——) at each period: * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

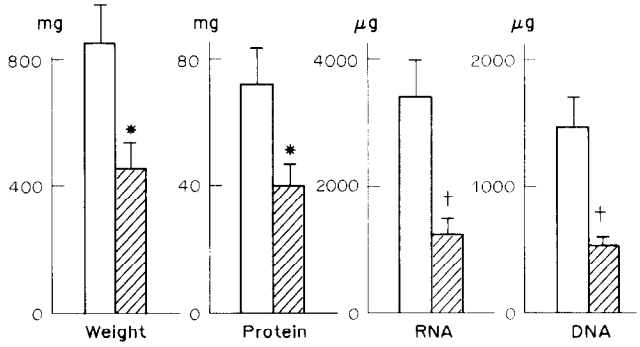

Fig. 2. Tumour weight, total protein, RNA and DNA content in octreotide-treated (hatched bars) and control animals (open bars) after a 14-day treatment. * $P < 0.05$, † $P < 0.01$.

contained cystic spaces and vesicles filled with brown fluid rich in amylase and lipase activities. In large tumours, areas of necrosis were common and viable tumour tissue was present mostly at the periphery. All subcutaneous transplants grew only locally and did not metastasise. Although the volume of the tumour was reduced in the octreotide-treated group compared with the control group, the general appearance of the tumour was not different.

Tumour growth and biochemical composition of the tumour

Octreotide, infused at the dose of 40 μg/kg per day, led to a constant octreotide plasma level of 2–4 ng/ml. As illustrated in Fig. 1, this treatment reduced the volume of the tumour by 80% ($P < 0.001$) during the first 2 days. On the following days the reduction of tumour volume was smaller, being 30–45% between the 4th and 15th day. After a 14-day treatment, the tumour weight, protein, RNA and DNA content had decreased by 52% ($P < 0.05$), 46% ($P < 0.05$), 63% ($P < 0.01$) and 64% ($P < 0.01$), respectively (Fig. 2). Amylase, chymotrypsin and lipase content decreased by 76% ($P < 0.01$), 60% ($P < 0.01$) and 34% (NS), respectively (Fig. 3). Thus the decrease in protein and enzyme content paralleled that in DNA content. However, when enzyme activities were expressed per mg of DNA, there was no significant difference between control and octreotide-treated groups. These results suggest that a 14-day treatment with octreotide reduced the number of tumour cells without markedly affecting the enzyme content of each cell.

Histological and ultrastructural observations

When observed by light microscopy, the pancreatic tumour appeared as a disorganised tissue in which the ratio of nuclei to

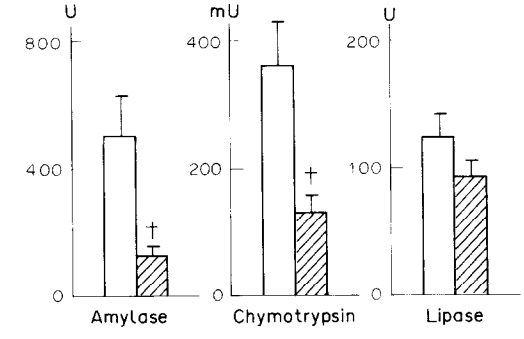

Fig. 3. Total amylase, chymotrypsin and lipase content of tumours from octreotide-treated (hatched bars) and control animals (open bars) after a 14-day treatment. † $P < 0.01$.

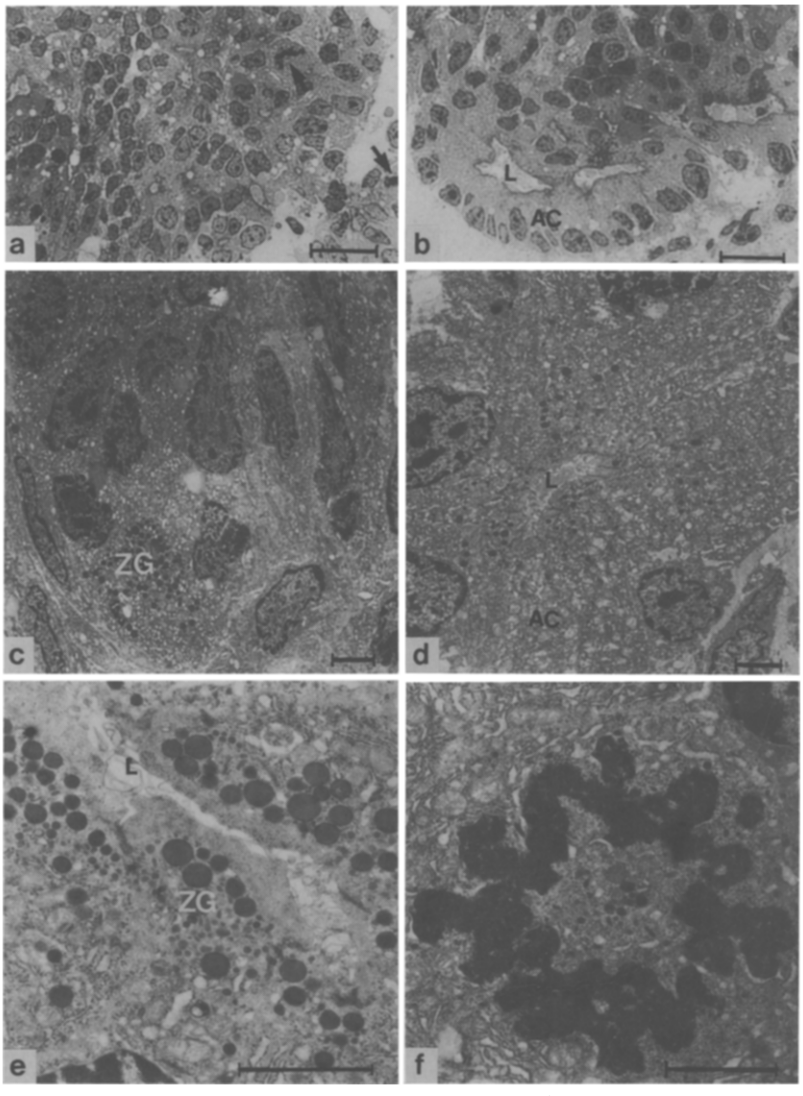

Fig. 4. Histological (a,b) and ultrastructural (c–f) appearance of the azaserine-induced pancreatic carcinoma transplanted to the rat. Acinar tumour cells appeared generally disorganised (a,c) but in some areas they were more arranged as typical acini (AC) (b,d); cells are polarised around the acinar lumen (L). Arrows indicate mitoses. (e) High magnification of the apical pole of acinar cells with two types of granules (ZG). (f) Mitotic figure in an acinar cell. In (a) and (b) the bar represents 20 μm, in (c–f) 2 μm.

cytoplasm was markedly increased (Fig. 4a). In some areas, cells were arranged in acinar structures around more or less dilated lumina (Fig. 4b). Mitotic figures were frequently encountered. Electron microscopy showed that the disorganised areas were generally depleted or devoid of zymogen granules while the nucleus occupied most of the cell. Nuclei were prominent and pleomorphic (Fig. 4c). In acinar type areas, cells were cuboidal and well polarised with a round basal nucleus among a dense dilated rough endoplasmic reticulum (Fig. 4d). Golgi apparatus appeared poorly developed while zymogen granules were located in the apical part of the cell around the acinar lumen. Some granules were large and round in shape while others were small and pleomorphic (Fig. 4e). Bundles of microfilaments were often noted beneath these granules in the apical region of these cells. Mitoses were generally observed in the granulated cells (Fig. 4f).

When animals were treated with octreotide, there was no marked change in the structural appearance of the pancreatic tumour except for the mitotic figures which were less frequent.

Somatostatin receptor autoradiography

The tissue distribution of specific ^{125}I-SDZ 204-090 binding sites in sections of pancreatic tumour tissue is illustrated in Fig. 5. Control and octreotide-treated animals were investigated. All tumours expressed a high density of somatostatin receptors which appeared to be homogeneously distributed. Figure 5 shows tumour sections from untreated control (Fig. 5a–c) and from octreotide-treated animals (Fig. 5d–f). Binding of the radioligand to the tumour sections (Fig. 5b, e) was quantitatively displaced by coincubation with an excess of unlabelled octreotide (Fig. 5c, f). The high density of binding sites, all over the tumour, was unchanged after continuous treatment of the animals with octreotide for 14 days. The somatostatin receptors were located in the neoplastic cell area but not in the stromal part. Adjacent sections stained with haematoxylin–eosin are shown in Fig. 5a, d.

Biochemical characterisation of somatostatin receptors

Biochemical analysis of pancreatic tumour membranes showed the presence of specific and high affinity binding sites for ^{125}I-SDZ 204-090. The K_d for somatostatin was 0.16 nmol/l and the B_{max} equivalent to 100 fmol/mg protein (Fig. 6a). Interestingly, comparable results were obtained with tumour membranes which were prepared from octreotide treated rats (K_d = 0.17 nmol/l and B_{max} = 110 fmol/mg protein) (Fig. 6b). Thus a 14-day treatment with 40 μg/kg per day octreotide

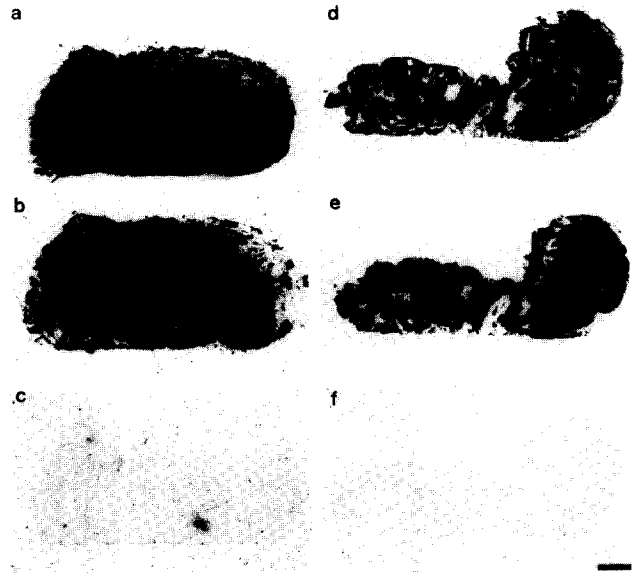

Fig. 5. Distribution of somatostatin receptors in an acinar cell tumour treated for 14 days with octreotide (d,e,f) or untreated (a–c). (a) and (d) represent the haematoxylin/eosin-stained section, (b) and (e), the corresponding autoradiograms after incubation of cryostat sections with ^{125}I-204-090 SDZ and (c) and (f) represent non-specific binding in the presence of an excess of octreotide. Bar 2 mm.

changed neither the number of somatostatin receptors nor their affinity on the pancreatic tumour. All tumours analysed in control and treated groups expressed specific and high-affinity somatostatin receptors.

DISCUSSION

The present investigation clearly indicates that the metabolically stable somatostatin analogue, octreotide, reduces growth of an acinar carcinoma of the pancreas transplanted into Lewis rats. The inhibitory effect was marked during the first 2 days of treatment (80%), then became less pronounced during the following 12 days (30–45%). Octreotide not only affected the size of the tumour but also its composition. Indeed, after a 14-day treatment with octreotide (40 μg/kg per day), the tumour weight and protein, RNA and DNA content decreased by 50–60%, suggesting partial growth inhibition. At the morphological level, there were also fewer mitotic figures observed in the atypical acinar cells.

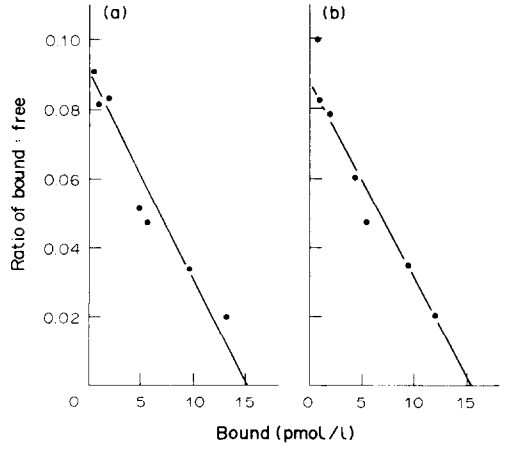

Fig. 6. Scatchard analysis of ^{125}I-SDZ 204-090 specific binding to pancreatic tumour membranes of control (a) and octreotide-treated animals (b). (a) K_d = 160 pmol/l, (b) K_d = 174 pmol/l.

Octreotide has also been shown to affect ductular carcinoma of the pancreas growing in nude mice [13]. However, the dose used by these researchers was larger than ours, being 300 μg/kg per day. In contrast to octreotide, other analogues of somatostatin exert various effects on pancreatic tumours. In Syrian hamsters bearing a ductal carcinoma, a cyclic hexapeptide analogue of somatostatin inhibited growth of the tumour while [L-5 Br-Trp^8]-SS-14 exerted a minor effect [12]. In contrast, in rats bearing the acinar tumour DNCP-322, this latter compound induced a marked reduction in tumour growth while the cyclic-hexapeptide-SS and SS-28 did not affect growth of the tumour [12]. The reason why these analogues exerted different effects on tumour growth is unknown but could indicate differences between the tumour models with respect to somatostatin receptor characteristics. However, these investigators did not report whether somatostatin-receptors were present on the tissue.

Somatostatin receptors have been demonstrated in a great number of tumours in the digestive system [29, 31]. Thus octreotide has been increasingly used in clinical trials such as for the treatment of multiendocrine tumours (carcinoid tumours, glucagonoma, insulinoma, nesidioblastosis, vipoma, gastrinoma) [32]. In some cases a reduction in the volume of tumours and metastases have been reported in long-term treated patients [17]. In experimental trials, octreotide has been shown to inhibit growth of different malignancies such as prostate tumours, osteosarcomas, chondrosarcomas and breast cancers [33].

Somatostatin could affect tumour growth by different ways: it could act indirectly by inhibiting the release of growth factors or peptidergic hormones involved in neoplastic processes. Probable candidates in the case of pancreatic tumours, may be CCK or bombesin-like peptides. As described by Lhoste and Longnecker [11], the latter stimulate azaserine-induced preneoplastic lesions in the rat pancreas.

Somatostatin could also inhibit tumour growth by altering its vascularisation. Indeed, as reported by Woltering *et al* [34], somatostatin, octreotide and RC-160 (another somatostatin analogue) inhibit angiogenesis in the chick chorioallantoic membrane in a dose-dependent manner. It is quite possible that inhibition of tumour vascularisation could explain in part growth inhibition induced by octreotide . However, a direct effect of this octreotide on pancreatic tumour cells was demonstrated by Viguerie *et al.* [35] who reported an antiproliferative effect on AR4-2J cells in culture, a cell line initially obtained from an azaserine-induced pancreatic tumour.

In this study, the presence of specific somatostatin receptors on tumour cells has been investigated by *in vitro* binding assays and by autoradiography. Biochemical studies indicated that receptors were of high affinity (0.16 and 0.19 nmol/l in non-treated and octreotide-treated tumours, respectively). Many reports indicated similar values in other kinds of tumours [29, 36]. By autoradiography, specific binding sites could be visualised only in neoplastic cell areas but not in non-neoplastic cells and connective tissue. The distribution of somatostatin receptors was quite similar in octreotide-treated and untreated tumours. These results obtained with two complementary techniques showed a good correlation and indicate that there was no desensitisation of somatostatin receptors under octreotide treatment. Our findings are in contrast with those of Koper *et al.* [37] who noted a desensitisation of cultured pituitary tumour cells after continuous exposure to octreotide. This loss of sensitivity was accompanied by a complete disappearance of somatostatin receptors.

Somatostatin and its analogues could also suppress cellular proliferation by inhibiting centrosomal separation [38] or opposing growth stimulation induced by EGF [39]. In fact, it was shown for another somatostatin analogue (RC-160) that somatostatin receptor activation stimulates a specific phosphatase leading to dephosphorylation of membrane EGF receptors [39]. However, there are no clues to the exact molecular mechanism of the growth inhibition.

In conclusion, the present study indicates clearly that octreotide treatment causes a partial suppression of the growth of transplanted acinar pancreatic tumours in the rat. In addition, we were able to demonstrate the presence of specific somatostatin receptors located on the tumour cells which may be involved in a direct inhibition of tumour growth, although an indirect effect cannot be excluded.

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