THE ROLE OF SOMATOSTATIN RECEPTORS IN BREAST AND PANCREATIC CANCER

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DE ROL VAN SOMATOSTATINE RECEPTOREN BIJ HET MAMMA- EN PANCREASCARCINOOM

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In memory to my father

y mi querida Charo

CONTENTS

CHAPTER I	Somatostatin, biology and clinical applications	9
CHAPTER II	Aim of the study	31
CHAPTER III	Somatostatin receptor scintigraphy in primary breast cancer	33
CHAPTER IV	Somatostatin receptor-positive primary breast tumours: genetic, patient and tumour characteristics	49
CHAPTER V	Role of tumour-derived fibroblasts in the growth of primary cultures of human breast cancer cells: effects of epidermal growth factor and the somatostatin analogue octreotide	63
CHAPTER VI	Use of radionuclide-labelled somatostatin analogues for visualization of islet cell tumours	77
CHAPTER VII	The use of somatostatin receptor scintigraphy in the differential diagnosis of pancreatic duct cancers and non-functioning islet cell tumours	85
CHAPTER VIII	Octreotide therapy for liver metastases after resection of a non-functioning islet cell tumour	95
CHAPTER IX	Somatostatin receptor dependent growth inhibition of liver metastases by octreotide	103
CHAPTER X	General discussion	115

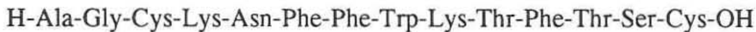
SUMMARY 119
SAMENVATTING 121
DANKWOORD 123
LIST OF PUBLICATIONS 125
CURRICULUM VITAE 127

CHAPTER I

SOMATOSTATIN, BIOLOGY AND CLINICAL APPLICATIONS

Biology

Somatostatin was isolated and characterized by investigators working in the laboratory of Guillemin at the Salk Institute in La Jolla, California in 1973 during a search for a growth hormone (GH, somatotropin) releasing factor in sheep hypothalamus, since physiological, experimental, and clinical observations had led to the concept that the hypothalamus controls and regulates the secretion of GH by the pituitary gland[2-3]. They observed that the addition of crude hypothalamic extracts in minute concentrations to the incubation medium of dispersed rat pituitary cells in monolayer cultures significantly decreases the basal secretion of immunoreactive GH[4]. This inhibition was related to the dose of hypothalamic extract added and was specific. They decided to attribute this inhibitory effect on the secretion of GH to a "somatotropin-release inhibiting factor" (SRIF) or somatostatin (SS). After the isolation of the postulated SS from the hypothalamic extracts, quantitative amino acid analysis and sequence determination, the primary structure of SS was characterized[5]. SS was found to be a tetradecapeptide (SS-14) with a disulphide bridge connecting both cysteine molecules (*fig.I*):



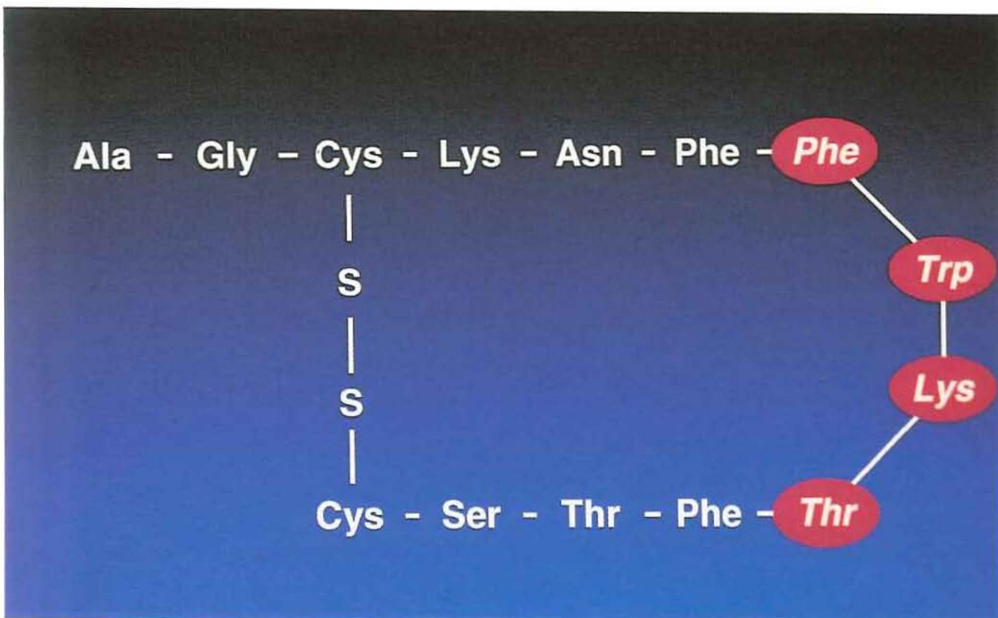
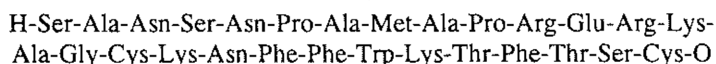


Figure I.
Somatostatin.

Several groups[6-8] synthesized SS after the structure of this tetrapeptide was established and its *in vitro* effects (eg.GH release inhibition) were confirmed in rat pituitaries. Since the primary structure of native porcine and sheep SS were identical, this suggested that SS also exists in other species.

It is now clear that SS is also widely distributed in the human body, being found first in high concentrations in the hypothalamus, later on it was discovered in other areas of the brain, thyroid, thymus, bronchi, in the stomach (antrum, corpus and fundus), the pancreas and the intestines. More than 90% of SS immunoreactivity in the human gut is present within mucosal endocrine cells. SS in the pancreas is located in the D cells at the periphery of the islets of Langerhans[9-12].

After the identification and purification of SS-14, precursor forms of greater molecular weight have been recognized[9,10,13]. Somatostatin-28 (SS-28), or prosomatostatin, is a 28-amino acid polypeptide with SS-14 making up the C-terminus[9].



H-Ser-Ala-Asn-Ser-Asn-Pro-Ala-Met-Ala-Pro-Arg-Glu-Arg-Lys-
Ala-Gly-Cys-Lys-Asn-Phe-Phe-Trp-Lys-Thr-Phe-Thr-Ser-Cys-O

This larger molecular form can be converted into the smaller SS-14 by a processing enzyme found in the mouse hypothalamus[14]. Preprosomatostatins are even larger precursor forms of 120 or more amino acids, with SS-28 located at the C-terminus[10]. All of these different forms exert biologic activity but they differ in their relative potency[15].

Various studies demonstrated mainly inhibitory effects of SS-14. These include inhibition of GH and thyroid stimulating hormone (TSH) release by the anterior pituitary gland[1,16], inhibition of endocrine (insulin, glucagon) and exocrine pancreatic function and of gastrin release[17-22].

The degree of inhibition of GH secretion by SS is determined by two critical factors: the concentration of the peptide in the hypothalamic portal plasma and the number of SS receptors (SS-R's) on the anterior pituitary gland.

The effects of SS are believed to modulate the GH responses to GH-releasing hormone (GHRH) secretion in the generation of pulsatile GH secretion[23,24]. The suppression of TSH is of a lesser degree as compared with that of GH. This may be explained by the differences in SS-R's on the GH and TSH producing cells in the anterior pituitary gland[23,25].

Because large numbers of SS-R's are also present on most GH-secreting pituitary adenomas in acromegalic patients[26], chronic SS analogue therapy causes a marked clinical improvement in these patients and the typical soft tissue swelling decreases after weeks[27,28]. Tumour shrinkage of the adenoma was found in more than 50% of these patients, which may facilitate selective transsphenoidal adenomectomy and/or external pituitary irradiation[29].

Although the mechanisms regulating the stimulation of secretion by the exocrine pancreas have been extensively studied, little is known about the physiological mechanisms involved in inhibition. SS was suggested to act inhibitory directly via specific cell surface receptors, because such receptors were identified on isolated guinea pig acinar cells[30-32]. Numerous studies, however, have failed to demonstrate inhibition by SS of amylase release by isolated acini during stimulation with cholecystokinin (CCK), vasoactive intestinal polypeptide (VIP), carbachol, or secretin[33,34]. Also in pancreatic lobule preparations in the isolated vascularly perfused pancreas SS was ineffective in inhibiting stimulated secretion[34]. In contrast, SS was an effective inhibitor of stimulated exocrine secretion *in vivo*. This was explained by Mulvihill et al.[35] who postulated the concept that SS acts indirectly via neural mechanisms (tetrodotoxin-sensitive, non-adrenergic, or non-cholinergic pathways).

The therapeutic use of SS is impractical because of its multiple actions and the short duration of its antisecretory effects; it's half-life in the circulation is about 3 minutes[36,37]. Studies were carried out by several groups to systematically design and synthesize SS analogues with selective enhanced and prolonged activities[36-38]. Analogues of SS-14 and SS-28 were expensive and not superactive *in vivo*[37,39-42]. Therefore Veber et al.[43] carried out conformation analysis and designed several analogues by replacing 9 of the 14 amino acids of SS with a single proline residue. However, these hexapeptide analogues, although more potent than SS in inhibiting GH, insulin and glucagon release, did not reveal antitumour activities. Bauer et al.[44] synthesized another series of highly potent analogues. They retained Phe-Trp-Lys-Thr and incorporated it with the tryptophan residue in the D configuration, into a cysteine-bridged octapeptide analogue (***fig.II***). This analogue, SMS 201-995 (Octreotide) was long acting and 45-70 times more potent than SS-14 in inhibiting GH secretion and more selective, since it suppressed insulin and glucagon to a lesser extent than GH. Also it did show a direct inhibitory effect on tumour growth[44-48].

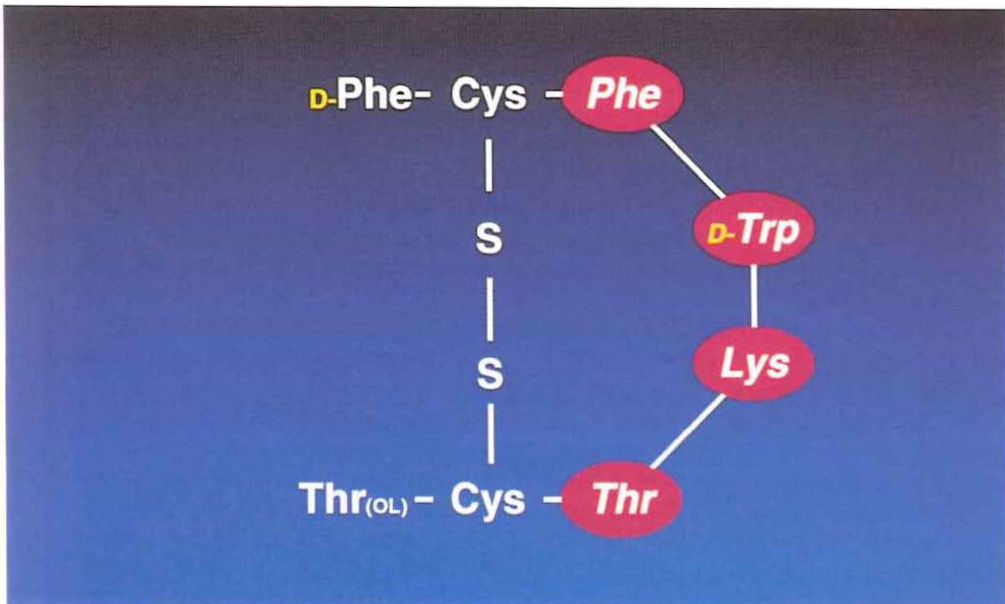


Figure II.
Octreotide.

The wide spectrum of gastrointestinal and endocrine actions of SS, mainly inhibitory in nature, are summarized in ***table 1***.

Table 1. *Summary of gastrointestinal and endocrine activities of somatostatin.*

Endocrine secretion

Inhibits secretion of gastrin, secretin, vasoactive intestinal polypeptide (VIP), pancreatic polypeptide, insulin and glucagon

Exocrine secretion

Inhibits secretion of gastric acid and pepsin, pancreatic enzyme and bicarbonate production, and intestinal epithelial electrolytes and water release

Absorptive function

Inhibits absorption of glucose, xylose and amino acids; increases absorption of electrolytes and water

Motility

Inhibits motor activity in stomach, small intestine and gallbladder

Haemodynamics

No effect on cardiac output: decreases coeliac, mesenteric and renal blood flow

Growth

Decreases gastric and intestinal mitotic index and deoxyribonucleic acid synthesis; inhibits trophic effect of gastrin

Clinical application

Due to the wide spectrum of action, the use of SS has been recently described in the treatment of a variety of gastrointestinal disorders, mostly of primary concern to the surgeon[49,50]. Several reports are now available describing the usefulness of SS-14 and/or octreotide in controlling upper gastrointestinal haemorrhage, especially that associated with portal hypertension[51-58]. SS-14 and/or octreotide were superior to vasopressin in these studies both in the initial control of bleeding, as well as in the number of hospital deaths in patients bleeding massively from oesophageal varices[55,56]. The effectiveness of SS-14 and/or octreotide in the treatment of upper gastrointestinal haemorrhage is probably mainly related to its effect on mesenteric bloodflow and pressure, but possibly also related to its concomitant cytoprotective effects and by the suppression of gastric acid secretion[58-61].

SS-14 inhibits hormone secretion from both normal and tumorous endocrine gastro-entero-pancreatic tissue. On the basis of this observation, octreotide has been used in the treatment for pancreatic islet cell tumours and carcinoids. Most of these tumours are malignant and have already metastasized at the time of diagnosis. These tumours are in general slow growing and most of the clinical distress in this type of patients is related to the hypersecretion of hormones which often incapacitates them and causes long and repeated hospital stays. The clinical use of octreotide in this type of patients is of considerable help in controlling clinical symptomatology. Debilitating diarrhoea (VIPoma), peptic ulceration (gastrinoma), skin rashes (glucagonoma), hypoglycaemic (insulinoma) and flushing attacks (carcinoid) were well controlled during chronic treatment with SS analogues[62-64]. There is no doubt that SS analogue therapy is of great benefit for most of these patients and improve their quality of life dramatically[62,65].

Clinical trials have been reported demonstrating the effectiveness of octreotide in pancreatitis[66]. Experimental evidence in animal models supports the usefulness of octreotide in pancreatitis: the reduction of exocrine pancreatic secretion by octreotide decreases local inflammation and necrosis[67,68]. In chronic pancreatitis octreotide is widely tested in ongoing trials in Europe. Case reports, however, suggest that octreotide may reduce the size of

pseudocysts, while decreasing pain (S.Jenkins, personal communication). Post pancreatectomy complication rates were reduced by intravenous somatostatin[69], and octreotide was similarly found to reduce fistula formation, as well as incidence of acute pancreatitis after pancreatic surgery[70-72] or pancreatic transplantation[73,74]. In a well conducted, randomized, double blind, placebo controlled, multicenter trial it was found that the perioperative application of octreotide reduces the occurrence after pancreatic resection, of postoperative complications such as pancreatic fistulas, abscesses and subsequent sepsis, particularly in patients with tumours[75].

Octreotide has few side effects. Ileus with nausea and vomiting has been reported in several patients, probable caused by inhibition of normal gastric and intestinal motility. Blood sugar levels should be frequently monitored during treatment, especially in type II diabetic patients, since octreotide inhibits insulin release and therefore may cause transient hyperglycaemia[76]. Due to its inhibitory effects on cholecystokinin release, gallbladder stones may develop during chronic treatment[77,78]. Only very few allergic reactions have been reported[79]. The safety of its use during pregnancy and lactation has not been determined.

Since it was known that SS and its analogues interfere with growth factors and possibly do have an antiproliferative effect on some tissues, several reports were published about an oncological application of octreotide[80-82]. Schally demonstrated in 1987 that the reduction of prolactin and GH levels by SS might contribute to the inhibition of the growth of breast and prostatic tumours[83,84]. Another example of an indirect effect of SS through growth factor inhibition was postulated after it was noticed that GH stimulates cell differentiation directly and clonal expansion indirectly through local production of insulin-like growth factor-I (IGF-I)[85-88]. On the other hand, preliminary evidence has indicated that SS analogues can selectively stimulate the formation of IGF-binding protein 1, thereby interfering with IGF-1 action at the receptor level[89,90]. SS may also have a direct antiproliferative effect and inhibits DNA synthesis and cell replication induced by epidermal growth factor (EGF) in some cells by preventing centrosomal separation[91]. In human pancreatic cancer cells, SS activates dephosphorylation of EGF receptors and thereby reverses the stimulative effect of EGF on cell growth[37,84,92].

Another possible mechanism by which SS analogues might inhibit tumour growth is interference with the synthesis of autocrine growth factors by tumour cells or by direct inhibition of angiogenesis[88,93-96]. Finally a change in natural killer cell activity was reported in man[99] as well as a stimulation of the reticuloendothelial and lymphopoietic system in rats. So SS induced changes in immunological activity may explain in part the tumour growth inhibitory effect[100].

SOMATOSTATIN RECEPTORS

After Schönbrunn and Tashjian first measured somatostatin receptors (SS-R's) in 1978 using the clonal pituitary cell line GH4C[99], SS-R's have been demonstrated on a variety of human tumours, using various iodinated SS analogues in homogenate ligand binding assays or autoradiography on tissue sections[100,101]. Large numbers of binding sites with high affinity for SS were found on most tumours with amine precursor uptake and decarboxylation (APUD) characteristics, as well as on meningiomas, well-differentiated brain tumours (astrocytomas), neuroblastomas and human breast tumours[102-118]. Examples of tumours with APUD characteristics, which are often SS-R positive are, growth-hormone-producing pituitary adenomas, endocrine pancreatic tumours, carcinoids, paragangliomas, small cell lung cancers,

medullary thyroid carcinomas and pheochromocytomas. In this thesis only the use of SS and its analogues in the diagnosis and treatment of breast and pancreatic tumours will be reported.

Critical to the action of SS is the presence of SS-R's, which like other membrane receptors subserve two functions: (1) to recognize the ligand and bind it with high affinity and specificity, and (2) to generate a transmembrane signal that evokes a biological response. Three receptor-linked effector systems have been identified[119-129]: (see ***fig.III***)

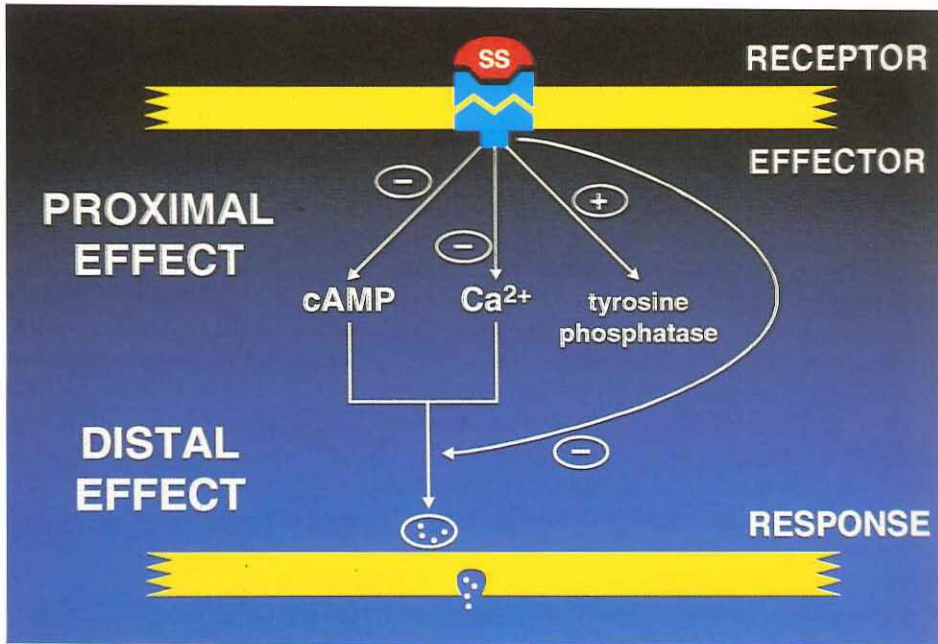


Figure III.

(1) Reduction of the intracellular mediators CAMP and Ca^{2+}, due to effects on adenyl cyclase and ionic channels (K^{+}, Ca^{2+}).

(2) Acting at a step distal to CAMP generation and Ca^{2+} entry,thereby blocking hormone secretion stimulated by CAMP and/or Ca^{2+} ionophores.

(3) Stimulation of tyrosine phosphatase, leading to dephosphorylation and inactivation of tyrosine kinase such as those activated by (EGF).

Pharmacological studies have suggested that the distribution of SS-R's is often heterogeneous and that there may be selective subtypes for both SS-14 and SS-28. The biological activity of the two peptides in general correlates with their potency for receptor binding in most systems, suggesting that their selective actions may result mainly from differential interaction with receptors[130-135]. Reubi et al. have identified pharmacologically SS-R subtypes in rat and human brain by means of their differential affinity for certain somatostatin analogues[136-138].

Recently, at least five different human SS-R types have been cloned. All subtypes bind SS-14 and SS-28 with high affinity, while their affinity for octreotide differs considerably. Octreotide binds with high affinity to subtype 2 ($SSTR_2$), while this analogue has a relatively

low affinity to $SSTR_3$ and $SSTR_5$ and shows virtually no binding to subtypes 1 and 4 ($SSTR_1$ and $SSTR_4$)[139-142]. The cloning of five human SS-R subtypes could provide the molecular basis for the mechanisms of regulation by SS of different functions and this may lead to the development of selective analogues for a variety of clinical applications. The majority of the SS-R positive human tumours expressed the $SSTR_2$ subtype, only a minority of tumours had exclusively other SS-R subtypes. One third of the insulinomas, 50% of the receptor positive medullary thyroid carcinomas and all SS-R positive ovarian tumours were not of the $SSTR_2$ type[141]. These SS-R positive tumours may poorly react to treatment with octreotide and they cannot be visualized *in vivo* with radiolabelled octreotide.

SOMATOSTATIN RECEPTORS IN BREAST CANCER

Since expression of SS receptors (SS-R's) was a known feature of tumours of the nervous system, neuroendocrine tissues and endocrine gastro-intestinal tumours, Papotti et al. correlated the expression of neuroendocrine markers in human breast cancer with the presence of SS-R's[116]. The presence of neuroendocrine cells in normal and in breast cancer in man has been a matter of controversy for a long time. As early as 1947, Vogler demonstrated argyrophilic cells in normal breast tissue[143]. Later on Feyrter and Hartmann suggested, on the basis of silver impregnation, the endocrine nature of mucoid carcinomas ("carcinoids") of the human breast[144]. However they were describing myoepithelial cells, which were not endocrine, as shown by several histochemical and electronmicroscopic studies[145,145]. Between 1977 and 1982 several reports demonstrated a variety of argyrophilic breast tumours containing dense-core secretory granules and showing the typical features of carcinoids. Bussolati et al. also found chromogranin-positive cells immunocytochemically in part of human breast cancers with the mouse monoclonal antibody LK2H10 directed against human chromogranin[147]. Immunoreactivity for neuron-specific enolase, which is present in neurons, neuroendocrine cells and tumours with neuroendocrine differentiation, was found in more than 30% in breast carcinomas. However, expression of this marker in mammary tissues does not appear to be at all related to endocrine differentiation, as defined by ultrastructural demonstration of secretory granules. Other markers such as chromogranin A and B and synaptophysin were later found to be more specific for neuroendocrine differentiation.

Neuroendocrine differentiation of tumours can be diagnosed on the basis of the following morphological and cytochemical criteria: argyrophilia (by Grimelius silver staining), presence of secretory granules (by electron microscopy (EM)), and expression of specific neuroendocrine markers, such as chromogranin A and B, synapthophysin and neuron-specific-enolase (NSE). Neuroendocrine differentiation in a subset of human breast carcinomas has been suggested in the light of these morphological and cytochemical criteria:

(a) argyrophilia by Grimelius staining	Azzopardi et al. 1982[148] Bussolati et al. 1987[149]
(b) secretory granules in EM	Cappella et al. 1980[150] Nesland et al. 1986[151,152]
(c) specific neuroendocrine markers	Bussolati et al. 1985, 1987[147,149] Buffa et al. 1988[153]

To correlate the existence of neuroendocrine differentiation with the presence of SS-R's in breast carcinomas, Pappoti et al. stained a series of 100 cases with the Grimelius silver staining procedure, carried out immunocytochemistry with specific neuroendocrine markers and compared the results with that of autoradiography for SS-R's[116]. A highly significant correlation was established between the expression of neuroendocrine markers and high SS-R density. This occurred, however, only in 7 of these 100 cases. In a large series Reubi et al.[114] evaluated the incidence of SS-R's expression in primary breast tumours. In a group of "small" tumour samples with a mean section surface of 14 mm^2, 21% of the tumours were SS-R positive. However in a group of "large" tumour samples with a mean section surface of 180 mm^2, 46% were SS-R positive and especially in this group often a non-homogeneous distribution was seen, which means that tumour regions within SS-R positive tumours were SS-R negative. In their study they also showed that metastases of SS-R positive primary tumours may often be SS-R positive[114].

Binding capacities and apparent dissociation constants for SS-R were determined by Fekete et al. in 500 breast biopsy samples using multipoint membrane receptor assays[115]. In 36% of the tumour samples SS-R's were present and no correlation was found between SS-R binding sites and binding capacities of other receptors like oestrogen, progesterone, EGF and LH-RH. A negative relationship between the expression of SS-R and EGF receptors in breast cancer samples was suggested by Reubi et al.[114].

SOMATOSTATIN RECEPTORS IN PANCREATIC CANCER

Pancreatic cancer may be divided in endocrine and exocrine tumours, which is based on the origin of these tumours. Endocrine pancreatic tumours have neuroendocrine properties, which can be confirmed using a combination of immunocytochemical staining with chromogranin A and neuron- specific-enolase and the Grimelius silver staining. The origin of endocrine pancreatic tumours either from multipotent cells in the ductular epithelium or islet cells remains to be established. They are hormone producing tumours, causing several clinical syndromes by hypersecretion of these hormones[154,155]. After SS-R's have been detected on the normal endocrine islet cells (A, B and D cells possess such receptors)[156], Reubi et al. demonstrated that most hormone producing endocrine pancreatic tumours also express a high density of SS-R's. Later on it was observed that these receptors were functional, because a positive correlation existed between SS-R status of hormone producing endocrine pancreatic tumours and the beneficial effect of a stable long-acting SS analogue on hormone release as well as growth of these tumours during therapy[157,158].

Although SS-R have been demonstrated on exocrine pancreatic cells in experimental animals mainly in the acinar cells[159-161], neither SS-R's nor neuroendocrine properties could be found on human exocrine pancreatic adenocarcinomas[162].

SOMATOSTATIN RECEPTOR SCINTIGRAPHY

The development of the SS receptor scintigraphy has recently been extensively described in the thesis by W.H. Bakker et al.[163] and therefore it will be summarized only briefly.

First of all I^{123} was bound to the SS analogue Tyr^3-octreotide (***fig.IV***) in which Phe has been replaced by Tyr in the active site of the molecule to make radio-iodination possible.

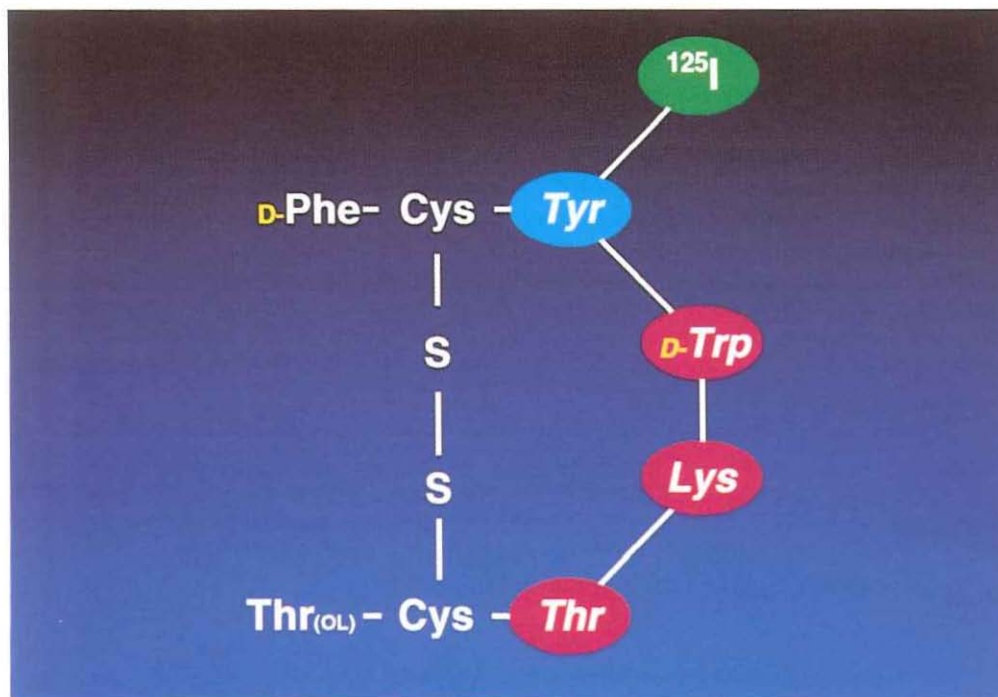


Figure IV.
[^{125}I-Tyr^{3}]-octreotide.

It was shown that this radioactive analogue was biologically active and bound specifically to SS-R's. After it was demonstrated that this radioligand could visualize SS-R positive tumours in rats after *in vivo* administration, it was given iv. to patients suspected to have a SS-R expressing tumour. SS-R positive tumours as well as their metastases could be visualized[164-166].

The clearance of [I^{123}-Tyr^{3}]-octreotide is mainly by the liver and bile. The bowel radioactivity makes interpretation of gastrointestinal tumours in the upper part of the abdomen difficult. Also for other technical reasons (^{123}I is not readily available world-wide, radiolabelling and purification is time consuming, the physical half-life of the radionuclide is short and there is rapid clearance from the blood resulting in a short effective residence time for accumulation in tumour tissue), [^{111}In-DTPA-D-Phe^{1}]-octreotide (*fig. V*) was developed.

In contrast to ^{123}I, ^{111}In is readily available and has attractive physical characteristics (physical half-life of 2.8 days, gamma emission, which is suitable for scintigraphy with the gamma camera). The diethylene-triaminopentaacetic acid (DTPA) had to be coupled to octreotide in order to make conjugation of octreotide with ^{111}In possible. [DTPA-D-Phe^{1}]-octreotide binds more than 95% of added ^{111}In in an easy, single-step labelling procedure without necessity of further purification. The specific SS-like biologic effect of these analogues was proven by the inhibition of GH secretion by cultured rat pituitary cells in a dose-dependent fashion by octreotide, [DTPA-D-Phe^{1}]-octreotide and non-radioactive [^{115}In-DTPA-D-Phe^{1}]-octreotide[167]. The binding of [^{111}In-DTPA-D-Phe^{1}]-octreotide to rat brain

cortex membranes proved to be displaced similarly by natural SS as well as by octreotide, suggesting specific binding of [^{111}In-DTPA-D-Phe^{1}]-octreotide to SS-R's.

Because of its longer half-life in the circulation, [^{111}In-DTPA-D-Phe^{1}]-octreotide is exposed longer to tumour tissue, which improves gamma camera imaging 24 hours after injection and its renal clearance without degradation *in vivo* contrasts to the hepato-biliary clearance of [I^{123}-Tyr^{3}]-octreotide and subsequent degradation *in vivo*. Therefore, [^{111}In-DTPA-D-Phe^{1}]-octreotide is also very suitable for use in single photon emission computed tomography (SPECT) of the abdomen, which is especially of importance in the localization of small endocrine gastroenteropancreatic tumours, which are obscured by radioactivity from other organs, eg. kidney and spleen[168,169].

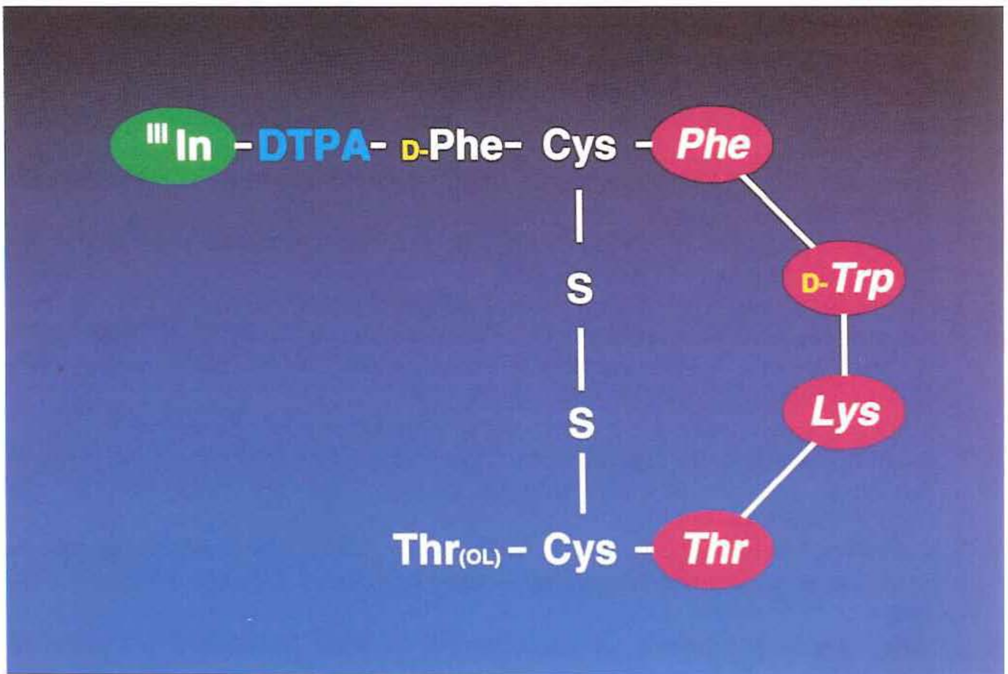


Figure V.
[' 'In-DTPA-D-Phe^{1}]-octreotide.

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CHAPTER II

AIM OF THE STUDY

Somatostatin receptors (SS-R's) have been found on a variety of neuroendocrine tumours like carcinoids, paragangliomas and meningiomas. Several *in vitro* studies also indicated the presence of SS-R on part of human breast cancers, on islet cell tumours, but not on adenocarcinomas of the pancreas. This finding led us to explore whether it was possible to visualize SS-R positive breast and islet cell tumours *in vivo*. In order to validate that indeed SS-R expressing tumours were visualized, we investigated the correlation between the *in vivo* results and the presence of SS-R's as demonstrated *in vitro* by autoradiography of the same tumour specimen.

The presence of SS-R's on part of human breast cancers raised two questions. First, it was investigated whether breast tumours expressing SS-R's form a distinct subgroup with particular genetic characteristics, and whether there is a correlation between SS-R expression and neuroendocrine differentiation of these tumours. For this reason we studied SS-R positive primary breast tumours for a number of clinical, genetic and pathological characteristics.

Secondly, growth inhibitory effects of somatostatin had been previously demonstrated in experimental breast cancer tumour models. The mechanism(s) of this effect is currently poorly understood. It might be due to inhibition the synthesis of several auto- and paracrine growth factors, which act directly on the tumour cells or indirectly via tumour-derived fibroblasts which in previous studies were found to be capable of stimulating the growth of breast cancer cells.

The culture of human breast cancer cells is difficult. It was decided to approach the problem of the preparation of primary cultures of breast cancer cells by incubating tumour associated fibroblasts and epithelial tumour cells separately. Thereafter the role of breast cancer derived fibroblasts was investigated on the proliferation of primary cultures of epithelial cells derived from the same tumour. Finally a potential inhibitory effect of the somatostatin analogue octreotide on tumour cell proliferation was studied.

In contrast to most islet cell tumours, pancreatic duct cancers were previously found to be SS-R negative. As most islet cell tumours can be visualized with [^{111}In-DTPA-D-Phe^{1}]-octreotide scintigraphy, it was investigated whether it is possible to differentiate preoperatively with this technique between these two tumour types and whether somatostatin receptor scintigraphy plays a role in the long term follow-up of these patients.

Most patients with islet cell tumours eventually die because of extensive liver metastases. As liver metastases remain visible at scintigraphy of patients with islet cell tumours on the basis of the presence of SS-R's on the tumour cells, we further evaluated the pathophysiological significance of the SS-R's on these tumour cells. We performed a study in rats, bearing transplantable SS-R positive pancreatic and SS-R negative colon tumour cells, in order to investigate whether it was possible to inhibit the growth of SS-R positive liver metastases by octreotide administration, and whether an inhibitory effect of octreotide on the growth of liver metastases is specific for SS-R positive tumours.

CHAPTER III

SOMATOSTATIN RECEPTOR SCINTIGRAPHY IN PRIMARY BREAST CANCER

Published in *The Lancet 1994:434:640-643*

Abstract

Somatostatin receptor scintigraphy successfully visualizes the primary tumours, as well as the distant metastases in most patients with carcinoids, islet cell tumours and paragangliomas. Previous *in vitro* studies indicated that somatostatin receptors (SS-R's) are present on part of human breast cancers. In this study we report the scintigraphic visualization with [^{111}In-DTPA-D-Phe^{1}]-octreotide of 39 of 52 primary breast cancers (75%) in 50 patients. Parallel *in vitro* autoradiography with [^{125}I-Tyr^{3}]-octreotide of 30 of these tumours showed a corresponding receptor status in 28 of them. A homogeneous, dense distribution of SS-R's detected *in vitro* resulted in a higher degree of accumulation of radioactivity at the tumour site *in vivo*. Non-homogeneous *in vitro* distribution of receptors pointed in most instances to the presence of a large non-invasive carcinoma component, mainly ductal carcinoma in situ (DCIS). Significantly more invasive ductal carcinomas could be visualized than invasive lobular carcinomas (85 vs 56%, resp., $p<0.05$). Also the number of T_2 tumours which were visualized was higher than the T_1 tumours (86 vs 61%, resp., $p<0.05$). Special detailed gamma camera images of the axillae visualized non-palpable tumour containing lymphnodes in 4 of 13 patients with histologically proven micrometastases.

In the follow-up after 2.5 years, SS-R scintigraphy in 28 of the 37 patients with an originally SS-R positive tumour, was positive in the 2 patients with clinically recognized metastases, as well as in 6 of the remaining 26 patients who were symptom-free at that moment. Ca 15-3 and CEA elevations were observed in only 2 and 1, respectively, of these patients during follow-up.

Conclusions: A majority of primary breast cancers can be visualized by SS-R scintigraphy. Especially invasive ductal carcinomas are positive. In the follow-up after 2.5 years 25% of the symptom-free patients who had originally SS-R positive primary tumours, showed distant metastases at scintigraphy, without elevations of serum CA 15-3 and CEA levels in the majority of these patients. It is anticipated that this technique will be of value in selecting patients for clinical trials with somatostatin analogues and/or other medical therapy. Furthermore, octreotide scintigraphy is more sensitive than measurements of the usual serum tumour markers for detecting recurrences of SS-R positive breast cancer.

Introduction

Somatostatin receptors (SS-R's) have been found on a variety of neuroendocrine tumours like carcinoids, islet cell tumours, paragangliomas, as well as on brain tumours, like meningiomas[1-5]. A number of studies also indicate the presence of SS-R's on part of human primary breast cancers[6-9] which are probably of neuroendocrine differentiated[8].

Direct antiproliferative effect of somatostatin analogues have been reported on the growth of a variety of experimental tumours, including human breast cancer cell lines and explants, via a somatostatin specific receptor mechanism[10-11].

Recently we introduced a new nuclear medical technique in which SS-R positive tumours could be visualized *in vivo*, after the administration of a radionuclide labelled somatostatin analogue followed by gamma-camera scintigraphy[12-16]. In the present study we investigated the value of this technique in the visualization of the primary tumours and their axillary metastases of 50 breast cancer patients, as well as of the use of SS-R scintigraphy in the follow-up of these patients. Furthermore, its value to the detect recurrent disease was compared with the two serum markers CA 15-3 and CEA, which are most commonly used for this purpose in breast cancer.

Methods

Patients

We studied 50 patients with 52 primary breast tumours (mean age 61 years [range, 38 to 93]). After clinical examination and mammography, all 52 tumours were cytologically confirmed to be primary breast cancer. When the patients gave informed consent to participate in this study, bloodsamples were taken for measurements of tumour markers and SS-R scintigraphy was performed on an outpatient basis. After this scanning procedure all patients were operated within two weeks, except for one patient, who received chemotherapy due to a T_4 breast tumour. Only physical examination and chest X-ray were done preoperatively. None of the patients showed evidence of metastatic disease. There were no clinical signs for spread of the disease, either. 27 patients were treated by lumpectomy and axillary dissection, 22 with modified radical mastectomy (2 patients had a bilateral modified radical mastectomy). The Scarff, Bloom, and Richardson grade (SBR) was assessed by one and the same pathologist (R.v.P.) in all patients. The presence of SS-R's in 30 of these tumours was measured by *in vitro* autoradiography (J.C.R.) on cryostat sections of tumour tissue, as has been described previously in detail for other breast tumours[1,9].

Materials

The somatostatin analogue [DTPA-D-Phe^1]-octreotide was obtained from Mallinckrodt Medical BV (Petten, The Netherlands). [DTPA-D-Phe^1]-octreotide was labelled with "ultra-pure" ^{111}Indium. The labelling procedure has been described elsewhere[18]. Doses ranged from 200 MBq to 272 Mbq [^{111}In-DTPA-D-Phe^1]-octreotide.

Scintigraphy

Planar images were obtained with a large field-of-view gamma camera (Counterbalance 3700, Siemens Gammasonics, Erlangen, Germany) equipped with a 190-KeV parallel-hole collimator. Generally, the field of view covered the chest and the very upper part of the abdomen. Starting 24 hours after injection of [^{111}In-DTPA-D-Phe^1]-octreotide planar chest images were obtained anteriorly and posteriorly, with additional images of the axillary region

with arms in elevated position. 500 Kcts were collected per image with a maximal counting time of 15 min. A simple high/moderate/low/negative system was used to define the accumulation of radioactivity by the tumours as visualized during the scanning procedure, carried out by E.P.K. and H.Y.O., who were not informed about the localization of the tumour.

Tumour marker assays

Serum samples were stored at -20°C before the assays were carried out. Serum CEA levels were determined by the enzyme immunoassay (ELISA) kits of Boehringer (normal upper limit normal concentration 10,0 ng/ml). Serum CA-15.3 was determined by ELISA kits of Centercor, Leiden, The Netherlands (normal upper normal concentration in serum 27 U/ml).

Results

[^{111}In-DTPA-D-Phe^{1}]-octreotide scintigraphy was carried out in 50 patients with 52 primary breast tumours. 39 of these 52 tumours were visualized. The intensity of scintigraphic visualization varied considerably between tumours. In ***figure Ia*** the SS-R scan is shown of a 70 yr old lady with a bilateral tumour. In ***figure Ib*** the corresponding receptor autoradiographic results obtained on sections of both tumours removed from this patient, show specific binding of [^{125}I-Tyr^{3}]-octreotide to these tumours.

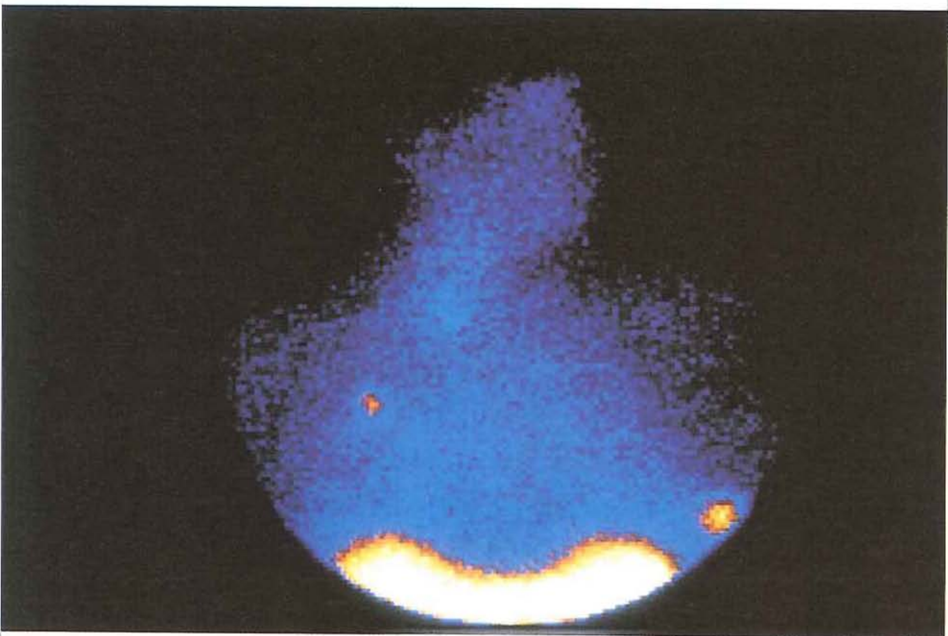

Figure I.
a. *[^{111}In-DTPA-D-Phe^{1}]-octreotide scintigraphy visualizing the bilateral breast tumours.*

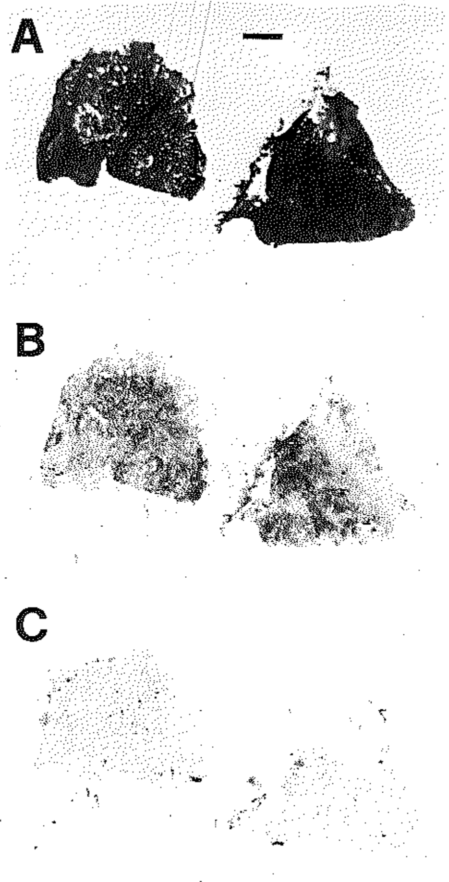

Figure I.
b. Distribution of somatostatin receptors in both breast tumour samples of the same patient:
(a) Haematoxylin-eosin stained sections containing mainly tumour tissue.
(b) Autoradiogram showing total binding of [^{125}I-Tyr^{3}]-octreotide.
(c) Autoradiogram showing non-specific binding of [^{125}I-Tyr^{3}]-octreotide (in presence of 10^{-6} M Tyr^{3}-octreotide).

In 30 of these 52 tumours autoradiographic studies of the surgically removed tumour tissue for the presence of SS-R could be done in parallel to the scintigraphy with [^{111}In-DTPA-D-Phe^{1}]-octreotide. SS-R's were present in 23 of these tumours. A comparison between the results of *in vivo* scintigraphy and *in vitro* autoradiography showed that receptors were found in both instances in 22 cases, while receptors were absent at both investigations in 6 cases. A discrepancy between the results obtained *in vivo* and *in vitro* was observed in 2 cases. In one tumour a non-homogeneously sparse distribution of SS-R's was found at autoradiography, while the tumour was not visualized *in vivo*. In the other case we could see low radioactivity on the scintigram of the breast containing a SS-R negative tumour according to autoradiographic examination. Two types of SS-R distribution were recognized at autoradiography of these tumours: in 16 cases the receptors were homogeneously and often

densely distributed over the tumour tissue, while they were found to be non-homogeneously scattered throughout the tumour tissue in 7 tumours. The non-homogeneous SS-R distribution was in all cases mainly related to a large non-invasive ductal carcinoma component, which was not found in the other SS-R positive tumours.

In retrospect especially those tumours showing a dense distribution of SS-R's *in vitro* had been visualized most clearly *in vivo*, whereas a low density *in vivo* coincides with a non-homogeneous distribution of receptors. As an example ***figure IIa*** shows the scan of a 56 yr old patient with a T_2 ductal carcinoma with a large non-invasive component. In ***figure IIb*** the *in vitro* autoradiographic study with [^{125}I-Tyr^3]-octreotide shows only specific binding of somatostatin throughout the non-invasive component of this tumour tissue. The *in vivo* [^{111}In-DTPA-D-Phe^1]-octreotide scan in this patient showed non-homogeneous, low radioactivity (see for comparison ***fig.Ia*** and ***fig.IIa***).

Figure III shows the scintigram of a 39 yr old patient with a T_2 invasive ductal carcinoma of the left breast. Physical examination did not reveal palpable lymphnodes in the axilla. The scan made 24 hours after injection of the [^{111}In-DTPA-D-Phe^1]-octreotide showed radioactivity in the axillary region, however. Indeed histological confirmation of axillary lymphnode metastases in the tissue removed at operation was made thereafter.

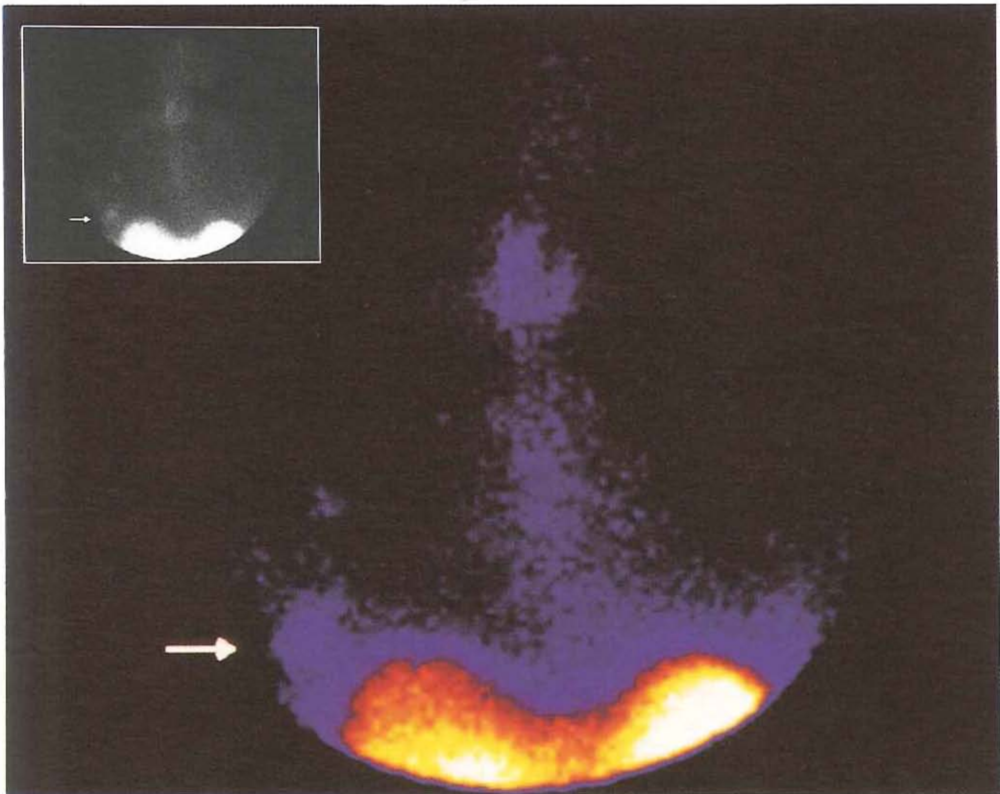

Figure II.

a. *[^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy of a 56 yr old patient visualizing the tumour in the right breast weakly (arrow).*

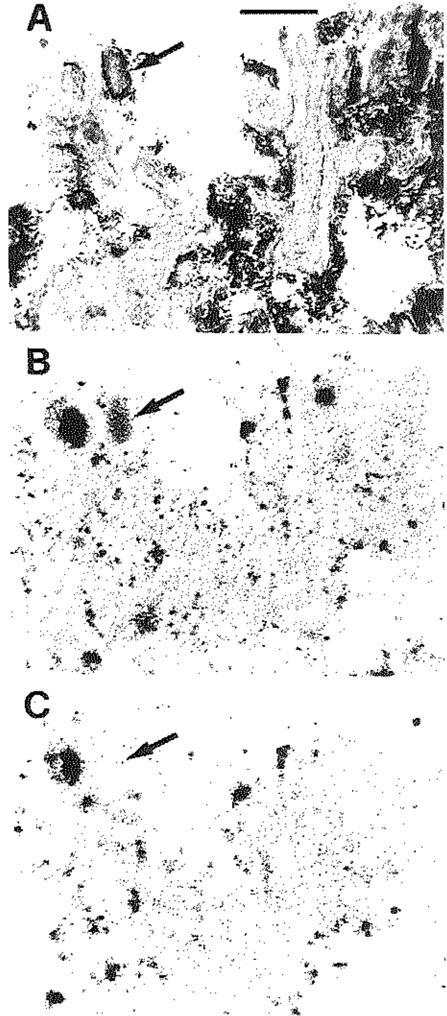

Figure II.

b. *Distribution of somatostatin receptors in a breast tumour sample of this patient:*

(a) Haematoxylin-eosin stained sections containing non-invasive tumour tissue, ductal carcinoma in situ (DCIS).

(b) Autoradiogram showing total binding of [^{125}I-Tyr^3]-octreotide. Only one limited region containing DCIS is strongly labelled.

(c) Autoradiogram showing non-specific binding of [^{125}I-Tyr^3]-octreotide (in presence of 10^{-6} M Tyr^3-octreotide).

Out of the subsequent 13 consecutive patients with histologically proven non-palpable axillary lymphnode micro-metastases and a positive SS-R scan of the primary tumour, these lymphnode metastases were visualized in 4 patients. None of the patients with a negative scan of the primary tumour showed abnormal radioactivity in the axillae or elsewhere in the body.

Further analysis of patient data and the results of SS-R scintigraphy showed no correlation between [^{111}In-DTPA-D-Phe^1]-octreotide visualization of the tumours *in vivo* and age (***table 1***). Eighty five % of the ductal carcinomas could be visualized and 56% of the

lobular carcinomas ($p<0.05$; ***table 2***). Also significantly more T_2 tumours were visualized than T_1 tumours ($p<0.05$;***table 3***).

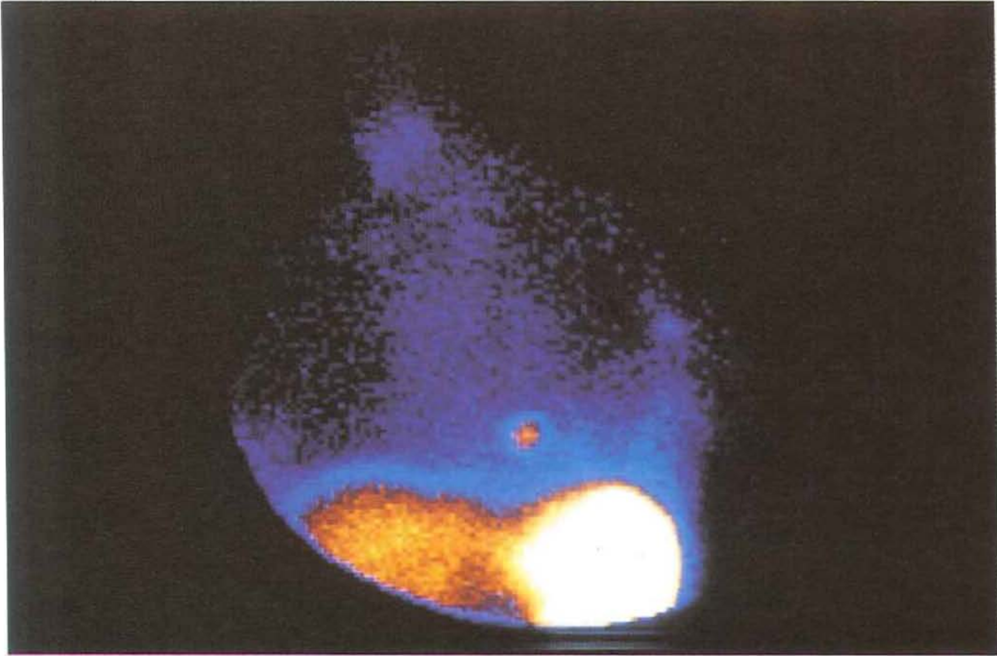

Figure III.
[^{111}In-DTPA-D-Phe^1]-octreotide scintigraphy of a 39 yr old patient visualizing the breast tumour on the left side and also its axillary lymphnode metastases.

Table 1. *Probability of finding [^{111}In-DTPA-D-Phe^1]-octreotide uptake in primary breast tumours, related to the age of the patients.*

Age	No. of tumours	Positive SS-R scan No. of tumours (%)
< 60 yr.	18	15 (83%)
> 60 yr.	34	24 (71%)

The 37 patients with 39 SS-R positive primary breast cancers were selected to undergo repeat SS-R scintigraphy 2.5 years after initial therapy (after 23-36 months). Of these patients 2 had died (one due to metastatic breast cancer), 3 were bedridden at home in a bad clinical condition (2 related to metastatic breast cancer). Two patients had been discharged from follow-up because of their age and 2 patients refused to undergo repeat scintigraphy.

Of the remaining 28 patients with an originally SS-R positive primary breast cancer 2 patients had already undergone a repeat scintigraphy because of symptoms suspected for

metastases. In one of these patients SS-R positive metastases had been visualized in the liver, lung and cervical spine and in the other patient both axillary and mediastinal lymphnode metastases were seen on the side of the original breast tumour.

Table 2. *Probability of finding [^{111}In-DTPA-D-Phe^{1}]-octreotide uptake in primary breast tumours, related to histology.*

Histology	No. of tumours	Positive SS-R scan No. of tumours (%)
Ductal carcinoma	40	34 (85%)
Lobular carcinoma	9	5 (56%)*
Medullar carcinoma	2	0
Tubular carcinoma	1	0
Total	52	39 (75%)

* $p<0.05$ vs ductal carcinomas

Table 3. *Probability of finding [^{111}In-DTPA-D-Phe^{1}]-octreotide uptake of primary breast cancer, related to tumour size and pathological stage.*

Tumour size	No. of tumours	Positive SS-R scan No. of tumours (%)
T_1	23	14 (61%)
T_2	28	24 (86%)*
T_4	1	1
Pathological stage		
T_1N_0	15	9 (60%)
T_2N_0	14	12 (86%)
T_1N_1	8	5 (62%)
T_2N_1	9	8 (89%)
T_2N_2	5	4 (80%)
T_4N_2	1	1

*$p<0.05$ vs T_1

The other 26 patients were symptom-free: both the clinical history, as well as physical examination were uneventful, while a yearly repeated mammogram was normal. In 6 of these 26 patients [^{111}In-DTPA-D-Phe^{1}]-octreotide scintigraphy showed lesions suspected for metastases which could be confirmed by other diagnostic tools (biopsy, bone scan, ultrasound, CT-scan): bone (n=4), liver (n=1) and pulmonary (n=2) as well as pleural (n=1) metastases were readily seen, as well as metastases in axillary (n=2), infraclavicular (n=1) and mediastinal (n=1) lymphnodes. In one patient, treated by lumpectomy and axillary dissection, a local recurrence was seen at SS-R scintigraphy. In ***figure IVa-c*** examples of the scans obtained in one patient are shown.

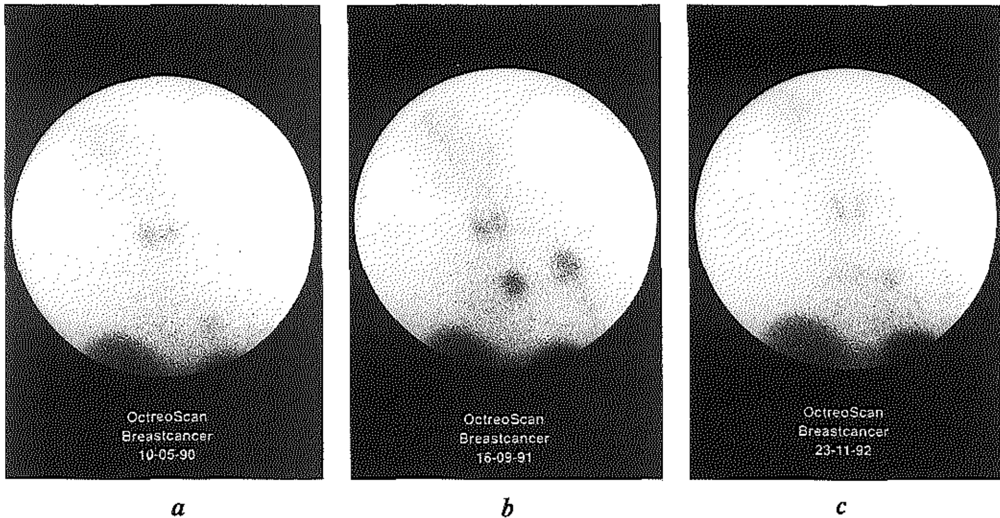

Figure IV.

a. *58 yr old patient with primary breast cancer of the left breast. Also the normal thyroid gland is visualized. (Histology: infiltrating ductal carcinoma, without axillary lymphnode metastases.*

b. *17 months later mediastinal and axillary lymphnode metastases were visualized at somatostatin receptor scintigraphy on the left side. (Cytology: axillary lymphnode showed ductal carcinoma).*

c. *Treatment with chemotherapy followed (6 cycles of Carboplatin 300 mg/m^2 and Etoposide 100mg/m^2 intravenously every 4 weeks). Thirteen months later there was a complete disappearance of the axillary tumour uptake and a lower uptake of the mediastinal tumour. The axillary lymphnode metastases were not seen any more at the CT-examination, while the mediastinal tumour had the same size as before.*

Fourteen patients with an originally SS-R positive primary tumour which had been visualized, showed a normal somatostatin scan in the follow-up. Slight scattered radioactivity distributed over one lung was seen in 6 patients after radiotherapy which had been given after lumpectomy and axillary dissection. The 13 patients with an originally SS-R normal scan were not rescanned after 2.5 years. None of these patients had died, all patients were symptom-free.

Serum CA 15-3 and CEA levels were normal at first presentation in patients with SS-R positive primary tumours in 35 and 37 patients, respectively. None of these tumour markers was elevated in patients with primary SS-R negative tumours, of which the SS-R status is based on SS-R scintigraphy. At follow-up only one of the symptomatic patients had elevated CA 15-3 and CEA levels, and also only one of the asymptomatic patients had an increased level of CA 15-3, which however was already elevated on admission.

All patients with a normal SS-R scan in their follow-up had serum tumour marker levels in the normal range except for one patient who had a slightly elevated CA 15-3 as was the case at first presentation (***table 4***).

Table 4. *CA 15-3 and CEA levels in patients with SS-R positive primary breast tumours and proven recurrent disease according to SS-R scintigraphy at follow up of 30 months.*

Patient category	CA 15-3(U/ml)		CEA (ng/ml)	
Symptomatic	Admission	Follow-up	Admission	Follow-up
(n=2)	17	23	2	2
	17	331	1	30
Asymptomatic				
(n=6)	11	14	1	1
	42	92	1	2
	11	11	1	1
	22	20	1	1
	22	22	8	8
	25	21	2	2

Symptomatic : Patients with clinically overt metastatic disease
Asymptomatic: Patients without clinically overt metastatic disease
Normal value: CEA < 10 ng/ml; CA-15-3 < 27 U/ml

Table 5. CA 15-3 and CEA mean levels in 29 patients with SS-R positive primary breast tumours and in 20 patients with normal SS-R scintigraphy at follow-up of 30 months.
Nine patients had no follow-up.

Tumour marker	Admission	Follow-up
CA 15-3 (U/ml)[1]	18 (7-38*)	17 (6-40*)
CEA (ng/ml)[1]	3 (1-6)	3 (1-8)

[1] mean levels
* values of the same patient

Table 6. CA 15-3 and CEA levels at first presentation in 13 patients with SS-R negative primary breast tumours.

Tumour marker	Mean Serum value (range)
CA 15-3 (U/ml)	15 (6-22)
CEA (ng/ml)	2 (1-5)

Discussion

The SS-R imaging technique has been shown to be successful in the visualization of the primary as well as metastatic tumour sites of a variety of neuroendocrine tumours like carcinoids, islet cell tumours and paragangliomas[12-16]. Validation of this technique was reached by the *in vitro* demonstration of high-affinity binding sites for somatostatin on those tumours, which had been visualized *in vivo*[14]. In parallel a positive SS-R scan closely predicted a beneficial effect of chronic octreotide therapy on hormonal hypersecretion by these tumours.

Also part of human breast cancers contain SS-R's as measured. In a group of 158 "small" breast cancer samples with a mean section surface of 14 mm^2 34 tumours (21%) were SS-R positive, while in a group of 72 "larger" tumour samples with a mean section surface of 180 mm^2 33 tumours (46%) were SS-R positive[6]. A subpopulation of SS-R containing breast tumours is probably neuroendocrine differentiated, as shown by Papotti et al.[8]. Studies by Bussolati et al.[17] and Papotti et al.[8] indicated a high variability of the incidence of neuroendocrine characteristics in breast cancer. The percentage of positive cases varied not only according to the specificity and sensitivity of the neuroendocrine markers used, but depended also on the number of tissue slices per tumour investigated microscopically[8].

In the present study we visualized 39 of 52 primary breast cancers (75%) with SS-R. There was a close correlation between the *in vivo* [^{111}In-DTPA-D-Phe^1]-octreotide scan and subsequent *in vitro* findings at autoradiography with ^{123}I-Tyr^3-octreotide, also confirming and validating the concept of receptor imaging in this kind of tumour. There was considerable

variability in the accumulation of radioactivity at SS-R scintigraphy. A higher density *in vivo* turned out to correlate in most instances with a homogeneous and dense distribution of SS-R's throughout the tumour at autoradiography, while a lower density of radioactivity over the tumour area *in vivo* in most instances corresponded with a non-homogeneous and often sparse distribution of these receptors. Interestingly, the low density of receptors *in vivo* and *in vitro* seemed to be due to the occurrence of a non-invasive carcinoma component, mainly being ductal carcinoma in situ.

The high incidence of SS-R's in these 52 carcinomas (as observed with the *in vivo* technique) might be related to several causes: firstly, it is hypothesized that *in vivo* SS-R visualization of the primary breast cancer is more sensitive than *in vitro* autoradiography using sections of parts of the tumour only, as this nuclear medical technique investigates in fact the presence of receptors three-dimensionally in the entire tumour. In accordance with this, statistically significantly more T_2 tumours than T_1 tumours were visualized *in vivo*. Secondly, our patients might represent a "selected" group in comparison with those found in other countries, as the incidence of ductal carcinomas amongst the patients with newly diagnosed breast cancers has increased over the last years in the Netherlands after the introduction of routine and especially repeated screening of the population[19]. Significantly more (locally) invasive ductal carcinomas were visualized with this technique.

Very little is known concerning the biological behaviour of SS-R positive breast cancer in man. In a retrospective study involving 110 patients Foekens et al.[20] suggested that the presence of SS-R might predict a longer disease-free survival. Also *in vitro* studies in more than 300 breast cancer samples showed an inverse relationship between somatostatin and EGF-receptor expression[6,9]. These observations suggest that patients with SS-R positive cancers might have a relatively good prognosis. This, however, seems not to be substantiated by our observations. In the follow-up after 2.5 years we found that from the 37 patients with a SS-R positive tumour at least 5 had extensive metastases (one had died, two were bed-ridden because of metastases and in two symptomatic patients the scan indicated the presence of multiple metastases), while also 6 of 26 symptom-free patients had (multiple) histologically proven metastases, as initially visualized at SS-R scintigraphy. It is unclear at present how much time might have elapsed before the disease would have become symptomatic in these cases.

CA 15-3 and CEA are the most commonly used tumour markers to monitor patients with recurrent breast cancer. Both markers are elevated in only 5-20% of women with primary breast cancer, but elevations between 61 and 84% have been recorded for women with extensive metastatic disease. CA 15-3 seems to be related to the extent of the metastases, the number of metastatic sites and survival, whereas CEA is only correlated with the extent of disease[21,22].

In this study we show a higher sensitivity of SS-R scintigraphy compared with these tumour markers to detect the development of recurrent breast cancer in patients with SS-R positive primary breast cancer. SS-R scintigraphy demonstrated recurrent disseminated breast cancer in 8 (only two of whom were symptomatic for recurrence) out of 28 patients with SS-R positive primary tumours. Six patients had normal CA 15-3 and CEA serum values (***table 4-6***). Another three out of these 28 patients had abnormal serum tumour markers, two of whom had an abnormal SS-R scintigram. The third patient, who only showed marginally elevated CA 15-3 serum levels both at first presentation and follow up, is clinically without evidence of disease 3.2 years after operation. Possibly the CA 15-3 in this patient is false positive for breast cancer.

Our data include a number of new aspects with regard to the diagnosis and therapy of breast cancer. We have presented evidence that the technique of peptide receptor visualization of primary breast cancer with a radionuclide-labelled somatostatin analogue is successful in 75% of cases. At the primary diagnosis the scintigraphic technique seems of minor value in the detection of axillary lymphnode metastases. Very often axillary lymphnode metastases include only microscopically recognized tumour cell nests.

The results sofar also suggest that the (distant) metastases of the primary somatostatin receptor breast tumours continue to express such receptors at 2.5 years after the initial therapy. The scintigraphic technique showed the unexpected presence of (multiple) metastases in nearly 25% of symptom-free initially SS-R positive breast cancer patients, who had normal CA 15-3 and CEA serum values. At the moment one can only speculate what the clinical relevance is to detect recurrent disease in an early stage.

Studies with breast cancer cell lines indicate a direct receptor-mediated inhibitory effect of somatostatin (analogues) on cell proliferation[10,23-25]. No prospective, controlled studies on the use of somatostatin analogues in patients with breast cancer have been published yet. It is concluded, that the technique of SS-R is effective in the early detection of SS-R positive breast cancer recurrence. Therefore it may be used in the future for the selection of patients who can be treated with somatostatin analogues and/or other forms of therapy. Also radiotherapy with an α- or ß-emitting radionuclide coupled to a somatostatin analogue might be considered, especially when disseminated disease is recognized.

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CHAPTER IV

SOMATOSTATIN RECEPTOR-POSITIVE PRIMARY BREAST TUMOURS: GENETIC, PATIENT AND TUMOUR CHARACTERISTICS

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Abstract

In a series of 87 primary breast tumours, somatostatin receptor (SS-R) expression was detected by *in vitro* autoradiography in 58 tumours. In 41 tumours the SS-R expression was homogeneous and in 17 it was heterogeneous. Although the tumours were not selected by the investigators upon entry in the study, examination of the tumour and patient characteristics showed that a pre-selection had taken place for small tumours. Eighty percent of the tumours were classified as stage pT1 or pT2 tumours. The large size of the tumour sections used for autoradiography can explain the high incidence of somatostatin expression in our series. Forty-three of these tumours, 30 SS-R positive and 13 SS-R negative, were tested for morphological and (immuno)histochemical markers of neuro-endocrine differentiation. Three SS-R positive tumours were also positive for 2 or more other markers of neuroendocrine differentiation, suggesting that neuroendocrine breast tumours and SS-R positive breast tumours are overlapping, but independent, subgroups of tumours. To test whether specific genetic alterations are associated with SS-R positive or SS-R negative breast tumours, we examined in a selected series of 47 SS-R positive and 32 SS-R negative breast tumours a number of known genetic markers by Southern blotting. Deletions or rearrangements of the retinoblastoma (*RB*) tumour-suppressor gene were observed in 5 SS-R positive and 5 SS-R negative tumours. In 4 SS-R positive and also in 4 SS-R negative tumours an amplification of the *neu* oncogene was observed. Amplifications of the *int*-2 oncogene were found in 2 SS-R positive and 1 SS-R negative breast tumour. In one SS-R positive tumour an amplification of the c-*myc* oncogene was observed and in another SS-R positive tumour a rearrangement of the L-*myc* oncogene was found. These results indicate that no correlation exists between SS-R expression and alterations of the RB gene or amplifications of one of the investigated proto-oncogenes.

Introduction

The study of breast carcinogenesis is complicated by the heterogeneity of the disease. One way of simplifying the matter is to subdivide these tumours into clinically relevant subgroups. There are indications that breast carcinomas which express the somatostatin receptor (SS-R) are such a distinct subset. For example, patients with a SS-R positive tumour have a better prognosis[1]. Tumours of these patients show a correlation between SS-R and oestrogen receptor expression and an inverse correlation between SS-R expression and epidermal growth factor (EGF) receptor expression[2,3]. Papotti and co-workers reported a correlation between SS-R expression and neuroendocrine differentiation in a series of 100 primary breast tumours[4].

Alterations in oncogenes and tumour-suppressor genes are the fundamental changes that can lead to tumour formation. In breast cancer a number of genetic changes have been

identified:loss of heterozygosity of the chromosome regions 1q, 3p, 11p, 13q14 (*RB* gene), 17p13, 18q and amplifications of the *int*-2, *myc* and *neu* oncogenes[5-9]. At present it is not clear whether these genetic alterations are equally important in all types of breast cancer or whether a particular combination of events is present in a histologically and clinically distinct subgroup of tumours[10,11]. The finding of *neu* amplifications predominantly in the comedo type ductal carcinoma *in situ*[5] and loss of heterozygosity of regions of chromosome 22q in lobular carcinoma[9] strongly support this latter possibility.

The aim of this study was to investigate whether breast tumours expressing SS-R are a distinct subgroup at the genetic level. To this end a series of primary breast tumours was collected and tested for SS-R expression and genetic alterations. We chose genetic markers that are frequently altered in both breast carcinomas and in neural or endocrine tumours such as neuroblastoma and small cell lung carcinoma. These are alterations of the retinoblastoma gene (*RB*) and/or amplification of members of the *myc* family of oncogenes. Amplification of the *neu* and *int*-2 oncogenes were also studied since these frequently occur in breast cancer.

Furthermore, clinical and pathological characteristics of the tumours were investigated. In addition, to test whether the reported correlation between SS-R expression and neuroendocrine differentiation was present in this population, 43 tumours were also examined for neuroendocrine differentiation characteristics.

Material and methods

Patient material

Eighty seven breast tumours were obtained from the surgical departments of the Dijkzigt Academic Hospital and the Dr. Daniël den Hoed Cancer Centre in Rotterdam between 1984 and 1990. Immediately after removal of the tumour, one part was taken for histological examination and the remainder was snap frozen and stored in liquid nitrogen until use. Genetic studies were begun on 27 tumours from our archives, that were selected for presence or absence of the SS-R. All tumours were histologically classified according to the criteria of Azzopardi et al.[12].

Patient and tumour characteristics were compared with SS-R status of the tumours. Data were collected on age at onset, oestrogen receptor status, lymphnode involvement, histology, stage and differentiation grade of the tumours. These data are summarized in ***table 1***.

Somatostatin receptor autoradiography

Frozen tumour samples were shipped on dry ice to the Sandoz Research Institute in Bern (Switzerland), where the SS-R's were measured by autoradiography on 10 μm cryostat sections. The size of the sections was over 100 mm^2. As a ligand, the iodinated stable analogue of somatostatin [^{125}I-Tyr^3]-octreotide was used. Incubation and washing conditions were as described[2]. Non-specific binding was determined by adding unlabelled [Tyr^3]-octreotide. Results were scored on a semi-quantitative scale. A tumour was counted as positive when specific binding was at least twice as high as the non-specific binding obtained by incubation in the presence of non-radiolabelled octreotide. Positive results were further

classified as homogeneously positive when the whole section of tumour tissue stained positive. A tumour was heterogeneously positive when patches of positive cells were present in the tumour section.

Table 1. Clinical and pathological data from 87 primary breast tumours.

		All	SS-R neg.	SS-R pos.		
				Total	Homogeneous	Heterogeneous
Number		87[6]	29	58	41	17
Age[1]		57.5	60	56.2	56.9	54.4
ER[2]		45/64(70%)	14/21(67%)	31/43(72%)	20/28(71%)	11/15(73%)
Nodes[3]		31/73(42%)	12/26(46%)	19/47(40%)	14/33(46%)	5/14(36%)
Stage[4]	pT1	30/81(37%)	8/29(28%)	21/52(40%)	17/37(46%)	4/15(28%)
	pT2	38/81(46%)	16/29(55%)	22/52(42%)	15/37(40%)	7/15(47%)
	pT3	6/81(7%)	1/29(3%)	5/52(10%)	3/37(8%)	2/15(13%)
	pT4	8/81(10%)	4/29(14%)	4/52(8%)	2/37(5%)	1/17(7%)
Grade[5]	I	15/84(18%)	4/29(14%)	11/55(20%)	6/38(16%)	5/17(29%)
	II	27/84(32%)	8/29(28%)	19/55(45%)	13/38(34%)	6/17(35%)
	III	42/84(50%)	17/29(59%)	25/55(45%)	19/38(50%)	6/17(35%)

[1]Mean age in years. [2]>10fmol/mg cytosol protein, measured by ligand binding assay or enzyme immunoassay.[3]Post-surgical regional lymphnodes present.[4]pT post-surgical primary tumour stage according to TNM classification.[5]I, highly differentiated;II, moderately differentiated;III, poorly differentiated.[6]For specific subgroups, the number do not add up to 87 due to missing values.

Histology and immunohistochemistry

Sections of 5 μm were cut from formalin-fixed, paraffin-embedded tumour tissue. For histological examination the sections were stained with either haematoxylin and azophloxin or haematoxylin and eosin. To examine the neuroendocrine differentiation the original Grimelius silver impregnation method was used[13]. Other neuroendocrine differentiation markers were examined using serial sections collected on poly-L-lysine treated slides and processed for immunohistochemistry. Antibodies against neuron-specific enolase (NSE) (DAKO, Copenhagen, Denmark), chromogranin A (ENZO, New York, NY, USA) and chromogranin B (Dr. H. Winkler, Innsbruck, Austria) were used as described[14].

Southern hybridizations

Between 5 and 10 μg of DNA, isolated from frozen tumour samples and peripheral blood lymphocytes (as control), were digested with *Hind*III restriction enzyme, separated by electrophoresis on 0.8% agarose gels, and transferred to Hybond N^+ nylon membranes (Amersham, Aylesbury, UK). The membranes were hybridized to ^{32}p-oligolabelled DNA probes. Hybridization and washing procedures were performed under standard conditions. After autoradiography on XAR films (Eastman Kodak, Rochester, NY, USA) for 1 to 7 days the membranes were stripped using 0.5% $NaDodSo_4$ (100^0C, 15') and re-hybridized. Hybridization to a myoglobin (MB) DNA probe served as an internal control for the amount

of DNA in each lane and the level of amplification.The following probes were used : *RB*, a 0.9 and a 3.8-kb *Eco*R1cDNA fragment; *int*-2, a 1.0-kb *Bam*H1-*Kpn*1 BK4 fragment; c-*myc*, a 1.6-kb *Cla*1-*Eco*R1 fragment; L-*myc*, a 1.8-kb *Sma*1-*Eco*R1 fragment; N-*myc*, a 1.0-kb *Eco*R1-*Bam*H1 fragment; *neu*, a 1.6-kb cDNA fragment and *myoglobin*, a 0.6-kb *Bgl*11-*Eco*R1 pcr-made fragment.

Results

Somatostatin receptor expression

Somatostatin receptor expression was assessed by autoradiography using a radiolabelled somatostatin analogue, [Tyr^3]-octreotide. In our series of 87 consecutive tumours, SS-R expression was detected in 58 tumours (67%). In 41 of these, (47%) SS-R expression was homogeneous and in 17 (20%) heterogeneous receptor expression was observed. The remaining 29 tumours (33%) showed no detectable SS-R expression. An example of an autoradiogram showing heterogeneous receptor expression is given in ***figure I***.

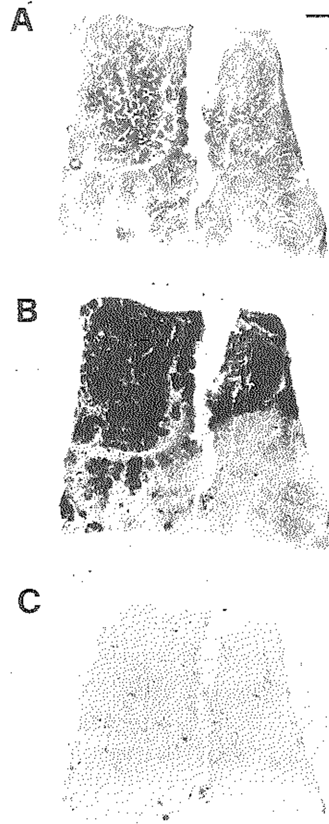

Figure I. *Heterogeneous expression of somatostatin receptors.*
- *(a) H&E stain of a section of a primary breast carcinoma, showing tumour tissue throughout the section*
- *(b) Autoradiogram showing total binding of the radiolabelled somatostatin analogue [^{125}I-Tyr^3]-octreotide to a serial section of the same tumour*
- *(c) Autoradiogram of the same tumour after incubation of labelled [^{125}I-Tyr^3]-octreotide in the presence of 10^{-6}M of unlabelled [Tyr^3]-octreotide, showing non-specific binding*

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Neuroendocrine differentiation

Neuroendocrine differentiation was tested in 43 samples from 52 non-selected tumours used in the genetic studies, from which paraffin embedded tumour tissue was available. The tumours were reviewed for neuroendocrine differentiation characteristics such as solid growth, lack of exocrine differentiation and monotonous cell morphology. In addition sections were stained by the Grimelius procedure and consecutive sections were incubated with antisera against neuroendocrine differentiation markers neuron specific enolase (NSE) and chromogranin A and B. The results are listed in ***table 2***.

Table 2. *Neuroendocrine markers in primary breast tumours*[1].

Case	Histology[2]	NSE	Chrom.A	Chrom.B	Grimelius	SS-R
1	-	+	-	-	-	-
3	-	+	-	-	-	+
6	-	+	-	-	-	+
15	-	-	-	5%	-	+
18	-	-	5%	-	3%	++
20	-	+	-	-	-	++
22	-	+	-	-	-	++
23	-	-	-	-	3%	-
25	-	+	-	-	-	+
26	-	+	-	-	-	+
27	-	+	-	-	-	+
28	-	++	-	?[3]	-	++
31	-	-	-	-	5%	-
37	-	+	-	-	-	-
39	-	+	-	-	-	++
43	-	+	-	-	20%	+
45	+	+	-	?[3]	-	+
49	-	+	-	-	-	++
50	-	+	-	-	-	++
51	-	+	-	-	-	+

[1]Only those tumours that were positive for one or more markers are listed.
[2]Presence of histological characteristics of neuroendocrine differentiation:
solid growth, lack of exocrine differentiation and monotonous cell morphology.
[3]Non-specific result.

NSE positivity was detected in 17 samples. In 4 tumour samples 3, 3, 5 and 20% of the tumour cells, respectively stained positive with the Grimelius procedure. In one of the tumours positivity for chromogranin A was detected in 5% of the cells. In another tumour 5% of the tumour cells were positive for chromogranin B.

We arbitrary scored a tumour as having a neuroendocrine differentiation when it was positive for at least 2 of these markers. Three tumours met this criterium: case 45 showed neuroendocrine morphological characteristics and was NSE positive, but negative for all other neuroendocrine markers;case 18 showed Grimelius and chromogranin A positivity in 3 and

5% of the cells, respectively;case 43 was NSE and Grimelius positive in 50 and 20% of the cells. These three tumours also expressed the SS-R and are regarded as having a weak neuroendocrine differentiation.

Genetic alterations

Genetic alterations of the *RB* gene and amplification or alteration of the *neu, int*-2, c-*myc*, L-*myc* and N-*myc* oncogenes were investigated on Southern blots in 79 primary breast tumours. Thirty six tumours expressed the SS-R homogeneously, 11 expressed the receptor heterogeneously and in 32 tumours no SS-R's could be detected. The tumours were not otherwise stratified. The results are shown in ***table 3***.

Deletion or rearrangement of the *RB* gene was detected in 10 tumours, of which 3 showed homogeneous SS-R expression, 2 heterogeneous SS-R expression and 5 were SS-R negative. *Neu* amplifications were detected in 4 SS-R positive and 4 SS-R negative tumours. In 2 SS-R positive and one SS-R negative sample, *int*-2 amplification was observed. One of these samples showed co-amplification of the *neu* and *int*-2 oncogenes, and one had both a deleted *RB* gene and *int*-2 amplification. C-*myc* amplification was observed in 1 SS-R positive tumour. Rearrangement of the L-*myc* oncogene was detected in 1 SS-R positive tumour. No alterations of the N-*myc* gene were detected. Compared to the hybridization signal of the myoglobin gene, 5 to 15 fold amplifications were observed.

Table 3. *Genetic alterations in 79 primary breast tumours in relation to SS-R expression.*

Gene alteration		SS-R expression		
		Homogeneous	Heterogeneous	Negative
RB	Yes	3(8%)	2(18%)	5(16%)
	No	33	9	27
Neu	Yes	4(11%)	0	4(12%)
	No	32	11	28
int-2	Yes	2(5%)	0	1(3%)
	No	34	11	31
c-*myc*[1]	Yes	1(3%)	1(12%)	0
	No	29	7	30
L-*myc*[2]	Yes	1(3%)[3]	0	0
	No	30	10	30
N-*myc*	Yes	0	0	0
	No	36	11	32

[1]68 tumours were tested for c-*myc* amplification and [2]71 tumours for L-*myc* alterations-[3]rearrangements

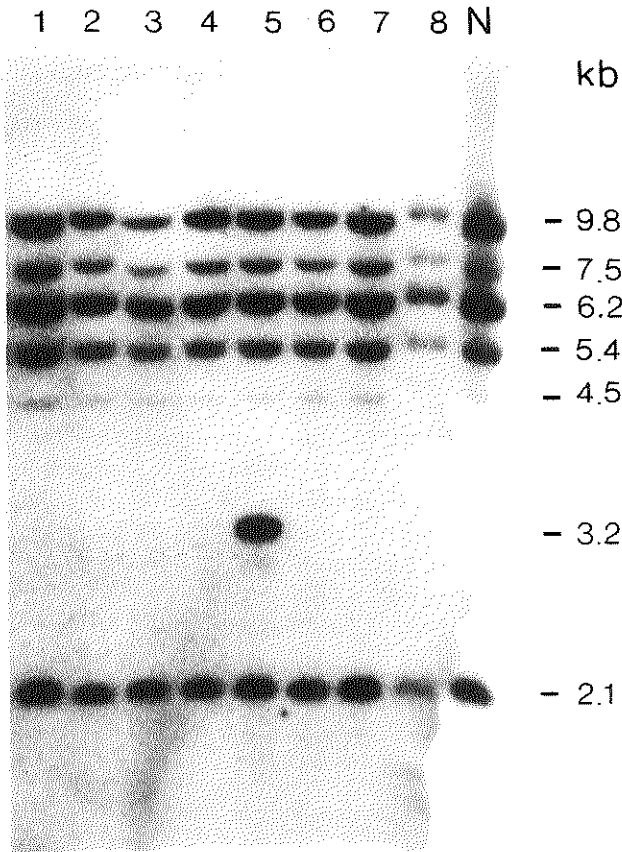

Figure II. *RB alterations in breast tumours.*
Autoradiogram of a Southern blot containing HindIII-digested DNA from 8 different primary breast tumours in lanes 1 to 8 and from lymphocyte control DNA in lane N. The blot was hybridized to a 3.8 kb-RB1 CDNA probe.

Examples of such rearrangements and amplifications on Southern blot are shown in ***figure II***and ***III***. In ***figure II*** lanes 1-8 contain *Hind*III digested tumour DNA's and lane N *Hind*III- digested control DNA from normal peripheral lymphocytes. This Southern blot was hybridized to a 3' *RB* CDNA probe. Two tumour DNA's show a clear rearrangement of the *RB* gene. The tumour in lane 5 shows an extra equimolar 3.2-kb fragment, and in lane 3 the top 2 hybridizing fragments of 9.8 and 7.8-kb are less intense then the other hybridizing fragments in this tumour, indicating a partial deletion of the *RB* gene. ***Figure III*** shows *Hind*III-digested tumour DNA's, hybridized with probes for the *neu* and c-*myc* oncogenes. The hybridization pattern of the myoglobin gene (MB) is included as a control for the amount of DNA. A *neu*-amplification is visible in lane d, where compared to the MB signal, the *neu*-signal is 5 times stronger.

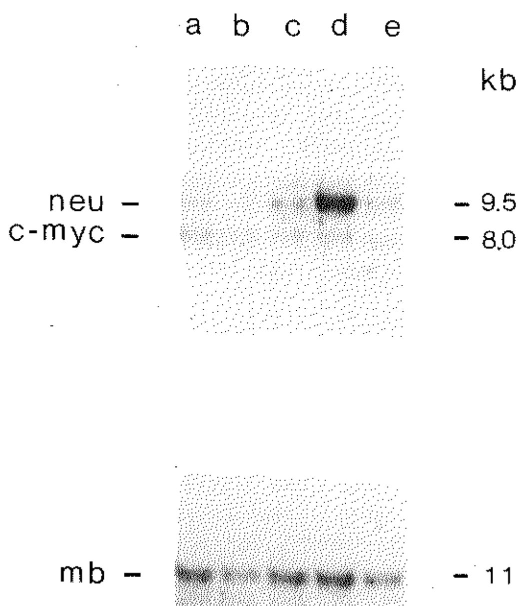

Figure III. *Oncogene amplfication in breast tumours*
Autoradiogram of a Southern blot containing HindIII-digested DNA from 5 different primary breast tumours, after hybridization to neu and c-myc gene probes. The DNA's were also hybridized with a myoglobin gene probe (MB) as a control for the amount of DNA.

Discussion

In our series of 87 primary breast tumours, we detected SS-R expression in 67% of the tumours. The incidence of SS-R expression was much higher than that reported in the literature to date. However, concurrently with this study, we screened a partly overlapping group of 50 patients for SS-R expression using an *in vivo* SS-R scanning technique prior to operation. In this group of patients, 75% of the tumours were positive for SS-R expression (Chap.III). Other studies on SS-R expression in breast cancer report an incidence of 10 to 47%. Fekete et al.[15] detected binding of somatostatin in 36% of 500 breast cancer biopsy homogenates. Using autoradiography Reubi and Torhorst[2] and Papotti et al.[4] detected specific somatostatin analogue binding in 17%-20% of primary breast tumours. However, in a more recent study Reubi et al.[3] detected somatostatin analogue binding in 46% of primary breast tumours. In that study much larger tumour sections (180$\pm$8 mm^2) were examined for SS-R expression than previously. Over 50% of the SS-R positive tumours showed heterogeneous SS-R expression, which had not been detected previously and which may explain the 2-fold difference in incidence upon comparison with earlier studies.

In the present study we also used large tumour sections ($>$100 mm^2) for autoradiography. However, in this series only 17 of the 58 SS-R positive tumours showed

heterogeneous SS-R expression. Thus, a lower detection limit as a result of the use of larger tissue sections and subsequent detection of more heterogeneous SS-R positive tumours cannot fully explain the high incidence of SS-R positive tumours in this series of breast tumours.

Compared with other reports[1-4] our tumour series showed a similar distribution of age at onset and oestrogen receptor expression. Moreover, no difference was observed between the SS-R positive and SS-R negative tumours. However, our tumour population contained a high percentage of T1/T2 stage tumours (83%). This high percentage was found in both the SS-R positive and the SS-R negative subgroups, but the frequency of T1 tumours was higher in the SS-R positive group. In only one other study on SS-R expression, has tumour size been reported[16]. In that report T1 and T2 stages made up 59% (16/27) of the tumours and 3/27 of these tumours were SS-R positive.

A trend towards smaller tumour size in the Dutch breast tumour population was also observed in a larger study by Coebergh et al.[17], who reported a 2-fold rise in incidence of pT1 tumours (28 to 42%) and a 50% reduction of pT3/pT4 tumours in the years 1970-1986. The authors suggested that this shift towards smaller tumours could be due to the earlier detection as a result of population screening programs. Another factor contributing to the low percentage of later-stage tumours in our series, was the policy of the Surgery Department of the Dijkzigt Academic Hospital not to operate on patients with a clinically assessed T4 tumour. These T4 tumours were therefore seldom available for study. This bias in our set of tumours should be born in mind when comparing the results of the present study with those of other studies.

Therefore, a possible explanation for the high incidence of SS-R expression reported in this study could be the combination of a high percentage of pT1/pT2 tumours and the large size of the tumour sections (not of the tumours themselves) used for SS-R autoradiography, which allowed the detection of heterogeneous receptor expression.

Small tumour size has been correlated to a favourable prognosis and recently also to a lower differentiation grade (*i.e.* better tumour differentiation)[18]. Our data show a tendency towards a lower differentiation grade in SS-R positive tumours. This is concordant with clinical data assigning a favourable prognosis to patients with SS-R positive tumours.

Since SS-R expression has been correlated to neuroendocrine differentiation in other tumour type[5], such as lung cancer, we tested 43 tumours for expression of other markers of neuroendocrine differentiation. Seven percent (3/43) of these tumours showed neuroendocrine differentiation, which is within the range of 5-10% reported by others[4,14]. Since we found a normal low frequency of neuroendocrine differentiation, but a high incidence of SS-R expression in the same series, we conclude that SS-R receptor expressing breast tumours and those showing neuroendocrine differentiation in this study are overlapping, but independent subgroups.

In a selected group of 79 tumours we investigated whether any of the genetic alterations that are frequently observed in breast tumours were specific for the SS-R positive or SS-R negative subgroups. The Southern-blot method, used to detect changes in the *RB* gene and the *neu*, *int*-2 and *myc* oncogenes, does not permit identification of all possible alterations in these genes, but is useful for detecting subgroup specificity of these alterations. Our results indicate that no significant correlations exist between the presence or absence of the SS-R and the loss of the *RB* tumour-suppressor gene. Similarly, none of the investigated oncogene amplifications or rearrangements are specific for the subgroup of the SS-R positive or SS-R negative breast tumours. The *RB* and *myc* genes were chosen because they were reported to be altered in both breast cancer and neuroendocrine tumours. Our observations that these

alterations also occur in SS-R negative tumours lacking neuroendocrine markers suggests that these genetic changes are not restricted to breast tumours with neuroendocrine differentiation.

Nesland et al.[19,20] reported that *neu* amplification and neuroendocrine differentiation are mutually exclusive in breast tumours. In our series we detected 3 breast tumours with weak neuroendocrine differentiation. None of these tumours contained an amplified *neu* oncogene. This observation is in line with that of Nesland et al.[20], although the numbers are small.

No other reports have been published on the relation between genetic alterations and expression of the SS-R in breast tumours. Some data are, however available on the relationship between amplification of the *neu* oncogene and expression of the EGF receptor. A correlation between *neu* over-expression and EGF receptor expression was reported by Marx et al.[21]. This observation was in contrast to the results of others who did not find such a correlation[22,23]. In the present study SS-R content of the tumours was assessed. However, we have reported earlier that SS-R and EGF receptor expression are mutually exclusive in breast cancer[3]. This implies that the observed lack of correlation between SS-R expression and *neu* amplification and EGF receptor expression, accord with each other.

It is worth while to test other genetic markers in the subgroup of SS-R positive breast tumours, because the SS-R expressing breast tumours are a clinically relevant subgroup, and also because a high percentage of early stage tumours in this subgroup gives an opportunity to study those genetic alterations that are involved in the initial steps of tumour formation.

Addendum

First of all the high incidence of the SS-R expression found in this study, using autoradiography in large tumour sections, correlates well with the numbers of tumours which could be visualized *in vivo* by the [^{111}In-DTPA-D-Phe^{1}]-octreotide scintigraphy (Chapter III). Although the data found in this study showed a tendency towards better differentiation of SS-R breast tumours, this by no means is an indication towards better prognosis. Out of the 37 patients with a SS-R positive tumour 5 patients were found to have, clinically overt, recurrent disease during 2.5 years of follow-up, which is in contrast with the 17 patients with an initially SS-R negative tumour, who were all without evidence of disease (Chapter III). Several retrospective studies suggested that the presence of SS-R might predict a longer disease free survival, however in these studies small breast samples were investigated and therefore low numbers of SS-R positive tumours were found. In order to define the biological behaviour of SS-R positive breast cancer in man, prospective studies have to be carried out with a much longer follow-up period, using the somatostatin receptor scintigraphy for identification of SS-R positive tumours, since this nuclear medical technique investigates the presence of the receptors in the entire tumour, and may directly stage the patient Therefore this *in vivo* visualization technique is preffered to *in vitro* detection of SS-R's by for instance autoradiography.

In the 11 patients with recurrent SS-R positive disease no specific alterations or amplifications of the investigated genes could be found and only 2 of the tumours from these patients showed neuroendocrine differentiation.

ACKNOWLEDGEMENTS
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CHAPTER V

ROLE OF TUMOUR-DERIVED FIBROBLASTS IN THE GROWTH OF PRIMARY CULTURES OF HUMAN BREAST CANCER CELLS: EFFECTS OF EPIDERMAL GROWTH FACTOR AND THE SOMATOSTATIN ANALOGUE OCTREOTIDE

Int J Cancer in press

Summary

In the present study we have investigated in a co-culture system using Transwell tissue culture inserts with microporous membranes, the role of human breast cancer-derived fibroblasts in the proliferation of primary cultures of epithelial cells derived from the same tumour. Fibroblasts and epithelial cells were enriched according to differences in their density on Percoll density gradients. The co-culture system was first established using MCF-7 breast cancer cells and a human fibroblast line (HF cells).

Insulin (10 μg/ml), 17β-oestradiol (E2; 10 nM), Epidermal Growth Factor (EGF; 10 ng/ml), and HF cells all significantly stimulated growth of MCF-7 breast cancer cells. The stimulatory effects by insulin, E2, and EGF were additive to the stimulatory effect by HF cells. These data suggest that (unique) factors, other than the above mentioned growth promoting compounds, are responsible for the growth promoting effects by fibroblasts.

In 3 out of 6 human breast cancers investigated tumour-derived fibroblasts stimulated tumour-derived epithelial cell proliferation. EGF (10 ng/ml) significantly stimulated epithelial cell proliferation in 4 out of 6 cultures. The stimulatory effects of fibroblasts and EGF were additive or synergistic, and were observed in the additional presence of fetal calf serum, again suggesting production of unique factors by the fibroblasts. In one culture the fibroblasts significantly inhibited epithelial tumour cell proliferation. In reverse, the epithelial cells significantly stimulated proliferation of fibroblasts in 3 out of 3 cultures. The somatostatin analogue octreotide (1 Nm) significantly inhibited epithelial cell proliferation by 46% in one tumour cell culture in the absence, but not in the presence of fibroblasts. Interestingly, in one culture octreotide significantly inhibited the proliferation of fibroblasts co-cultured with epithelial cells.

In conclusion, our study clearly emphasizes the need to investigate in breast cancer the role of stromal cells on epithelial cell proliferation, as well as their role in interfering with drug-induced manipulation of breast cancer cell growth.

Introduction

Culturing primary human breast cancer cells has prove to be difficult. The most successful method appears to be treatment of the tissue with collagenase, subsequent 1 x g sedimentation of epithelial clumps and culturing the cells in media with specific substitutes[1,2]. One of the problems underlying these difficulties in culturing human breast cancer cells may be the dependency of the individual cancer cells for an intact autocrine and paracrine growth regulatory apparatus which is proposed to exist *in vivo*[3,4]. Recent laboratory studies also point to an important role of stromal cells in the development and growth of breast cancer. Conditioned media from human breast cancer-derived fibroblasts have been shown to enhance the growth of several human breast cancer cell lines via the secretion of (unknown) growth

factors[5-7]. This stimulatory effect of stromal cells on the growth of malignant breast epithelial cells has also been demonstrated *in vivo*[8,9].

The importance of stromal-epithelial interactions in the growth of epithelial tumour cells led us to investigate the growth of primary cultures of human breast cancer cells in the absence and presence of the corresponding tumour-derived fibroblasts. For this, we first separated the epithelial cells and fibroblasts on discontinuous Percoll density gradients. This method of enrichment of breast cancer cell populations has been applied successfully before[10]. Thereafter we have co-cultured both cell populations using Transwell tissue culture inserts with microporous membranes. In addition we also studied the effects of EGF and the somatostatin analogue octreotide in this co-culture system. A non-homogeneous distribution of receptors for EGF and somatostatin has been demonstrated by autoradiography by Reubi et al.[11] in 46% of large tumour samples of human breast cancer tissues, while in the same study 25% of the somatostatin receptor (SS-R) positive breast tumours were shown to contain EGF receptors. These two receptor types were not topographically overlapping in the majority of the cases, however. The growth factor and the neuropeptide may be involved in positive and negative growth regulation of human breast cancer cells, respectively. More knowledge about the interrelationship between the direct and indirect effects of octreotide and EGF on the growth of human breast cancer cells is of clinical importance in the light of recent clinical trials in which patients with advanced breast cancer are treated with octreotide[12]. The co-culture system using Transwell tissue culture inserts was first established using MCF-7 human breast cancer cells and human fibroblasts (HF) cells.

Materials and methods

Cell dispersion and separation of cells according to differences in their density.

Fresh tissue from 6 malignant breast tumours was obtained within 30 minutes after surgical removement. Histological diagnosis of tumour tissue was confirmed by routine histopathological examination. All specimens were diagnosed as infiltrating ductal carcinomas. The tissue was minced into pieces of approximately 1 mm^3, washed twice with isolation medium (see below), centrifuged at 100 x g for 5 min., and incubated overnight in culture medium containing 2 g/L collagenase. Thereafter, the remaining cell pellet was washed twice with isolation medium and incubated for another 1 hour at 37°C with a mixture of collagenase and dispase (1 g/L and 2.4 x 10^3 U/L, respectively) in order to obtain a single cell suspension. After this incubation period the cells were washed twice with isolation medium. The remaining cell suspension consisted of single cells now. An aliquot of this suspension was separated, the cells were counted and plated in multiwell plates (see below) and represents the original cell suspension. The remaining cells were layered on a discontinuous Percoll density gradient (1.04-1.05-1.06-1.07-1.08-1.10 g/ml) and centrifuged to isodensity during 20 min. at 800 x g. The cells present in the different fractions (interphases) were collected, washed twice with isolation medium, counted and cultured as described below. Percoll was obtained from Pharmacia (Uppsala, Sweden).

Cell culture

The human epithelial tumour cells with equilibrium densities of >1.07 g/ml[10], were resuspended in culture medium, counted and seeded in multiwell plates in a concentration of

25.000 cells per well. Fibroblasts with an equilibrium density of <1.05 g/ml[10] were resuspended in culture medium, counted and seeded in a number of 25.000 cells per Transwell in Transwell microporous membranes (0.4 μm; Costar Europe Ltd., Badhoevedorp, The Netherlands). The Transwells were transferred into wells containing 1 ml of culture medium without or with epithelial cells. Thereafter, the epithelial cells (without or with fibroblasts) were incubated without or with test-substances for 5-7 days. During the last 24 hours 3.7 x 10^4 Bq of [methyl-^{3}H]-thymidine was added to the wells. The medium was removed, the cells were washed twice with ice-cold 0.15 mol/L NaCl, solubilized with 1 mol/L NaOH and transferred to vials for scintillation counting of the incorporated radioactivity.

The isolation medium was Hanks' Balanced Salt Solution (HBSS) supplemented with 10 g/L human serum albumin, penicillin (10^5 U/L) and sodium bicarbonate (0.4 g/L). Culture medium consisted of DMEM/F-12 (1:1) supplemented with non-essential amino acids, fetal calf serum (10%), human transferrin (10 μg/L; Sigma), ascorbic acid (50 μM) and sodium selenite (6 μg/L). Media and supplements were obtained from Gibco Brl (Paisley, Scotland). Human transferrin and ascorbic acid were obtained from Sigma Chemical Company (St. Louis, MO, USA), sodium selenite from Merck (Darmstadt, Germany).

MCF-7 human breast cancer cells were kindly provided by Dr. C. Quirin-Stricker (Institute de Chimie Biologique, Faculté de Médecine, Strasbourg, France). Fibroblasts isolated from human foreskins (HF cells) were kindly provided by Dr. M. Ponec (Academic Hospital Leiden, Leiden. The Netherlands). MCF-7 and HF cells were cultured in culture medium and were passaged twice a week using trypsin (0.05%) and EDTA (0.02%). In the experimental incubations, the MCF-7 cells (25.000 cells seeded per well) were cultured in multiwell plates in 1 ml culture medium with or without HF cells (2 x 10^5 cells per Transwell). After 1 day of culture, the medium was replaced by serum-free culture medium. After another 1 day of incubation, this medium was replaced by medium containing 10% growth factor inactivated fetal calf serum and the cells were incubated for 2 days without or with test-substances. Under these conditions optimal stimulation of MCF-7 cell proliferation by mitogens such as insulin or insulin-like growth factors is found[13]. At the end of the incubation the DNA content of the cells was measured using the bisbenzimide fluorescent dye (Behring Diagnostics, La Jolla, USA) as described previously[14]. Insulin (bovine) was purchased from Sigma, epidermal growth factor from Bissendorf Biochemicals (Hannover, Germany).

Immunocytochemical detection of keratin and BrdU

For keratin- and BrdU- staining the epithelial cells were cultured on glass coverslips. For keratin-staining, the cells were fixed at the end of the incubation period during 10 min. with methanol. Staining for keratin was done using a DAKO PAP KIT™ System (code K518; DAKO A/S, Glostrup, Denmark). For BrdU- staining, the cells were incubated for 60 min. with 5-bromo-2'-deoxy-uridine, washed twice with PBS, and fixed during 20 min. at -20°C in 70% ethanol in glycin buffer (50 mmol/L, pH 2.0). Staining for BrdU was done using a BrdU- labelling and detection Kit II from Boehringer (cat. no. 1299 964; Boehringer Mannheim B.V., Almere, The Netherlands). The cells were double-stained for keratin using the DAKO PAP KIT™ System as described above.

Statistical analysis of data

All values are expressed as mean ± SE, n=3 wells per treatment group. All data were analyzed using analysis of variance (ANOVA) to determine overall differences between treatment groups. When significant overall effects were found by ANOVA, a comparison between treatment groups was made using the Newman-Keuls test[15]. $P<0.05$ was considered to be statistically significant.

Results

Fibroblast-epithelial cell interactions in cell lines

We first established the co-culture system using transwell tissue culture inserts in MCF-7 human breast cancer cells co-cultured with human fibroblasts (HF cells). In initial experiments we determined the number of HF cells that were required to significantly stimulate MCF-7 cell proliferation. ***Figure 1*** shows that a low number of HF cells (10.000-20.000 HF cells seeded per transwell) only slightly stimulated MCF-cell proliferation, while a higher number (200.000 cells) stimulated MCF-7 cell proliferation by 76%. In the further co-culture studies we therefore used this higher number of HF cells.

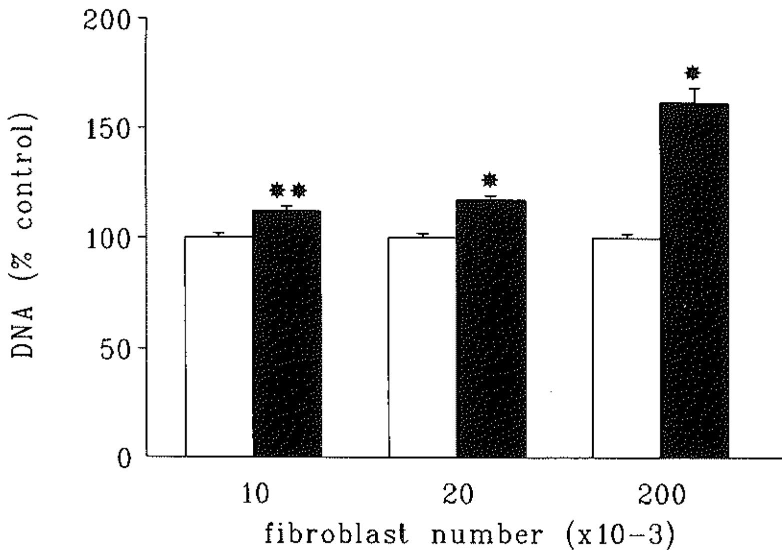


Figure 1.
The effect of human fibroblasts (HF cells) on the growth of MCF-7 breast cancer cells. HF and MCF-7 cells were co-cultured during 4 days using Transwell tissue culture inserts with microporous membranes as described in the Materials and Methods section. Open bars: MCF-7 cells without HF cells, filled bars: MCF-7 cells with HF cells; $^{*}p<0.01$ *and* $^{**}p<0.05$ *vs MCF-7 cells without HF cells.*

In *figure II* the effects of 17β-oestradiol (E2) and insulin on MCF-7 cell growth with or without co-culture with HF cells is shown. The HF cells significantly stimulated proliferation of MCF-7 cells (+61%; $p<0.01$ vs cells without HF cells). E2 (10 nM) and insulin (10 μg/ml) alone significantly stimulated MCF-7 cell proliferation by 76 and 75 %, respectively ($p<0.01$ vs control cells). The effects of E2 and insulin were additive and not synergistic to the stimulatory effect of the HF cells on MCF-7 cell proliferation. EGF (10 ng/ml) had, under these conditions, only a marginally stimulating effect (+25%, $p<0.01$ vs control cells). Again, the effect of EGF was additive to the effect of HF cells on MCF-7 cell growth (data not shown).

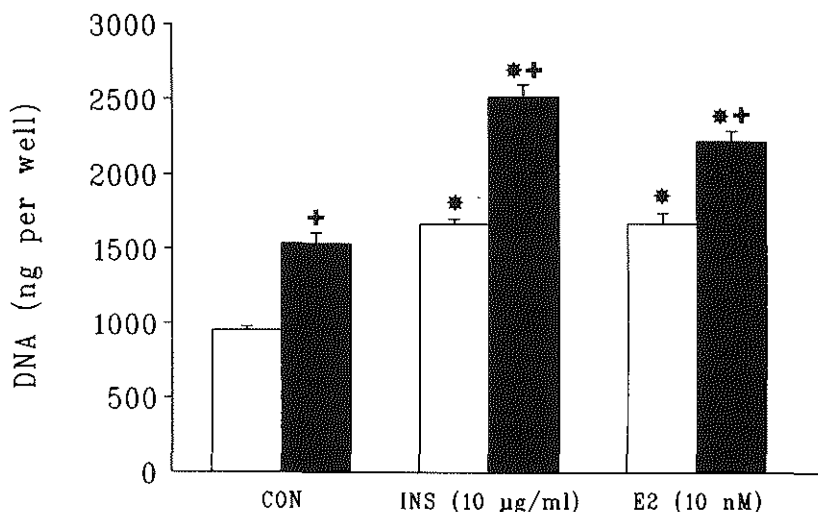


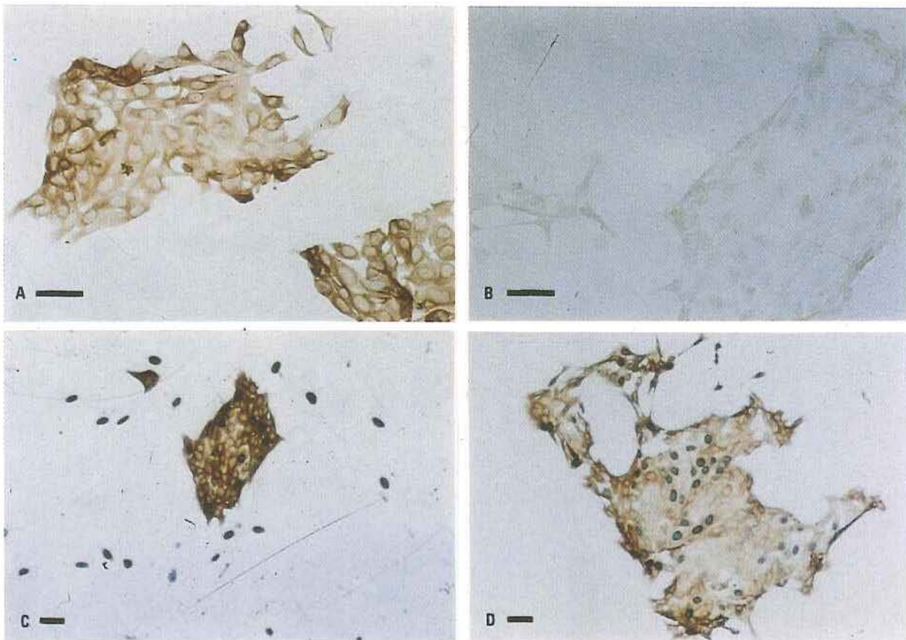
Figure II. *The effect of insulin (INS) and 17β-oestradiol (E2) on MCF-7 cell growth, with (filled bars) or without (open bars) co-culture with human fibroblasts (HF cells). MCF-7 and HF cells were co-cultured during 4 days as described in the Materials and Methods section. Insulin (10 μg/ml) and E2 (10 Nm) were added on day 3 of culture. $^*p<0.01$ vs control; $^+p<0.01$ vs cells without HF cells.*

Staining for keratin and incorporation of BrdU in primary cultures of human breast cancer cells.

In initial experiments, we have cultured cells from all fractions of the Percoll density gradients. On the basis of haematoxylin stained preparations of these fractions we found that cells with densities of less then 1.05 g/ml had a fibroblast-like appearance, while cells with a density of more then 1.07 g/ml were clearly epithelial-like. The intermediate density fractions showed a mixture of both cell types. In the further studies we therefore used cells with densities of < 1.05 g/ml (enriched in fibroblasts) and > 1.07 g/ml (enriched in epithelial cells) only. The original, unseparated cultured cell suspension consisted of both cell types, with a preponderance of fibroblasts. We also observed in most cultures that the Percoll-enriched fraction of epithelial cells grew best in the presence of fibroblasts and EGF (10

ng/ml). ***Photomicrograph 1A*** shows an example of the staining for keratin of an epithelial cell population co-cultured with fibroblasts and EGF (10 ng/ml). Virtually no fibroblasts (keratin-negative) are present. ***Photomicrograph 1B*** shows absence of staining using non-immune rabbit serum instead of rabbit antiserum to human keratin proteins.

Photomicrographs 1C and ***1D*** show an example of a double-staining for keratin and BrdU in proliferating cells from the same tumour. ***Photomicrograph 1C*** shows that in the original, unseparated cell suspension both proliferating fibroblasts (blue nucleus, keratin-negative) and epithelial cells (blue nucleus, keratin- positive) are present. In contrast, ***photomicrograph 1D*** shows that in the enriched epithelial fraction, mainly proliferating epithelial cells are present.



Photomicrograph 1:

Photomicrograph of immunocytochemical staining for keratin and/or BrdU of human primary breast cancer cells co-cultured with stromal cells derived from the same tumours. *A*:breast cancer-derived Percoll-enriched epithelial cells co-cultured with fibroblasts and EGF (10 ng/ml), keratin staining (red-brown); ***B***:cells as in A, negative control (normal rabbit serum) keratin staining; *C*:original cell suspension from breast cancer specimen as in A, cultured in the presence of EGF, double-staining for keratin (red-brown) and BrdU (blue nuclei); ***D***:epithelial enriched cells, co-cultured with fibroblasts and EGF as in *A*, double-staining for keratin and BrdU. Magnification bar = 50 μm.

Therefore, measurement of ^{3}H-thymidine incorporation in the enriched epithelial cell fraction will indeed represent proliferation of epithelial cells, and we performed further studies on the effects of fibroblasts on the proliferation of enriched epithelial breast cancer cells. Using this co-culture system also the effects of EGF (10 ng/ml) and the somatostatin analogue octreotide (1 Nm) were studied.

The effect of tumour-derived fibroblasts on ^{3}H-thymidine incorporation in tumour-derived epithelial cells.

Figure III shows the effect of Percoll-enriched fibroblasts (F) on ^{3}H-thymidine incorporation of Percoll-enriched tumour-derived epithelial cells of six breast cancers. The fibroblast fraction significantly stimulated ^{3}H-thymidine incorporation in 3 out of 6 cultures (no. 1, 2 and 5), and inhibited ^{3}H-thymidine incorporation in one (no. 3).

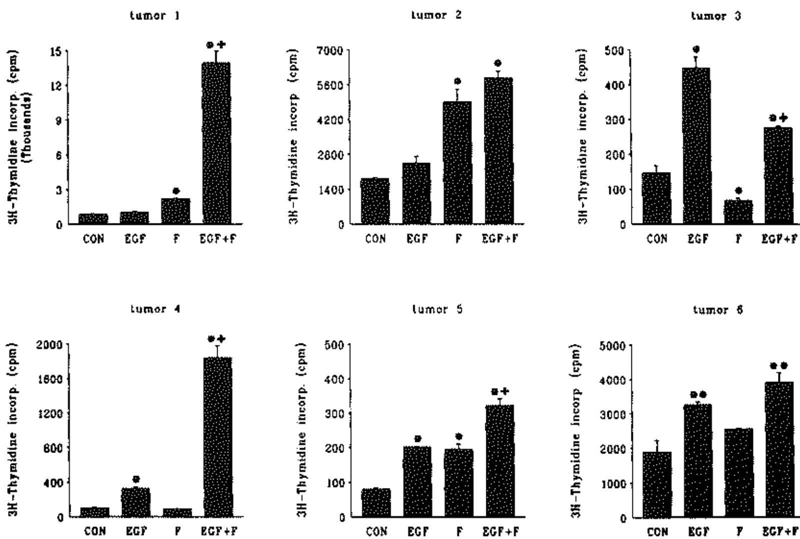


Figure III. *The effects of breast cancer- derived fibroblasts (F) and 10 ng/ml epidermal growth factor (EGF) on ^{3}H-thymidine incorporation by Percoll-enriched epithelial cells derived from the same tumours. Fibroblasts and epithelial cells were co-cultured using Transwell tissue culture inserts as described in the Materials and Methods section. $^*p<0.01$ and $^{**}p<0.05$ vs control; $^+p<0.01$ vs EGF alone. CON=control, EGF= 10 ng/ml EGF, F=fibroblasts.*

EGF (10 ng/ml) alone significantly stimulated ^{3}H-thymidine incorporation in 4 out of 6 cultures (no. 3, 4, 5, 6). The effects of fibroblasts and EGF were significantly synergistic in 2 cultures (no. 1 and 4), and additive in one culture (no. 5). In contrast, in the culture of tumour no. 3 the fibroblast fraction significantly inhibited EGF-stimulated ^{3}H-thymidine incorporation.

In two cultures from histologically proven non-malignant tissues (one blunt duct adenosis and one chronic granulomatous inflammation) we found that the fibroblast fraction inhibited epithelial cell growth, while in one the fibroblast fraction completely blocked EGF-induced stimulation of epithelial cell proliferation (data not shown).

The somatostatin analogue octreotide (1 Nm) had no statistically significant effect on epithelial cell proliferation either in the absence or in the presence of fibroblasts in tumour no. 1, 2, 4, 5 and 6, whereas in tumour no.3 octreotide significantly inhibited epithelial cell proliferation by 46% ($p < 0.01$ vs control) only in the absence of fibroblasts (data not shown).

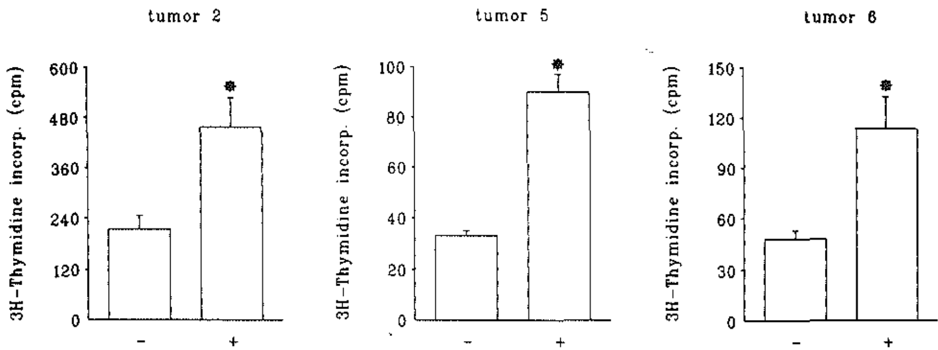


Figure IV. *The effect of Percoll-enriched epithelial breast cancer cells on the growth of fibroblasts derived from the same tumours. Both cell types were co-cultured using Transwell tissue culture inserts as described in the Materials and Methods section. - = without epithelial cells, + = with epithelial cells; *$p < 0.01$ vs fibroblasts without epithelial cells.*

In three cultures (tumour no. 2, 5 and 6) we also studied ^{3}H-thymidine incorporation in fibroblasts cultured in the absence and in the presence of epithelial cells. In addition to the stimulatory effect of tumour-derived fibroblasts on epithelial cell proliferation, we found that the Percoll-enriched epithelial cells also significantly stimulated fibroblast proliferation. This is shown in ***figure IV***. In the presence of epithelial cells, EGF (10 ng/ml) significantly stimulated fibroblast proliferation in all cultures (***table I***). In fibroblasts, co-cultured with epithelial cells, octreotide significantly inhibited ^{3}H-thymidine incorporation in 1 out of 6

cultures (no.5). There were not sufficient stromal cells obtained after Percoll-gradient separation in order to also study the effects of EGF and octreotide on the growth of fibroblasts that were not co-cultured with tumour-derived epithelial cells.

Table 1. *The effects of EGF and octreotide on ^{3}H-thymidine incorporation of fibroblasts co-cultured with primary breast cancer cells.*

	^{3}H-thymidine incorporation (cpm per dish)		
Tumour no.	Controls	EGF (10ng/ml)	Octreotide (1nM)
1	428±110	1450±153[a]	685±279
2	457±70	1417±86[a]	349±23
3	29±2	58±5[a]	33±3
4	77±11	177±7[a]	56±5
5	90±7	126±6[a]	58±1[a]
6	114±19	235±20[a]	138±31

[a]$p<0.01$ vs control;values are mean ± SE, control=fibroblasts co-cultured with primary breast cancer cells.

Discussion

Previous studies have demonstrated that human breast cancer-derived stromal cells secrete factors capable of stimulating the proliferation of breast cancer tumour cell lines[5-7]. While mammary tumour epithelial cell growth is regulated by steroid hormones and polypeptide growth factors[3,4,16], the nature of stromal factors responsible for stimulation of epithelial cell growth is still unclear. In the present study we investigated in a co-culture system using transwell microporous membrane inserts, the role of human breast cancer-derived fibroblasts in the growth regulation of epithelial cells derived from the same corresponding tumours. This co-culture system was first established using MCF-7 breast cancer cells co-cultured with a human fibroblast line (HF cells). In line with other studies[6,7,19] we found that fibroblasts are capable of stimulating the proliferation of MCF-7 breast cancer cells.

Most studies on stromal-epithelial interactions in human breast cancer have been performed using conditioned media from breast cancer-derived stromal cells capable of stimulating human breast cancer cell line proliferation[5-7]. In the present study we provide preliminary evidence for the existence of such a paracrine-growth stimulatory mechanism in a co-culture system of primary human breast cancer cells and stromal cells derived from the same tumours, although there was a considerable variability among the tumours. In 50% of the human tumour cell cultures we found that tumour-derived stromal cells stimulated the proliferation of epithelial cells derived from the same tumour. In one culture, however, the

stromal cells even inhibited epithelial cell proliferation. In two tumour cell cultures we showed that fibroblasts and EGF acted synergistically on epithelial cell proliferation, while in three other cultures the EGF-stimulatory effect was only slightly additive and not synergistic to the fibroblast-stimulated epithelial cell proliferation. Finally, in one culture the tumour-derived fibroblasts even significantly inhibited EGF-stimulated epithelial cell proliferation. In two cultures from "non-malignant" breast tissue-derived fibroblasts and epithelial cells we also observed an inhibitory effect of fibroblasts on epithelial cell proliferation. These data are partly in agreement with the results of a recent study by Roozendaal et al.[7], who suggested a higher stimulatory response of breast cancer cell lines to conditioned media of breast cancer-derived fibroblasts as compared to that of normal breast tissue derived-fibroblasts. They suggested that this was most likely determined by the phenotypic characteristics of the fibroblast cells involved, which may be different between normal tissue and tumour-derived fibroblasts. Moreover, Adams et al.[6] also found that normal tissue-derived fibroblast conditioned medium had an inhibitory effect on MCF-7 cell proliferation, while conditioned medium from tumour- derived fibroblasts was strongly stimulatory. Other investigators have shown either stimulatory[5,7,8,17] or inhibitory[18] effects of normal tissue- derived fibroblasts on epithelial cell proliferation. In the same co-culture system which we used in the human primary tumour cell cultures we found that normal human foreskin-fibroblasts were only stimulatory on human MCF-7 cell proliferation. It seems very important therefore, to study stromal-epithelial interactions on cells derived from the same tissue specimen.

Interestingly, we found that the tumour-epithelial cells also had a stimulatory effect on fibroblast proliferation. This suggests that within human breast cancer an autocrine growth regulatory route exists in which fibroblasts stimulate epithelial cells and epithelial cells stimulate fibroblasts. Breast cancer cells have been shown to secrete many growth factors (i.e. tumour growth factor (TGF)-α, TGF-β and platelet derived growth factor (PDGF)), while also fibroblasts are capable of secreting growth factors[4], particularly insulin-like growth factors (IGFs)[19]. In the present study we did not investigate the nature of the epithelial cell and fibroblast-derived factors responsible for the observed autocrine growth stimulation. However, the results of our study indicate that fibroblasts and growth factors (i.e. insulin, EGF and E2) act additive or synergistically on the proliferation of malignant breast cancer cells. In addition, in the human primary cell cultures we found that fibroblasts have a stimulatory effect on breast cancer cells, even when both cell types are grown in the presence of EGF and serum. This suggests that factors, other than the above growth promoting compounds, are responsible for the growth promoting effects induced by fibroblasts. The observed opposite effects of "non malignant breast tissue"- and " breast tumour tissue"-derived fibroblasts on epithelial cell proliferation suggest that the ratio's of growth stimulatory and growth inhibitory factors secreted may be different between "normal" and "tumour" fibroblasts, since it is unlikely that fibroblasts produce one specific growth factor only.

In approximately 50% of human breast cancer specimens receptors for somatostatin have been demonstrated, while EGF receptors were found in 25% of the samples containing somatostatin receptors (SS-R's). In only 8% of these cases the two receptor types were topographically overlapping[11]. On the basis of these data Reubi et al.[11] suggested that in the majority of breast tumours it is unlikely that tumour biology is influenced by a direct interaction of somatostatin and EGF through specific receptors in a particular cell. In a wide variety of experimental tumour models, including breast cancer, growth inhibitory effects by long-acting somatostatin analogues have been demonstrated *in vivo* and *in vitro*[20,21] The *in vivo* tumour-growth inhibitory effects by somatostatin analogues may act indirectly via the

inhibition of angiogenesis, immune modulatory effects, and via the inhibition of the secretion "endocrine" growth factors as growth hormone, prolactin and IGF-I, as well as via direct effects on tumour cells via specific SS-R's. These latter effects include inhibition of EGF-receptor activity via the stimulation of a tyrosine-phosphatase or inhibition of the secretion of autocrine and paracrine growth factors[21]. Because we found clear evidence in our study for the existence of a paracrine growth regulatory mechanism, we also studied the effect of the somatostatin analogue octreotide on epithelial cell and fibroblast proliferation with or without co-culture. We found that octreotide had no effect on basal and fibroblast-stimulated epithelial cell proliferation in 5 of 6 tumours, while in one tumour octreotide inhibited epithelial cell proliferation in the absence of fibroblasts only. Surprisingly, we found in one culture that octreotide significantly inhibited the proliferation of fibroblasts co-cultured with epithelial cells. Although we did not investigate the SS-R status of the tumours, it can be speculated that octreotide inhibits the secretion of growth factors by the epithelial tumour cells, thereby indirectly inhibiting growth of the co-cultured fibroblasts. In the 7-day co-culture period which we used in our study, this growth inhibitory action by octreotide on fibroblast proliferation did not result in an indirect growth inhibition of epithelial cells. Therefore, more studies are needed to evaluate a possible role of the somatostatin analogue octreotide in the treatment of breast cancer.

In *conclusion*, our study emphasizes the need to investigate in breast cancer the role of stromal cells on epithelial cell proliferation, as well as their role in interfering with drug-induced manipulation of breast cancer cell growth.

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CHAPTER VI

USE OF RADIONUCLIDE-LABELLED SOMATOSTATIN ANALOGUES FOR VISUALIZATION OF ISLET CELL TUMOURS

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Abstract

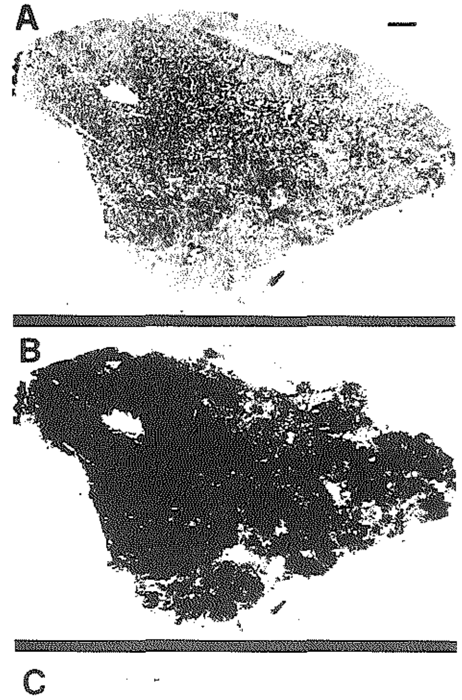
The results of visualization of the islet cell tumours of 25 patients after intravenous administration of two isotope-labelled somatostatin analogues ([^{123}I-Tyr^{3}]-octreotide and [^{111}In-DTPA-D-Phe^{1}]-octreotide) are described. The primary tumours, as well as previously unrecognized distant metastases were visualized in 20 of the 25 patients (80%). Parallel *in vitro* detection of somatostatin receptors (SS-R's) on those tumours that had also been visualized *in vivo* indicates that the ligand binding to the tumour *in vivo* indeed represents binding to specific SS-R's. It is an easy, quick, harmless procedure that is available for localization of primary endocrine pancreatic tumours and their often radiologically and clinically not yet recognized metastases.

Introduction

SS-R's have been shown to remain present on a variety of tumours which arise in tissues also containing these receptors in the normal state. High numbers of high affinity SS-R's have been found on most growth hormone secreting pituitary adenomas as well as on most metastatic islet cell tumours and carcinoids[1-3]. In parallel chronic therapy with octreotide normalizes clinical symptomatology as well as the biochemical abnormalities in most acromegalic patients: both the hyper-secretion of growth hormone, and the elevated circulating levels of insulin-like growth factor I (virtually) normalize in most instances[4]. Hormonal hypersecretion from (metastatic) endocrine pancreatic tumours and carcinoids is also well controlled during octreotide treatment in most patients, while in parallel clinical symptomatology greatly improves. Interestingly, evidence for control of tumour growth during somatostatin analogue treatment has been observed in part of these patients[5]. These results led to an instant improvement in the quality of life of these patients, making the clinical introduction of octreotide a major breakthrough in the treatment of these endocrine cancers.

It was remarkable to us that in the *in vitro* autoradiographic studies of most of these endocrine tumours (pancreatic and carcinoids) was virtually always a very high density of SS-R's was present within these tumours in contrast to the virtual absence of visible binding sites in the surrounding "normal" tissue which was known also to contain these receptors (*fig.Ia-c*). The presence of higher numbers and/or a higher affinity of the SS-R's on many of these tumours seemed also to be reflected by the clinical observation that tumorous hormone secretion (i.e. in acromegalic and/or gastrinoma patients) was much longer suppressed after the single subcutaneous administration of octreotide than normal growth hormone and/or gastrin secretion in healthy individuals[6].

Figure 1.
Somatostatin receptors in a case of a human gastrinoma
(a) Haematoxylin-eosin stained section.
(b) Autoradiogram showing total binding of ^{125}I-Tyr^{3}-octreotide.
(c) Autoradiogram showing non-specific binding in the presence of 1 μg Tyr^{3}-octreotide.
Bar = 1 mm.



These considerations led us to explore, whether it might be possible to detect SS-R positive tumours *in vivo* after the administration of a radioactive iodine labelled analogue[7]. In a way one might call this approach an " *in vivo* autoradiography" of SS-R positive tumours.

Material and Methods

Tyr^{3}-octreotide is a somatostatin analogue with tyrosine in position 3, where phenylalanine is present at that place in octreotide. The biological activities of octreotide and Tyr^{3}-octreotide are similar. We coupled Tyr^{3}-octreotide to ^{123}I and injected about 555 MBq [^{123}I-Tyr^{3}]-octreotide intravenously in patients which were suspected to have SS-R positive

tumours, while planar or SPECT (single photon emission computed tomographic) images were made with a gamma camera.

After the bolus injection of radioiodinated Tyr^3-octreotide rapid accumulation of radioactivity was seen in the liver. About 50 percent of the activity was cleared from the blood pool within 2 minutes after injection and the localization of a variety of tumours and their metastases was possible.

After the initial success of the visualization with [^{123}I-Tyr^3]-octreotide of the primary tumours as well as the metastases of a variety of SS-R positive tumours, we were concerned about several drawbacks to this technique: the cyclotron-produced radionuclide ^{123}I is not readily available in many parts of the world. Its short half-life of about 12 hours hampers its use. while we found that very high-quality specifications of the isotope are required in order to ensure its successful use in scintigraphy. ^{123}I is also very expensive. Another problem, especially in the visualization of islet cell tumours and abdominal carcinoids is that [^{123}I-Tyr^3]-octreotide is excreted via the liver, gall bladder and bile ducts into the gastro-intestinal tract, making these parts of the body so "hot" with radioactivity that small primary tumours in this region can easily be missed, especially if no SPECT (single photon emission computed tomography) pictures are made.

In order to circumvent these drawbacks we designed an alternative peptide: DTPA-octreotide (DTPA=diethylenetriaminopenta-acetic acid), which can be very easily conjugated with ^{111}In. [^{111}In-DTPA-D-Phe^1]-octreotide has a much longer half-life (of about 3 days), and it is excreted via the kidneys[8]. Our experience in over 250 patients with different tumours indicates that [^{111}In-DTPA-D-Phe^1]-octreotide even better visualizes SS-R positive tumours than [^{123}I-Tyr^3]-octreotide. Physiological organ accumulation of [^{111}In-DTPA-D-Phe^1]-octreotide is seen in the kidneys, spleen, liver, bladder, and the thyroid and pituitary glands.

Results and Discussion

The [^{123}I-Tyr^3]-octreotide scanning procedure revealed the localization of the primary tumour and/or its previously unknown metastases in 7 of 9 patients with islet cell tumours. In 5 of the 7 positive tumours we could subsequently investigate the surgically removed tumour[9]. There was a close relationship between the *in vitro* detection of SS-R's in these tumours using autoradiography and the gamma camera pictures obtained after injection of [^{123}I-Tyr^3]-octreotide. This indicates that the ligand binding to the tumour *in vivo* represents binding to specific SS-R's. In addition we carried out preoperative *in vivo* hormonal studies and *in vitro* experiments with cultured tumour cells. Again there was a close parallel between the presence of SS-R's on these tumours and the *in vivo* and *in vitro* effects of octreotide on hormonal secretion by these tumours. This means that a positive scan predicts a beneficial effect of therapy with octreotide on hormonal hypersecretion by these tumours. Furthers arguments in favour of the conclusion that indeed membrane receptors for somatostatin were visualized with this scintigraphic technique are that *in vivo* competition was observed between radiolabelled octreotide with regard to binding to SS-R positive tumours[10].

In ***table 1*** we compared the results of *in vitro* autoradiography of a group of 35 islet cell tumours with the results of *in vivo* scintigraphy in 25 patients with these tumours. SS-R imaging in these patients was carried out initially in the first nine of these patients with [^{123}I-Tyr^3]-octreotide, and in the rest of them with [^{111}In-DTPA-D-Phe^1]-octreotide.

Table 1. *Incidence of somatostatin receptors on endocrine pancreatic tumours in vitro (as demonstrated by autoradiography) and in vivo (as demonstrated by scintigraphy.*

	Incidence of somatostatin analogue receptors	
	in vitro	*in vivo*
VIPomas	7/8 (87%)	1/1
Gastrinomas	5/5 (100%)	10/11 (91%)
Glucagonomas	3/3 (100%)	1/1
Insulinomas	8/11 (72%)	5/8 (63%)
GRF-omas	4/4 (100%)	-
"non-functioning" islet cell tumours	4/4 (100%)	2/2
Somatostatinomas		1/2

Eight of these tumours have been investigated both *in vitro* and *in vivo*. In all cases the results *in vitro* and *in vivo* investigations were parallel.

The application of SS-R imaging turned out to be highly successful. The primary endocrine pancreatic tumours as well as their often previously not yet recognized metastases could be visualized in 20 of these 25 patients (80%). Metastases were actually scintigraphically evident in 11 patients. Ten of these patients were subsequently operated upon in our hospital, allowing surgical evaluation of the scintigraphic results. In one gastrinoma patient no lymphnode metastases on the aortic arch was not found at operation, despite the fact it was suggested at CT scanning. In another gastrinoma patient, who had familial multiple endocrine adenomatosis type I, the scintigraphically detected tumour was indeed found in the tail of the pancreas, but during operation an additional islet cell tumour was found in the corpus of the pancreas. This tumour had not been seen on the scan. Unfortunately no somatostatin autoradiography of this tumour was done. Examples of the gamma pictures obtained in two patients are shown in ***figure II*** and ***III***.

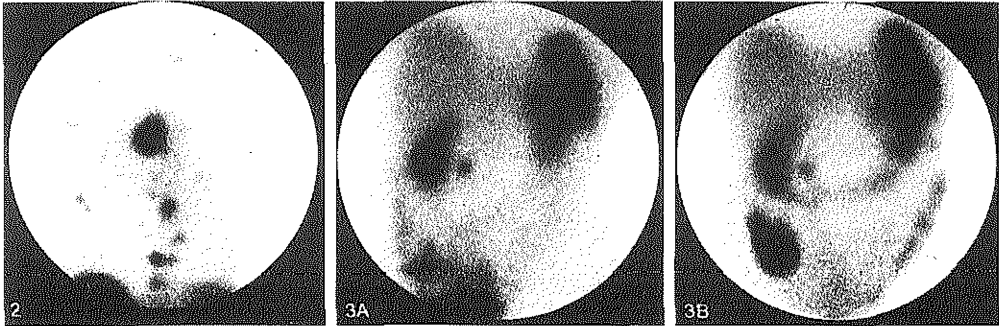


Figure II.

[^{111}In-DTPA-D-Phe^{1}]-octreotide scan of a 34 yr old patient with a metastatic somatostatinoma. Gama camera picture from the posterior view after 24 hours shows multiple bone metastases in the vertebrae and ribs, as well as a lymphnode metastases on the left part in the neck.

Figure III.

[^{111}In-DTPA-D-Phe^{1}]-octreotide scans of a 1,5 cm insulinoma in the head of the pancreas.

(a) *Anterior view of the abdomen obtained at 24 hours.*

(b) *Anterior view of the abdomen obtained at 48 hours.*

Both planar images demonstrate a hot spot just medial to the right kidney. Note the different distribution of the radioactivity in the intestine visualized on the two images.

The visualization of insulinomas with this technique turned out to be more difficult. We localized the tumours on the scan in only 5 of 8 patients investigated sofar. *In vitro* autoradiography showed that all insulomas contained receptors for somatostatin-14 and -28, but that octreotide receptors were absent on the tumours, which could not be visualized *in vivo*.

In *conclusion*, islet cell tumours are (in most instances) malignant tumours originating from endocrine cells pancreatic islet cells. The primary tumours, as well as the spread of the disease are often difficult to localize with current radiological techniques[11]. Somatostatin receptor scintigraphy is an easy, painless non-invasive new diagnostic technique in patients with this type of tumours, which in our hands considerably helps in localizing the primary tumours, as well as their metastases. Prospective controlled studies comparing this procedure with other currently used investigative techniques should further delineate its sensitivity and specificity.

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CHAPTER VII

THE USE OF SOMATOSTATIN RECEPTOR SCINTIGRAPHY IN THE DIFFERENTIAL DIAGNOSIS OF PANCREATIC DUCT CANCERS AND NON-FUNCTIONING ISLET CELL TUMOURS

Submitted to the *Ann Surg*

Abstract

Somatostatin receptors (SS-R's) are present on most islet cell tumours, while previous *in vitro* studies indicate the absence of these receptors on pancreatic duct cancers. Somatostatin receptor scintigraphy (SRS) has become a technique which has shown to localize the primary as well as metastatic spread of islet cell tumours with a high sensitivity.

In the present study the potential value of SRS was evaluated in the preoperative differential diagnosis of pancreatic duct cancer, as well as in the follow-up of these patients. In none of the 26 patients suspected of pancreatic duct cancer SRS was positive. In correspondence also all 16 tumour samples obtained from these patients, which could be investigated with *in vitro* autoradiography did not have SS-R's.

Out of 62 patients with pancreatic duct cancer, who had been operated upon between 1985 and 1990, 12 patients were alive for more than 3 years. Five of these 12 patients were known to have metastases. SRS was negative in 7 patients who were considered to be cured, while SRS was positive in 5 patients who had metastatic disease. In most of them SRS also visualized additional previously unknown tumour sites. Revision of the tissue samples with specific neuroendocrine staining techniques demonstrated that all 5 patients had "non-functioning" islet cell tumours.

This study suggests that SRS might be of use in the preoperative differential diagnosis between islet cell tumours and pancreatic cancer, as well as in the follow-up, especially in those cases in which the pathological examination of the tumour tissue had not included special staining techniques for neuroendocrine characteristics.

Introduction

Preoperative differentiation between pancreatic duct carcinomas and islet cell tumours is of importance, as palliative surgery in patients with islet cell tumours is not only of value to relieve clinical symptoms but also because a decrease in the tumour burden, which might enhance the effect of treatment[1].

Recently we developed a technique for the visualization of islet cell tumours which is based on the presence of somatostatin receptors (SS-R's) on these tumours[2-9]. The primary tumours, as well as previously often unrecognized distant metastases were visualized at somatostatin receptor scintigraphy (SRS) in 80% of 25 consecutive patients[9]. In the present study the value of this technique was evaluated in 26 patients with suspected exocrine pancreatic duct carcinomas. The results of this *in vivo* technique were correlated with the results of *in vitro* receptor autoradiographic studies in tumour specimen. Our previous studies did not demonstrate the presence SS-R's in human pancreatic duct adenocarcinomas[10].

The prognosis of exocrine and endocrine pancreatic cancer differs considerably. Because of the difference in biological behaviour, a three year survival of patients with known metastases from pancreatic duct cancers is rare[11-14], while in general it can be expected for

islet cell carcinomas[15-17]. Therefore we carried out SRS in the 12 survivors, who were alive three years or more after subtotal (Whipple procedure) or total pancreaticoduodenectomy performed for pancreatic duct adenocarcinoma.

Patients

Twenty-six patients with primary pancreatic duct cancers (mean age 64 years [range 42 to 81]) were studied. After clinical and laboratory investigations in search for a primary tumour in the pancreas all patients underwent ultrasonography, CT scanning and ERCP. Whenever possible cytology of the tumour was obtained. After informed consent to participate in this study, SRS was performed after bowel preparation on an outpatient basis. Without taking into consideration the result of the scintigraphy, 22 patients were subsequently operated upon for suspected pancreatic duct carcinoma. Four patients were not operated because of cytologically proven metastatic disease.

Tumour tissue specimens from these 22 operated patients were stained with haematoxylin-eosin and examined for homogeneous cell structure and characteristic growth patterns of exocrine and endocrine tumours. The Grimelius silver staining technique was used, as well as immunocytochemical staining with antisera against neurone-specific enolase (NSE) and chromogranin A to confirm a possible endocrine nature of the tumours. In addition immunocytochemical staining with antisera against insulin, gastrin, pancreatic polypeptide (PP), vasoactive intestinal polypeptide (VIP), somatostatin, glucagon and neurotensin was performed. Tumours were histologically classified by one pathologist, who was not informed about the SRS results. Whenever enough tumour tissue was available, the presence of SS-R's were studied by *in vitro* autoradiography on cryostat sections as has been described previously[18].

Another group of twelve patients who were still alive more than 3 years after subtotal (n=10) or total (n=2) pancreaticoduodenectomy performed for pancreatic duct adenocarcinomas also underwent a SRS. Five of these patients were known to have metastatic disease at the time when SRS was performed. Revision of the tumour blocks of these patients which had been previously reviewed by other pathologists took place in the same manner as described above. No autoradiography could be done any more on these tumour specimens.

Materials and Methods

Material

The somatostatin analogue [DTPA-D-Phe^1]-octreotide was obtained from Mallinckrodt Medical BV, Petten, Netherlands. [DTPA-D-Phe^1]-octreotide was labelled with "ultra-pure" ^{111}Indium obtained also from Mallinckrodt Medical BV (Petten, The Netherlands). The labelling procedure has been described elsewhere[19]. Doses ranged from 222 MBq to 272 Mbq [^{111}In-DTPA-D-Phe^1]-octreotide.

Scintigraphy

After bowel preparation (2 litre PEG [polyethyleneglycol]) planar or SPECT (single photon emission computed tomographic) images were obtained with a large field of view gamma camera (Counterbalance 3700 , Siemens Gammasonics, Erlangen, Germany) equipped with a parallel-hole collimator as described previously[20-21].

Results

[^{111}In-DTPA-Phe^{1}]-octreotide scintigraphy (SRS) was carried out preoperatively in 26 patients with suspected pancreatic duct carcinomas. None of these tumours or their metastases could be visualized. Twelve patients were subsequently treated by a Whipple procedure, 2 by total pancreatectomy, 2 patients had a palliative procedure (gastroenterostomy and/or choledocho-duodenostomy) after transduodenal biopsy was performed and 6 patients had a biopsy only been done. Four patients were not operated since cytology of suspected lesions in the liver showed adenocarcinoma. Pancreatic duct carcinoma was found in 18 patients operated upon. None of these tumours showed neuroendocrine characteristics. In 16 of these tumours autoradiographic studies of the surgical removed tumour tissue could be done and no SS-R's were present. In 2 patients the tumour was so extensive that no resection could be done, transduodenal biopsies in these two patients however showed atypia without any evidence of infiltrating tumour cells, which must be considered a sampling error. The other 2 patients underwent a Whipple procedure for chronic pancreatitis. In these patients also no abnormalities were detected at SRS. No autoradiography was done on the tissue of these patients.

Since long term survival of patients with pancreatic duct carcinoma is rare we looked in retrospect at our group of long-term survivors after surgery for pancreatic duct carcinomas. Long term survivors were defined as patients who lived more then 3 years after the primary operation. Out of 62 patients, who had been operated between 1985 to 1990, 12 patients were found to be alive for three or more years. Five of these twelve patients were known to have metastases. These 12 patients underwent SRS. In 7 patients no abnormalities were seen, while also other investigations suggested that these patients were tumour-free at that moment. However in 5 "survivors" in whom the presence of metastases was known, all metastatic lesions, as well as a number of additional tumour sites were visualized at SRS. Examples of the scintigram obtained in one of these patients is shown in ***figure I***.

Revision of the original tumour blocks including the additional staining procedures mentioned above demonstrated that these 5 patients in retrospect were operated primarily for "non-functioning" islet cell tumours. In the group of 7 patients without known metastases, and without abnormalities at SRS revision of the pathology confirmed that these patients indeed had been cured from pancreatic duct cancer.

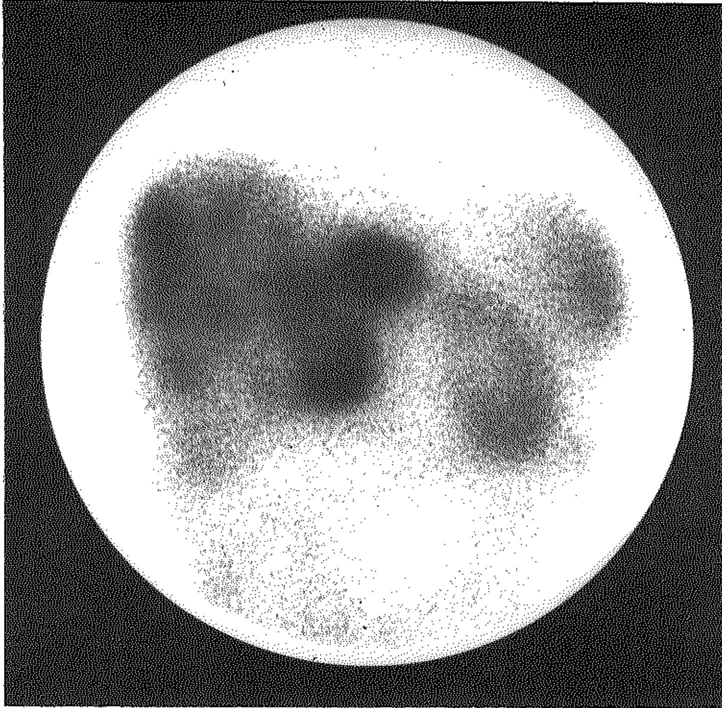

Figure I.

24 hours planar anterior abdominal and upper thoracic, right lateral skull [^{111}In-DTPA-D-Phe^{1}]-octreotide scintigram of a patient with a metastasized "non-functioning" pancreatic tumour

(a) Note the hotspot in the pancreatic region and the non-homogeneous distribution of radioactivity in the liver, indicating the presence of the primary pancreatic tumour and the presence of liver metastases.

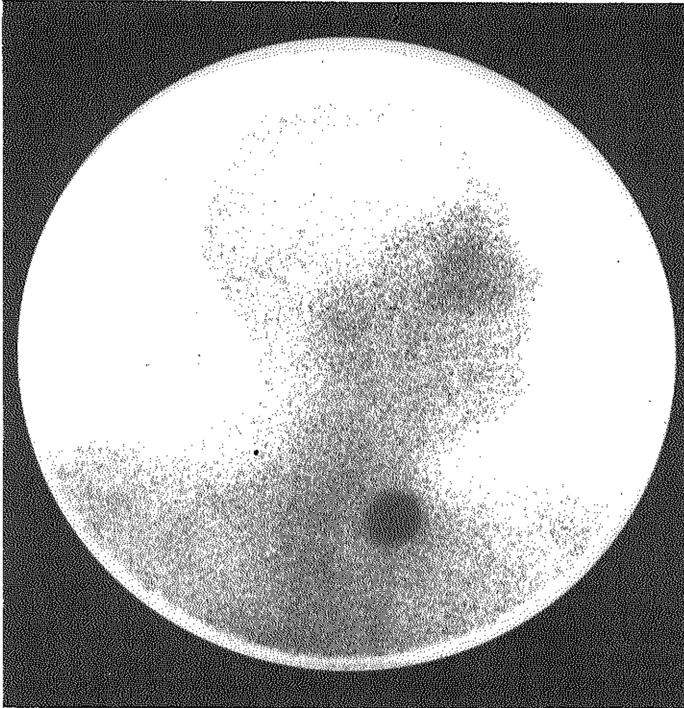

Figure I.
(b) Left Supraclaviculair lymphnode (Virchow) metastases in the same patient.

In ***figure II*** the scintigram of a 81 year old patient is shown, who was known to have a pancreatic tumour. The accumulation of radioactivity in the pancreatic region indicates an endocrine nature of the pancreatic tumour. The survival of more than 3 years and the good clinical condition after the tumour was diagnosed in this patient confirms the hypothesis. The patient refused to undergo an operation or biopsy.

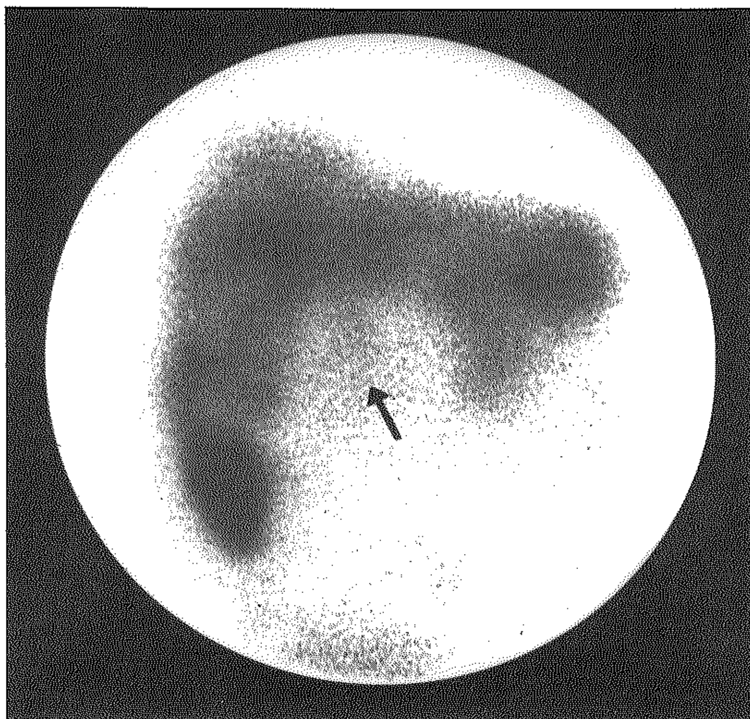

Figure II.
24 hours planar anterior abdominal [^{111}In-DTPA-D-Phe^{1}]-octreotide scintigram of a patient one year after a pancreatic tumour was diagnosed. The modest accumulation of radioactivity between the kidneys (arrow) indicates the presence of a neuroendocrine tumour in the region of the pancreas.

Discussion

It has been previously hypothesized that the *in vivo* visualization of SS-R positive tumours with SRS is more sensitive than *in vitro* autoradiography because the first technique investigates the presence of these receptors three-dimensionally in the entire tumour, whereas in the second technique sections of parts of the tumour are studied only two-dimensionally[20]. Therefore in the present study we investigated whether it might be possible to detect SS-R's

in vivo on human pancreatic duct cancers, despite the fact that no SS-R's had been found *in vitro* in a previous study[10]. The present results demonstrate the absence of SS-R's in all tested human pancreatic duct adenocarcinomas *in vivo* and *in vitro*. In both instances studies were carried out with the somatostatin analogue octreotide which has been reported to bind only to part of the SS-R subtypes, cloned in human tissues[23-24]. Previous studies by Schally's group suggested that human pancreatic duct cancers express low numbers of low affinity SS-R's which bind other analogues like RC-160[25]. Therefore our studies do not allow a definitive conclusion for the absence of certain SS-R subtypes on this cancer type. The finding of SS-R's on metastases of the islet cell tumours in 5 patients who originally were thought to have ductal cancers but who were still alive 3 years or more after the initial operation supports the concept that this type of clinically "non-functioning" neuroendocrine tumours are rather slow-growing, well differentiated tumour types. Most islet cell tumours (75%) present clinically with symptoms and signs related to the hormonal hypersecretion by these tumours[26,27]. Less frequently symptomatology is related to tumour mass, as is often the case in patients with pancreatic duct tumours. Preoperatively it is difficult to differentiate between ductal and islet cell tumours. Since it is well known that patients with islet cell tumours may benefit from palliative surgery[1], preoperative differentiation is of importance. In our previous study[9] all "non-functioning" islet cell tumours contained SS-R's and could be visualized at SRS.

The present study supports the concept that SRS may have a place in the preoperative differential diagnosis of endocrine "non-functioning" islet cell tumours and pancreatic duct cancers, as well as in the follow-up, especially in those cases in which the pathological examination of the tumour tissue had not included special staining procedures for neuroendocrine characteristics. Our results also indicate that the evaluation of the results of investigations on the role of surgery and/or radiochemotherapy in pancreatic duct cancer have to be interpreted with caution, if no histology and staining for neuroendocrine characteristics was performed.

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CHAPTER VIII

OCTREOTIDE THERAPY FOR LIVER METASTASES AFTER RESECTION OF A NON-FUNCTIONING ISLET CELL TUMOUR

Eur J Surg Oncology in press

Abstract

A case of chronic gastrointestinal bleeding occurring from a "non-functioning" islet cell tumour is reported. Surgical resection was performed although liver metastases were found during operation. Postoperative treatment was started with octreotide based on proven somatostatin receptors found on the primary tumour.

The case reviewed is notable for its long symptom free interval and supports a role for somatostatin and its analogues in the diagnosis and treatment of " non-functioning " islet cell tumours.

Introduction

Endocrine pancreatic tumours or islet cell tumours constitute a small, but important group of pancreatic tumours. The annual incidence of islet cell tumours was found to be 0.4/100.000 population[1]. Most of these tumours produce multiple hormones, but usually excessive secretion of one hormone predominates, causing specific clinical symptomatology [2,3]. However, about 15-40% of islet cell tumours do not give rise to hormone-related symptoms and are therefore called "non-functioning"[4,5]. The reasons why they are clinically silent include, the absence of the release of produced hormones, the production of biologically inactive pro-hormones, the production of hormones which do not cause clinical symptoms (pancreatic polypeptide,chromogranin A), downregulation of hormone receptors and/or the production of inhibitory hormones such as somatostatin[6]. Most patients with "non-functioning" islet cell tumours have rather uncharacteristic symptoms at presentation. The correct diagnosis is therefore made at a late stage when extensive tumour growth has occurred[2,7,8]. In general islet cell tumours grow slowly, but the significantly shorter survival of patients with "non-functioning" islet cell tumours in comparison with those which cause signs and symptoms of hormonal secretion indicates a more malignant behaviour of this subtype[2], but might also be due to the fact that they are diagnosed later. In 70% to 80% of the cases liver metastases are present at the time of diagnosis. In early stages, curative resections can be achieved, but here we report a case, which suggests that palliative surgery is not only of value to relieve clinical symptoms but that a decrease in tumour burden also facilitates the effect of adjuvant therapy.

Case report

In july 1991 a 59 year old man presented with a history of weariness, nausea and tiredness. Weight loss during the last 4-6 months amounted to 10 k. He was pale, had a low haemoglobin (6.8 mM/l) and a positive faecal haemocult (3x). Gastroduodenoscopy revealed an ulcerating tumour in the second part of the duodenum. Ultrasonography and CT scan showed a tumour in the head of the pancreas involving part of the duodenum. No signs of lymphnode or liver metastases were seen. Serum gastrin levels were normal. Biopsies taken during duodenoscopy showed a neuroendocrine tumour, which was positive for chromogranin A, neuron specific enolase, synaptophysin and somatostatin, while the tumour was also positive in the Grimelius silver strain. Somatostatin receptor (SS-R) scintigraphy with 222 MBq [^{111}In-DTPA-D-Phe^1]-octreotide has been performed as previously described[9]. Planar scintigraphy visualized an area of increased activity in the region of the primary tumour but no distant metastases were found, especially the liver showed a homogeneous distribution of radioactivity. However, single photon emission computerized tomography (SPECT) with radiolabelled octreotide demonstrated several hotspots, both in the region of the pancreas and in the liver.(*fig.I*) Laparotomy was undertaken and multiple small lesions (< 5mm) were seen macroscopically throughout the liver. Frozen liver sections showed the same type of tumour as found in the duodenum and in the head of the pancreas. To prevent further blood loss from this tumour a pyloric preserving pancreaticoduodenectomy was performed.

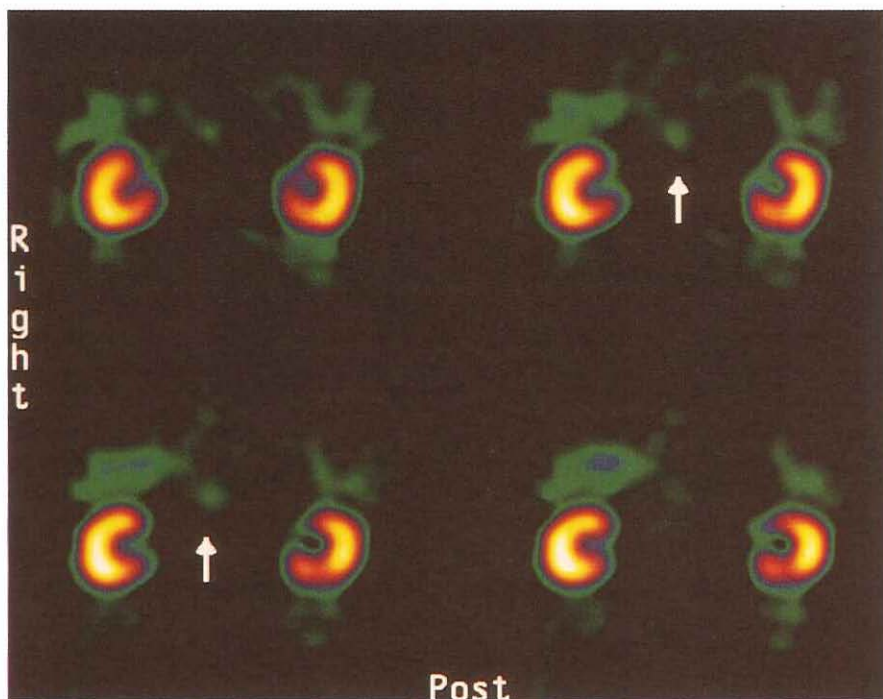

Figure I.

(a) 24 hours transversal SPECT images of a patient with a metastasized "non-functioning" islet cell tumour showing a hotspot at the side of the primary tumour in the caput of the pancreas (arrow) between the kidneys.

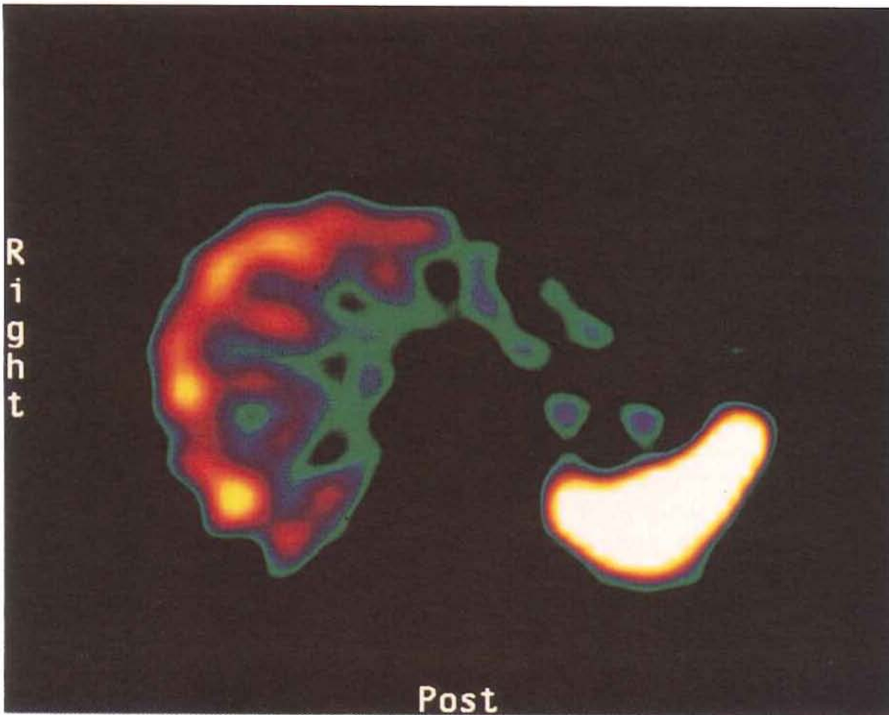

Figure I.
(b) Many hot spots (yellow) in the liver representing metastases (spleen in white).

Histology demonstrated a pancreatic islet cell tumour (*fig.II* and *fig.III*) with multiple small lymphnode metastases. Autoradiography showed a dense and homogeneous distribution of somatostatin-14 and octreotide receptors throughout the tumour. After an uncomplicated postoperative course the patient started with adjuvant therapy with 100 mcg octreotide 3 times daily, subcutaneously. Routinely performed ultrasonography every 3 months still did not reveal liver metastases or evidence of locoregional recurrence.

In november 1993 the patient was in a good clinical condition. He did not have side effects of the octreotide treatment, but he had some complaints from a hernia cicatricialis. During the surgical correction of this hernia the abdomen was reexplored in order to investigate whether there was evidence of locoregional tumour recurrence. Peroperatively no local recurrence was found. However the liver metastases were still present with a similar size as observed in july 1991. Multiple liver biopsies were taken which showed the same characteristics as before.

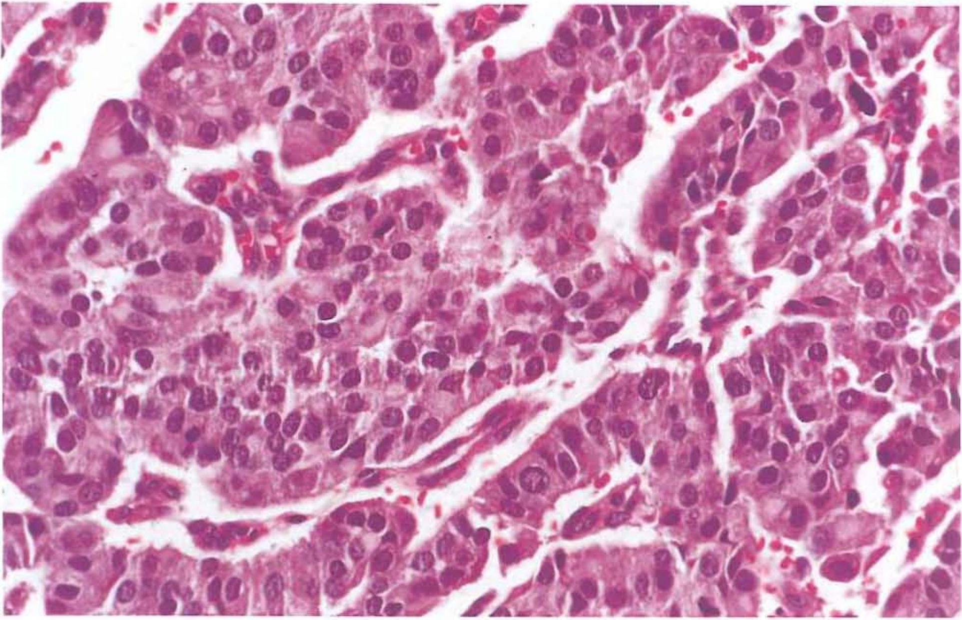

Figure II.

Histology of the resected specimen revealed a neuroendocrine tumour infiltrating the duodenal wall. The tumour shows epithelial cells in a trabecular pattern with a richly vascular stroma. The cells sometimes are slightly atypical but are mostly monomorphous. Sparse mitoses are found. (HE staining, 400x).

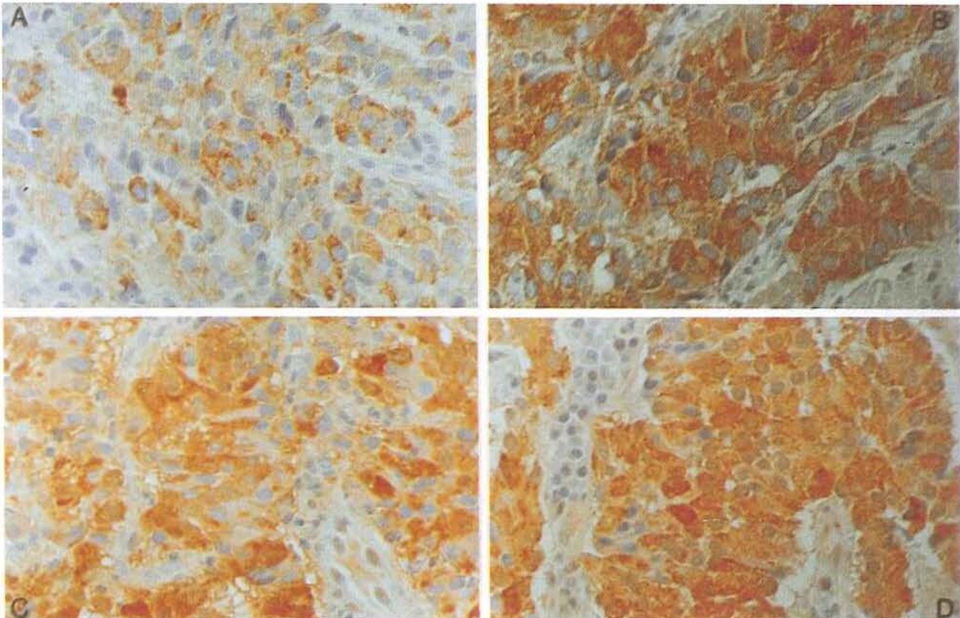

Figure III.

Neuroendocrine tumour: metastases in the liver. Immunoperoxidase staining for Chromogranin A (A), Synaptophysin (B), NSE (C), and Somatostatin (D) shows a distinct positive staining for all four antigens (300x).

Discussion

Pancreatic islet cell tumours have in general a better prognosis than exocrine, mostly pancreatic duct adenocarcinomas[10,11]. However, in spite of improvements in diagnostic methods, the median delay between the appearance of the first symptoms and the diagnosis has not been reduced and most patients are encountered when the tumour has reached an advanced stage[2,7,8]. As pointed out by Eriksson et al.[12] there is a need for optimalization of screening patients with uncharacteristic abdominal symptoms for the presence of islet cell tumours. In addition to the measurements of plasma levels of chromogranin A and pancreatic hormones, we use SS-R scintigraphy to demonstrate the presence of such tumours. Also previously unknown metastases can be visualized by this method, since in most instances not only the primary tumour expresses SS-R's but also its metastases[13-15].

Octreotide has been shown to exert a suppressive effect on hormonal hypersecretion by islet cell tumours, which contain SS-R's[16-19]. There is little clinical evidence however that octreotide can control the growth of islet cell tumours. This might be related to the fact that mostly patients with advanced disease were studied[20]. In a late stage such tumours may lose their SS-R's due to a loss of differentiation into somatostatin negative tumour cell clones[21,22]. Several *in vitro* studies did show an antimitotic effect of octreotide in cultured islet cell tumours[23,24]. We recently also showed *in vivo* a SS-R dependent growth inhibitory effect of experimental liver metastases by octreotide[25].

Octreotide only causes minimal side effects, and might therefore be preferred before the use of chemotherapeutic agents and/or interferon[26]. The mechanism of action of octreotide in the control of tumour growth inhibition might involve a direct inhibitory effect and/or an indirect one which affects autocrine tumour growth factors[27]. The reported case shows an aggressive surgical approach in the management of a patient with metastatic islet cell carcinoma, which resulted in a long stable disease interval after palliative pancreaticoduodenectomy. This is in accordance with the suggestions of Norton et al.[28], who observed a longer survival of this type of patients after (extensive) debulking surgery. The experience in this patient suggests an inhibitory effect of octreotide on further growth of SS-R positive islet cell tumour cells. This, however, cannot be concluded as yet on the basis of one case, as some "non-functioning" islet cell tumours grow relatively slowly, and the follow-up of the effect of octreotide therapy is only 30 months.

Also, this study points to the importance of the preoperative differentiation between islet cell tumours and ductal adenocarcinomas of the pancreas, since patients with liver metastases of the latter type have a median survival of only 4 months[29]. For this differentiation we believe that SS-R scintigraphy might be useful.

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CHAPTER IX

SOMATOSTATIN RECEPTOR DEPENDENT GROWTH INHIBITION OF LIVER METASTASES BY OCTREOTIDE

Br J Surg in press

Abstract

Rats were treated for 4 weeks by intraperitoneal injection of the somatostatin analogue octreotide, (15 μg twice a day). After intraportal injection of somatostatin receptor (SS-R) positive pancreatic tumour cells (CA-20948) and SS-R negative colon tumour cells (CC-531). Octreotide significantly inhibited the growth and development in the liver of SS-R positive CA-20948 tumour cells. The mean liver weight decreased from 17.9 $\pm$ 3.0 g (controls) to 14.5 $\pm$ 3.7 g ($p<0.05$). No effect of octreotide treatment was found on the growth and development of SS-R negative CC-531 tumour cells in the liver. Mean liver weight was 11.8 $\pm$ 4.5 g (controls) versus 14.0 $\pm$ 5.7 g.

Since no difference was observed between control and octreotide treated rats in serum levels of growth hormone (GH), prolactin (PRL) and insulin-like growth factor (IGF-I), the growth inhibition of SS-R positive CA-20948 tumour cells was not likely to be due to inhibition of the secretion of one of these tumour growth factors.

Our findings suggest that octreotide may be useful for treatment of patients with SS-R positive hepatic metastases, which can be demonstrated by somatostatin receptor scintigraphy.

Introduction

Several possible mechanisms of action have been postulated for the tumour growth-inhibitory effect of somatostatin *in vitro* analogues [1-11]. Schally et al. in 1987 demonstrated that the reduction of circulating PRL and GH levels by somatostatin might contribute to the inhibition of breast and prostate tumour growth[12]. Also somatostatin might inhibit local growth factor activity, as GH stimulates cell differentiation directly and clonal expansion indirectly through local production of IGF-I[13-16]. Somatostatin can also have a direct antiproliferative effect and it inhibits DNA synthesis and cell replication induced by epidermal growth factor (EGF) by preventing centrosomal separation[17]. In human pancreatic cancer cells, somatostatin activates dephosphorylation of EGF receptors and thereby reverses the stimulatory effect of EGF on cell growth[18,19]. Another possible mechanism by which somatostatin analogues might inhibit tumour growth is interference with the secretion of autocrine growth factors by tumour cells[17,20,21] or by direct inhibition of angiogenesis[22,23].

Following treatment with somatostatin analogues an increase in natural killer cell activity was reported in man[24] and a stimulation of the activity of the reticuloendothelial system in rats, indicating that a change in immunological activity might also explain part of the tumour growth inhibitory effects[9].

Octreotide has been found to significantly reduce the growth of tumour in the liver following intraportal injection of Walker cells into rats but it had no effect on the growth of tumours after injection of these cells into the thigh. This was explained by postulating a stimulating effect of octreotide on the reticulo-endothelial system (RES), and/or a reduction

in hepatic and tumour blood flow[25]. RC-160, another somatostatin analogue, also significantly inhibited the incidence and growth of liver metastases of colon 320 DM and WidR human colon-cancer cells in nude mice[26].

Recently a technique for the *in vivo* visualization of SS-R positive endocrine tumours and their metastases has been developed. A parallel between the presence of SS-R on tumours and the *in vivo* and *in vitro* effects of octreotide on hormonal release from these tumours was found, suggesting that a positive scan predicts a good suppressive effect of somatostatin analogues[27]. Therefore in the present study we investigated: 1) whether the growth and development of hepatic metastases could be inhibited by octreotide, 2) whether this was related to the presence of SS-R on the tumour cells and 3) whether this could be due to the inhibition of possible tumour growth factors.

Material and Methods

Animals

Male rats of the inbred WAG and Lewis strains, weighing 250-300g, were obtained from Harlan-CPB (Austerlitz, the Netherlands). The animals were bred under specific pathogen-free conditions and were 10 to 14 weeks old when used. The animals were kept under standard laboratory conditions (12 hours light/12 hours dark) and were fed a standard laboratory diet (Hope Farms, Woerden, The Netherlands).

Tumours

CC-531 is a 1,2 dimethylhydrazine-induced, moderately differentiated colon adenocarcinoma, transplantable in syngeneic WAG rats[28]. The tumour was maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (virus and mycoplasma screened), 1% penicillin (5000 U/ml), 1% streptomycin (5000 U/ml) and 1% L-glutamine (200mM). All supplements were obtained from Gibco (Breda, The Netherlands). Before their use cells were trypsinized (5 min.,37°C), centrifuged (5 min., 700xg), resuspended in RPMI 1640 and counted. Viability was measured with trypan-blue exclusion (0,3% in a 0,9% NaCl-solution). Viability always exceeded 95%. The tumour is immunogenic as determined by the immunization-challenge method of Prehn and Main[29].

The pancreatic tumour, CA-20948, which we derived from Dr. Klijn[3], was originally induced by azaserine. The tumour is of acinar origin and is maintained in our laboratory as a transplantable tumour in Lewis rats. The tumours were removed from the rats, washed in ice-cold RPMI 1640 supplemented with antibiotics and chopped into small fragments which were passed through a 18 gauge needle. The resulting slurry was incubated for 10 min. in RPMI 1640, centrifuged at 500 g for 5 min. and washed twice with cold medium. The pellet was resuspended and adjusted to a suspension containing 200×10^6 living cells/ml.

SS receptor binding studies

Reaction conditions were the same as those described by Reubi[30]. The radioligand used in the binding studies was the radiolabelled somatostatin analogue [^{125}I-Tyr^{3}]-octreotide (SMS 204-090; Sandoz, Basel, Switzerland). Briefly, membrane preparations (corresponding to 50 μg protein) of CC-531 colon and CA-20948 pancreatic tumour cells were incubated in a total volume of 100 μl RPMI at room temperature for 90 min. with 30,000-50,000 cpm radioligand and increasing concentrations of unlabelled Tyr^{3}-octreotide in HEPES buffer (10mM HEPES, 5mM $MgCl_2$ and 0,2g/l bacitracin, pH 7,6) containing 0,2% BSA (Sigma). After the incubation, 1 ml ice-cold HEPES buffer (Ph 7,6) was added to the assay mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min. at 14,000 rpm in an Eppendorf microcentrifuge[31]. The remaining pellet was washed twice with ice-cold HEPES buffer, and the final pellet was counted in a gamma counter. Specific binding was taken to be total binding minus binding in the presence of 10 μM unlabelled Tyr^{3}-octreotide. Unrelated compounds, such as thyrotropin-releasing hormone (TRH), luteinizing hormone-releasing hormone (LHRH), and epidermal growth factor (EGF), added in a 1000-fold excess were not able to displace [^{125}I-Tyr^{3}]-octreotide binding. SS-R receptor binding data were analyzed by the method of Scatchard plot[32].

Induction and measurement of liver metastases

Metastatic tumours in the liver were established for both cell lines in 16 rats. After each rat was anaesthetized, a 2.5 cm median incision was made to expose the portal vein, then $1.2x10^{6}$ viable CA-20948 cells in 0.6 ml HBSS or $5x10^{5}$ CC-531 cells in 0.5 ml HBSS were injected slowly into the portal vein, and the abdominal wall was closed using one layer of continuous silk suture.

After injection of tumour cells, the rats were divided into several groups as follows: (1) CC-531 liver metastases treated with octreotide (8 WAG rats); (2) CC-531 liver metastases used as controls (8 WAG rats); (3) CA-20948 liver metastases treated with octreotide (8 Lewis rats): (4) CA-20948 liver metastases used as controls (8 Lewis rats).
After 28 days all rats were killed by an overdose of ether for evaluation of the incidence of liver metastases. The livers were dissected and fixed in 10% formalin overnight. The visible tumours on the surface of each liver (up to 500) were counted and scored and finally all livers were weighted.

Treatment with the somatostatin analogue octreotide

The somatostatin analogue octreotide (D-Phe-Cys-Phe-D-Trp-Lys-Thr-Cys-Thr-ol) (Sandoz, Basel) 15 μg was injected intraperitoneally twice daily (approximately $\pm$50 μg/kg) starting on the first day after tumour implantation in rats of groups 1 and 3. The dose of octreotide was chosen on the basis of a previous study[33] on the effects of octreotide on growth of 7315a pituitary tumour cells in rats.

Assays

Plasma of 16 rats (8 controls and 8 treated with octreotide 15 μg twice daily i.p.) were collected 4, 11 and 18 days after starting the administration. PRL and rat GH concentrations were determined by a double antibody radioimmunoasay, as described previously[34,35]. Concentrations of IGF-I were measured by radioimmunoassay using a commercially available kit from Medgenix Diagnostics (Brussels, Belgium).

All data are expressed as means (s.e.m.). Statistical analysis was performed using the Mann-Whitney U test. $P<0.05$ was considered significant.

Results

SS receptor binding studies

Specific binding of the ^{125}I-labelled somatostatin analogue Tyr^3-octreotide to membrane preparations of CA-20948 pancreatic tumour cells was demonstrated, but not to the CC-531 colon tumour cells. ***Figure 1*** shows the binding [^{125}I-Tyr^3]-octreotide to membranes of CA-20948 (○) and CC-531 (△) tumour cells and on rat brain cortex (□), which were functioning as a control. Binding of [^{125}I-Tyr^3]-octreotide to CA-20948 membranes was specific and could be displaced in a dose-dependent manner with unlabelled Tyr^3-octreotide. Scatchard analysis of these data revealed a single class of high affinity binding sites with an apparent dissociation constant (K_d) of 0.6 nmol/l and a maximum binding capacity (B_{max}) estimated to be 110 fmol/mg per membrane protein.(***table 1***)

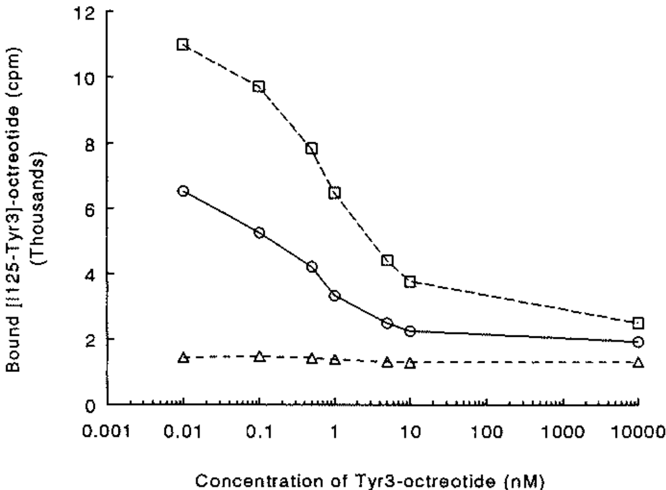

Figure 1.
Dose dependent displacement of [^{125}I-Tyr^3]-octreotide binding to membranes of rat brain cortex (□), CA-20948 pancreatic (○) and CC-531 colon (△) tumour cells by unlabelled Tyr^3-octreotide.

Table 1. Dissociation constant (K_d) and maximum binding capacity (B_{max}) of SS-receptors.

Cell type	K_d(nmol/l)	B_{max} (fmol per mg membrane protein)
CA-20948	0.6	110
CC-531	n.d	n.d
Brain cortex (control)	1.2	243

n.d.:not detectable

Effect of octreotide on the incidence of liver metastases

The number of hepatic metastases of CA-20948 pancreatic tumour cells was significantly reduced after 28 days of treatment with octreotide. The median number of liver tumours was 286 (range 146 to greater than 500) in treated rats and more than 500 (range 250 to in excess of 500) in controls ($P<0.05$). This difference in tumour load also recurred as a difference in mean (s.e.m.) liver weight (14.5 (3.7) g in animals given octreotide and 17.9 (3.0) in controls).(***table 2***) Regression analysis of liver weight and the number of metastases produced a correlation coefficient of 0.95 ($P<0.01$).

Rats injected with colonic tumour cells (CC-531) showed no significant difference in the number of liver tumours (median (range) 6.5 (0-425) in the treated animals and 11.0 (0-475) in the controls) and the mean (s.e.m.) liver weight (14.0 (5.7) g in the treated animals and 11.8 (4.5) in the controls).

Table 2. Incidence of hepatic metastases of CA-20948 and CC-531 cancer cells treated with octreotide.

Tumour cell line and treatment	Mean(s.e.m.)liver weight (g)	No. of tumours Median (range)	No. of animals with liver tumours			
			0 tumour	1-100 tumours	100-500 tumours	≥500 tumours
CA-20948						
Controls(n=8)	17.9 (3.0)	>500(250->500)	0	0	3	5
Octreotide 15μg twice daily(n=8)	14.5 (3.7)*	286(146->500)*	0	0	7	1
CC-531						
Controls(n=8)	11.8 (4.5)	11.0 (0-475)	2	4	2	0
Octreotide 15μg twice daily(n=8)	14.0 (5.7)**	6.5 (0-425)**	2	3	3	0

*$P<0.05$, **not significant (*versus* controls, Mann-Whitney U test)

Effects of octreotide on GH, PRL and IGF-I release

Plasma levels of growth hormone (GH), prolactin (PRL) and insulin-like growth IGF-I) factor-I were not affected by chronic administration of octreotide.(***table** 3*).

***Table 3.** Effect of the administration for 28 days of octreotide (2x15 μg/day) on plasma PRL, GH and IFG-I levels.*

	Prolactin (μg/l)	Growth hormone (μg/l)	Insulin-like growth factor-I (nnmol/l)
Day 4			
Controls(n=8)	12.4(8.6)	10.4(6.3)	108.0(11.5)
Octreotide(n=8)	11.4(8.1)	9.5(6.7)	99.8(10.6)
Day 11			
Controls(n=8)	9.6(6.4)	3.4(1.7)	108.9(11.0)
Octreotide(n=8)	11.7(7.5)	2.8(2.0)	100.9(10.7)
Day18			
Controls(n=8)	10.3(6.9)	5.6(4.0)	103.8(11.6)
Octreotide(n=8)	9.7(7.10)	5.1(3.1)	92.1(13.3)

Values are means (s.e.m.)

Discussion

Several studies have demonstrated that somatostatin analogues inhibit tumour growth in experimental tumour models[1-9]. Most tumours used in these experiments contain somatostatin receptors and were studied *in vitro* and *in vivo* as a transplantable tumour. Nott et al. reported on the effects of octreotide on a hepatic tumour derived from intraportal administration of Walker cells[10,25]. They suggested that the inhibitory effect by octreotide was related to the location of the Walker cells within the liver, since they showed that octreotide stimulated the activity of hepatic RES by 300%. Kupffer cells and large granular lymphocytes form an important natural defence against malignant cells in the liver. A reduced portal venous flow and splanchnic vascular resistance in octreotide treated animals, both decreasing the supply of nutrients in the early stages of development of hepatic tumour, was also found by these authors[25]. RC-160 can also inhibit the incidence and growth of liver metastases of two human colon cancers, by reducing the bromodeoxyuridine labelling index and DNA and protein contents of the tumour suggesting a cytostatic effect of RC-160 on cellular proliferation[26].

Another possible mechanism of antitumoural action of somatostatin analogues includes the interference with growth factor secretion such as PRL, GH and IGF-I. The reduction in

PRL levels produced by the administration of octreotide in animals may contribute to the inhibition of the growth of experimental breast and prostate tumours[12]. The fall in GH levels could also directly or indirectly be of importance for the inhibition of growth of various tumours. Secretion of IGF-I, which mediates most of the effects of growth hormone at tissue levels and also induces differentiation and proliferation of mesenchyme derived cells is also indirectly inhibited by somatostatin analogues[13-16]. A decrease in the concentration of IGF-I or suppression of its activity might therefore reduce or prevent its mitogenic effects shown.

The results of our study show that octreotide significantly reduces the growth and development of hepatic tumours derived from intraportal injection of SS-R positive CA-20948 pancreatic cells, but not of SS-R negative CC-531 colon cells. Since no difference was found between serum levels of PRL, GH and IGF-I during treatment with the somatostatin analogue, the mechanism by which tumour growth inhibition of CA-20948 cells was observed seems to be SS-R dependent and not only influenced by the stimulation of the RES system or by reduction in portal flow because no inhibition of growth was seen of SS-R negative CC-531 hepatic metastases. If any immunomodulation would have taken place, growth inhibition of CC-531 cells might have been expected since they are known to be immunogenic[36,37].

Recently we described the use of isotope labelled somatostatin analogues for the visualization of neuroendocrine tumours as well as their metastases[27]. *In vitro* detection of SS-R on these tumours indicated that the ligand binding to the tumour *in vivo* indeed represents binding to specific SS-R's. The detection of SS-R predicted a good suppressive effect of octreotide on hormonal hypersecretion by these tumours. The results of the present study indicate that octreotide might be considered as a potentially useful agent for the treatment of patients with SS-R positive liver metastases from neuroendocrine tumours, especially since no side-effects were observed in patients using octreotide[38].

In *conclusion*, we have demonstrated that the somatostatin analogue octreotide inhibits the growth and development of hepatic tumours of SS-R positive CA-20948 pancreatic cells. Our findings suggest that this is mediated via a SS-R dependent mechanism, since no inhibition could be found of SS-R negative CC-531 colon cells and no difference in circulating hormones and growth factor was observed. Further clinical studies are required to investigate the potential use of octreotide in patients with SS-R positive liver metastases.

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CHAPTER X

GENERAL DISCUSSION

Some 20 years after the isolation and characterization of somatostatin, five subtypes of somatostatin receptors (SS-R's) were also identified. Ligand-binding and autoradiographic studies demonstrated SS-R's on a variety of human, mostly neuroendocrine tumours. In this thesis the *in vivo* detection of SS-R's (probably only $SSTR_2$-R's) in breast cancer, and islet cell tumours is described, while aspects of the pathophysiological role of these SS-R's was studied.

For the visualization of SS-R positive tumours we used [^{111}In-DTPA-D-Phe^1]-octreotide, since there are several arguments that octreotide scintigraphy represents SS-R imaging. First of all pretreatment with high doses of unlabelled octreotide prevents tumour uptake of [^{111}In-DTPA-D-Phe^1]-octreotide in rats bearing SS-R positive tumours. Autoradiographically it was also found that only the anterior lobe of the rat pituitary, which is the only part of this organ with SS-R's, showed specific binding of the radioligand after its injection. As has been found in our study as well, a close relation exists between the presence of SS-R's, demonstrated with *in vitro* autoradiography and the visualization of tumours and diseases by *in vivo* octreotide scintigraphy.

Despite the many promising reports on the potential benefit of radiolabelled monoclonal antibodies for *in vivo* tumour detection, their widespread application has been hampered for several reasons. The low tumour background ratios achieved with this technique is due to the large molecules often used, which lead to a high background. Because of the rapid clearance of the small sized [^{111}In-DTPA-D-Phe^1]-octreotide by the kidneys, much higher tumour to background ratio can be obtained. Antibody formation, which can often be found after administration of monoclonal antibodies, is extremely rare in patients treated with octreotide. Therefore the SS-R imaging can easily be repeated without any risk of an anaphylactic reaction.

On the basis of specific binding between octreotide and its receptor it turned out to be possible to visualize primary breast cancers, as well as islet cell tumours with radiolabelled octreotide scintigraphy. Also undetectable metastases could be detected.

For breast cancer it was demonstrated that somatostatin analogue receptor scintigraphy can visualize recurrent disease very early, visualization at a stage when patients are without clinical symptoms, and when commonly used serum tumour markers are (still) normal. At present it is unclear whether early detection of recurrent disease is of clinical value. Theoretically early detection is beneficial because it is well known that chemotherapy is more effective in patients with a low tumour burden, although "low" has not been defined.

Somatostatin has been shown to exert tumour growth inhibitory effects, probably mainly via membrane SS-R's. Therefore a randomized controlled prospective study is justified comparing chemotherapy or tamoxifen alone versus chemotherapy or tamoxifen in combination with octreotide in patients with SS-R positive recurrent metastatic disease. The effect of chemotherapy on SS-R expression will be important for the results of such a trial, since one might expect no significant different between the two groups if chemotherapy

causes loss of SS-R expression. Moreover a study concerning radiotherapy with a radionuclide coupled to somatostatin might be considered in such patients.

The pathophysiological significance of SS-R's on breast cancer remains uncertain. In the studies described in chapters III and IV the expression of the somatostatin receptor in human breast cancer was investigated *in vitro* and *in vivo*, respectively. The consecutively collected tumours that were investigated in chapter IV do in part derive from the patients under investigation in chapter III. Due to a small difference in the periods that the tumours were collected the overlap is not complete. However, the patient and tumour characteristics in both studies are comparable. Also the high incidence of somatostatin receptor expression *in vitro* and *in vivo* corresponds well. Strikingly, the results of both studies suggest opposite implication for the patients prognosis. In chapter IV it was suggested that SS-R expression might correlate with a favourable prognosis. This was based on the correlation of SS-R expression with smaller tumour size. Also, earlier retrospective investigations had reported a favourable prognosis in SS-R positive breast cancer. In contrast to these data are the first follow up data of the *in vivo* study that suggest more aggressive disease: out of the 37 patients with a SS-R positive tumour, 11 patients were found to have recurrent disease during the 2.5 years of follow-up, whereas the 17 patients with an initially SS-R negative tumour were all without evidence for recurrent disease. These 11 patients were not characterized by any common patient or tumour characteristic that differentiated these patients from the other patients with a SS-R positive breast tumour, nor could any specific genetic alteration or amplification being found in the tumours. It appears that although SS-R positive tumours show a tendency toward better differentiation and smaller tumour size, this by no means implicates a better prognosis. A multivariate analysis, with a great number of tumours and longer follow-up period is required to show the significance of SS-R expression, and it possible correlation with genetic alterations and/or amplifications and prognosis of patients with primary breast cancer.

A relation between neuroendocrine differentiation and SS-R expression has been previously reported. In parallel with observations in metastasized colonic and prostatic cancer neuroendocrine differentiation might point to a worse prognosis. However, in our study no such correlation could be found. In addition, at the genetic level no relation could be observed between SS-R expression and alterations and/or amplification of frequently used genetic markers in breast cancer. Since no SS-R's seem present in the majority of normal breast tissue, SS-R expression might be acquired by tumour cells due to a neoplastic event, which results in further proliferation of tumour cells at subsequent stages of differentiation, expressing SS-R's. It might also be possible that SS-R expression of tumour cells is caused by an alteration in the expression of certain cellular genes whose normal function involve the regulation of cell growth.

In chapter III and IV the expression of the SS-R in human breast cancer was investigated *in vitro* and *in vivo*, respectively. In both studies a high incidence of SS-R expression was found. In order to investigate any growth inhibitory effect of the somatostatin analogue octreotide on SS-R expressing breast tumours *in vitro*, it was first necessary to culture the collected primary breast cancer cells which has been proved to be difficult. Secondly, since a stimulative effect of tumour-derived fibroblast has been demonstrated on malignant breast epithelial cells, we wanted to separate these cells because octreotide may be involved in negative growth regulation through tumour-derived fibroblasts. In chapter V the results showed the existence of a paracrine growth stimulative mechanism between tumour-derived fibroblasts and epithelial breast cancer cells. However the pathophysiological role of SS-R's and octreotide could not be established but with the possibility of culturing tumour-

derived fibroblast and human breast cancer cells separately, further studies in this field could now be developed.

The observation made in our study in rats showing SS-R dependent growth inhibition of liver metastases by octreotide might contribute to the initiation on of new clinical trials as above mentioned for SS-R positive breast cancer patients but also in patients after surgery for malignant islet cell tumours. However, since SS-R's are not always found on all tumour cells, and might be lost during further dedifferentiation of a tumour, one might only expect temporary growth inhibitory effect of somatostatin analogues.

The sensitivity of the SS-R scintigraphy in localizing islet cell tumours was high, except in the case of insulinomas. The presence of other subtypes of SS-R's in this tumour is well known and necessitates the development of other isotope-labelled somatostatin analogues. Other factors, which in general may interfere with the visualization of neuroendocrine tumours include the presence of unlabelled somatostatin e.g. by auto-, para-, or endocrine production of somatostatin, or somatostatin analogue administration. In both cases this may result in receptor blockade or "down"regulation of the receptor.

Finally, the difference in SS-R expression between islet cell tumours and pancreatic duct cancers seems to offer the possibility to differentiate between these tumour preoperatively. This is important, as palliative surgery in patients with islet cells tumours is not only of value to relieve clinical symptoms but also because a decrease in tumour burden might enhance the effect of medical treatment, resulting in a longer survival. The use of the SS-R scintigraphy as a diagnostic technique for suspected islet cell tumours, is favoured by its harmless, non-invasive nature. In islet cell tumours ultrasound and CT are usually limited to the pancreas and liver region, thereby missing possible metastases. Intra-operative ultrasound and surgical palpation are probably the best methods to localize the tumour, therefore the SS-R scintigraphy should not be performed to localize the tumour but only to support diagnosis of an islet cell tumour and for directly staging the patient.

SUMMARY

Somatostatin, a hormone which has an inhibitory influence on several physiological processes, is a small peptide consisting of 14 amino-acids, and was first isolated from the hypothalamus of the rat. Soon after, somatostatin was found in numerous other organs, such as the brain, stomach, intestines, pancreas, thyroid, thymus and bronchi, from which it could be extracted. Natural somatostatin has a very short half-life and can only be administered intravenously. The development of several longacting analogues of somatostatin facilitated diagnostic procedures and therapy involving somatostatin and its receptors.

One of the first and best known effects of the hormone somatostatin is inhibition of the release of growth hormone by the pituitary gland. Other, mainly inhibitory effects of somatostatin have been described recently, including a direct inhibitory effect on the growth of tumour cells. Somatostatin receptors (SS-R's), present on tumour and pituitary cells, mediate this inhibitory effect as well as release of growth hormone. The analogue octreotide, used in this study, binds to SS-R's on normal and tumour cells. Autoradiography, using ^{125}I-Tyr^{3}-octreotide, was utilized to localize receptors for somatostatin in vitro. These receptors were found in several neuroendocrine tumours, as well as in breast carcinomas and islet cell tumours of the pancreas.

The aim of this study was to assess the possibility of visualizing SS-R's *in vivo*, using octreotide labelled with radioactive ^{111}Indium and diethylene-triaminopentaacetic acid (DTPA) as a carrier (octreoscan).

The octreoscan visualized the primary tumour in 37 out of 50 patients with primary breast carcinoma. Autoradiography of the resected tumours confirmed the findings of the octreoscan. During follow-up, the octreoscan proved able to detect metastases in several patients, some of whom were asymptomatic or exhibited normal serum levels of tumour markers.

The octreoscan could detect islet cell tumours in 80 % of patients tested. In some cases, unknown metastases of these islet cell tumours were visualized by the octreoscan.

To differentiate pre-operatively between endocrine tumours of the pancreas and pancreatic adenocarcinomas, the value of the octreoscan was investigated. In contrast to islet cell tumours, none of the pancreatic adenocarcinomas could be visualized by the octreoscan. Autoradiography of the resected pancreatic adenocarcinomas did not reveal SS-R's.

The visualization of SS-R's *in vivo* can play an important clinical role. Patients with a SS-R positive primary tumour can be staged simply and rapidly, early detection of metastases is possible and the presence of SS-R's on tumours *in vivo* may correlate with a suppressive effect of octreotide on growth of and/or hormone production by these tumours. The octreoscan can help to differentiate preoperatively between endocrine pancreatic tumours and pancreatic adenocarcinomas. This differentiation is important because aggressive surgical therapy of endocrine pancreatic tumours can improve palliation and prolong survival, as illustrated in the case report in chapter VIII.

To investigate whether some genetic alterations or neuroendocrine differentiation would be correlated with SS-R expression on breast cancer cells, SS-R positive as well as SS-R

negative breast tumours were analyzed. A correlation between the presence of SS-R's and the absence of the retinoblastoma gene (tumour suppressor gene) or altered proto-oncogenes was not found. Neuroendocrine differentiation, assessed by Grimelius silver staining and immunohistochemical markers, was not seen more frequently in SS-R positive tumours. Other genetic alterations may be responsible for SS-R expression. These genetic alterations could influence the biological behaviour of this clinically relevant subgroup of tumours.

The mechanism of inhibition of growth of tumour cells by octreotide was studied in two experiments.

In the first experiment, fresh tissue of primary breast tumours was obtained. The tumour cells and the fibroblasts were separated on a gradient, and subsequently cultured. The inhibitory effect of octreotide on the growth of tumour cells, due to paracrine and/or autocrine growth factors produced by tumour cells or fibroblasts, derived from the same tumour, was studied. Fibroblasts proved to promote, as well as inhibit tumour cell growth. When epidermal growth factor (EGF), insulin or oestradiol were added to fibroblasts a synergistic effect on the promotion of tumour growth was observed. In one out of six breast tumours octreotide inhibited the growth of tumour cells in absence of fibroblasts. In another tumour octreotide inhibited the growth of fibroblasts. The model of separating and culturing tumour cells allows detailed investigation of the interaction of tumour cells and tumour derived fibroblasts, as well as studying a possible inhibitory indirect effect of octreotide on tumour growth.

In a second experiment, the effect of octreotide on the growth of tumour cells in the liver was studied. A rat liver metastases model was used. Liver colonies of tumour cells were induced by injecting tumour cells in the portal vein. In one group of rats SS-R positive tumour cells were used, in another group, SS-R negative tumour cells were injected. After intraportal injection of tumour cells, half of the rats was treated with octreotide, the other half, with saline. Tumour colonies could be found in the liver of both groups after four weeks. The growth of tumour cells in the liver was significantly less in the group of rats with SS-R positive tumour cells, that had been given octreotide. No changes of levels of growth factors were observed. Therefore, the inhibition of growth of SS-R positive tumour cells in the liver seems to be a receptor dependent mechanism.

SAMENVATTING

Somatostatine is een hormoon dat een remmende invloed heeft op verschillende fysiologische processen. Het is een klein eiwit bestaande uit 14 aminozuren en werd voor het eerst geïsoleerd uit de hypothalamus van de rat. Spoedig na deze ontdekking werd somatostatine ook in andere organen gevonden, zoals hersenen, pancreas, darm ,schildklier, thymus, darmen en bronchi, en kon het worden gesynthetiseerd. Een van de eerst ontdekte en meest bekende werkingen van somatostatine is remming van de afgifte van groeihormoon door de hypofyse.

Naast somatostatine werden verscheidene langwerkende analogen ontwikkeld, omdat het natuurlijke somatostatine een zeer korte halfwaarde tijd heeft en alleen intraveneus kan worden toegediend. Andere, voornamelijk inhiberende, effecten van somatostatine zijn de laatste jaren beschreven, waaronder een direct groeiremmend effect op tumorcellen. Zowel dit groeiremmende effect, als het inhiberend effect op groeihormoon release wordt tot stand gebracht via somatostatine receptoren (SS-R's), aanwezig op hypofyse- en tumorcellen. Het analogon octreotide, 8 aminozuren lang, bindt eveneens aan SS-R's op zowel normale als tumorcellen.

Receptoren voor somatostatine kunnen op cellen worden aangetoond met behulp van autoradiografie met [^{125}I-Tyr^{3}]-octreotide. Op vele neuroendocriene tumoren maar ook op mammacarcinomen evenals eilandjes tumoren van het pancreas werden deze receptoren gevonden.

Doel van ons onderzoek, beschreven in dit proefschrift, was in eerste instantie de mogelijkheid te bestuderen of SS-R's ook *in vivo* gevisualiseerd konden worden. Hiervoor werd gebruik gemaakt van het analogon octreotide waaraan met behulp van diethyleen-triaminopentaazijnzuur (DTPA) radioactief ^{111}Indium werd gekoppeld (octreoscan).

Bij 50 patiënten met een primair mammacarcinoom was het in 75% van de gevallen mogelijk om de primaire tumor te visualiseren. Autoradiografie op de na operatie verkregen tumoren valideerde dat daadwerkelijk SS-R's zichtbaar werden gemaakt. Ook metastasen van patiënten met een primair SS-R positieve tumor konden tijdens de follow-up worden gevisualiseerd, in sommige gevallen nog voordat de patiënten symptomen hadden, of tumormarkers verhoogd waren. Eilandjes tumoren van het pancreas konden in 80% van de gevallen worden gezien evenals vaak nog niet eerder bekende metastasen.

Om preoperatief te kunnen differentiëren tussen endocriene en exocriene pancreas tumoren werd de waarde van de octreoscan onderzocht. Alle exocriene pancreas tumoren (adenocarcinomen) waren in tegenstelling tot de endocriene tumoren, niet te visualiseren en ook bij autoradiografie waren geen SS-R's aanwezig. Het visualiseren van SS-R's *in vivo* heeft mogelijk als klinische betekenis dat, patiënten met een primair SS-R positieve tumor eenvoudig en snel kunnen worden gestageerd, vroege detectie van metastase mogelijk is en dat de aanwezigheid van SS-R's *in vivo* op tumoren, een voorspellende waarde heeft voor een suppressief effect van octreotide op groei van en/of hormoonproduktie door deze (eilandjes) tumoren. Ook kan in vele gevallen preoperatief reeds gedifferentieerd worden tussen endocriene en exocriene pancreastumoren. Dit is van belang is voor de therapie, daar een

agressief chirurgisch ingrijpen bij endocriene tumoren niet alleen van palliatieve betekenis is, doch ook daadwerkelijk de overleving van patiënten kan verlengen. Een voorbeeld van deze benadering wordt gegeven in het case report beschreven in hoofdstuk VIII.

Om te onderzoeken of bepaalde genetische afwijkingen, dan wel neuroendocriene differentiatie gecorreleerd zijn met SS-R expressie op mammacarcinoom cellen, werden zowel SS-R positieve als SS-R negatieve mammatumoren geanalyseerd. De aan- of afwezigheid van SS-R's bleek niet gecorreleerd met de afwezigheid van het retinoblastoma gen (tumor-suppressor gen), of met, bij het mammacarcinoom veel voorkomende afwijkingen van verschillende proto-oncogenen. Neuroendocriene differentiatie, onderzocht met de Grimelius zilverkleuring en immunohistochemische markers, werd ook niet vaker aangetoond bij SS-R positieve tumoren. Waarschijnlijk zijn andere dan de door ons onderzochte genetische afwijkingen verantwoordelijk voor de SS-R expressie en bepalen ook deze afwijkingen het biologisch gedrag van deze klinisch relevante subgroep.

Om een groeiremmend effect van octreotide op tumorcellen aan te tonen werd een tweetal experimenten uitgevoerd, welke nog niet eerder zijn beschreven. Allereerst werd bestudeerd of het groeiremmend effect van octreotide veroorzaakt werd door inhibitie van para- en/of autocriene groeifactoren geproduceerd door tumorcellen zelf of door fibroblasten afkomstig van dezelfde tumor. Hiertoe was het noodzakelijk om primaire tumoren in kweek te brengen. Primaire mammacarcinomen werden hiervoor vers aangeleverd, waarbij de tumorcellen zelf en de van de tumor afkomstige fibroblasten na zuivering apart werden geincubeerd. Deze fibroblasten bleken zowel tumorcel groei te kunnen stimuleren als te kunnen remmen. Bekende groeifactoren, als epidermal growth factor (EGF), insuline en oestradiol hadden een synergistisch effect met deze fibroblasten op tumorcel groei. Octreotide remde in een enkel geval de tumorcel groei alleen in afwezigheid van fibroblasten en bleek in een andere cultuur de groei van fibroblasten te kunnen remmen.

Deze eerste studie toont duidelijk het belang aan van de mogelijkheid om primaire mammatumoren in kweek te kunnen brengen en de tumorcellen apart te kunnen incuberen, gescheiden van fibroblasten afkomstig van dezelfde tumor. Op deze manier wordt het mogelijk om de rol van deze fibroblasten bij tumorcel proliferatie te bestuderen. Ook een eventueel indirect effect van octreotide kan op deze manier worden geanalyseerd.

In een tweede experiment werd onderzocht of remming van tumorgroei in de lever door octreotide mogelijk was, en afhankelijk was van de aanwezigheid van SS-R's, of dat dit het gevolg was van inhibitie van verschillende bekende groeifactoren, zoals groeihormoon, prolactine of insuline-afhankelijke groeifactor (IGF-I). Hiervoor werd een lever metastase model bij de rat gebruikt. Door in een groep ratten SS-R positieve en in een andere groep ratten SS-R negatieve tumorcellen in de vena porta te injecteren, groeiden na enige weken, in beide groepen kolonies tumorcellen in de lever. Na intraportale injectie van tumorcellen werd de helft van de ratten behandeld met octreotide en de andere helft met fysiologisch zout. De uitgroei van leverkolonies was significant minder in de groep van SS-R positieve tumorcellen behandeld met octreotide, zonder dat dit het gevolg was van inhibitie van een van de eerder genoemde groeifactoren. De inhibitie van SS-R positieve tumorcellen in de lever lijkt dus een receptor gebonden mechanisme en verdient derhalve klinische evaluatie.

DANKWOORD

De laatste jaren heb ik ervaren, dat samenwerking tussen verschillende disiplines binnen een Academisch ziekenhuis zeer plezierig en vruchtbaar kan zijn. Na het begin van het tweede deel van de opleiding tot chirurg, was in eerste instantie **Hans Jeekel** degene die mij stimuleerde om wetenschappelijk onderzoek in de oncologie te gaan doen. Hij is ook verantwoordelijk geweest voor mijn komst naar het Dijkzigt ziekenhuis, wat ik tot op heden nog geweldig waardeer. De keuze om mijn vervolg opleiding in het Dijkzigt ziekenhuis te willen doen, onder leiding van **Kieje Bruining**, is nooit een verkeerde geweest. Naast een vaak koele heldere klinicus, heb ik hem gelukkig de laatste jaren ook leren kennen als een levensgenietende "tuinman".

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