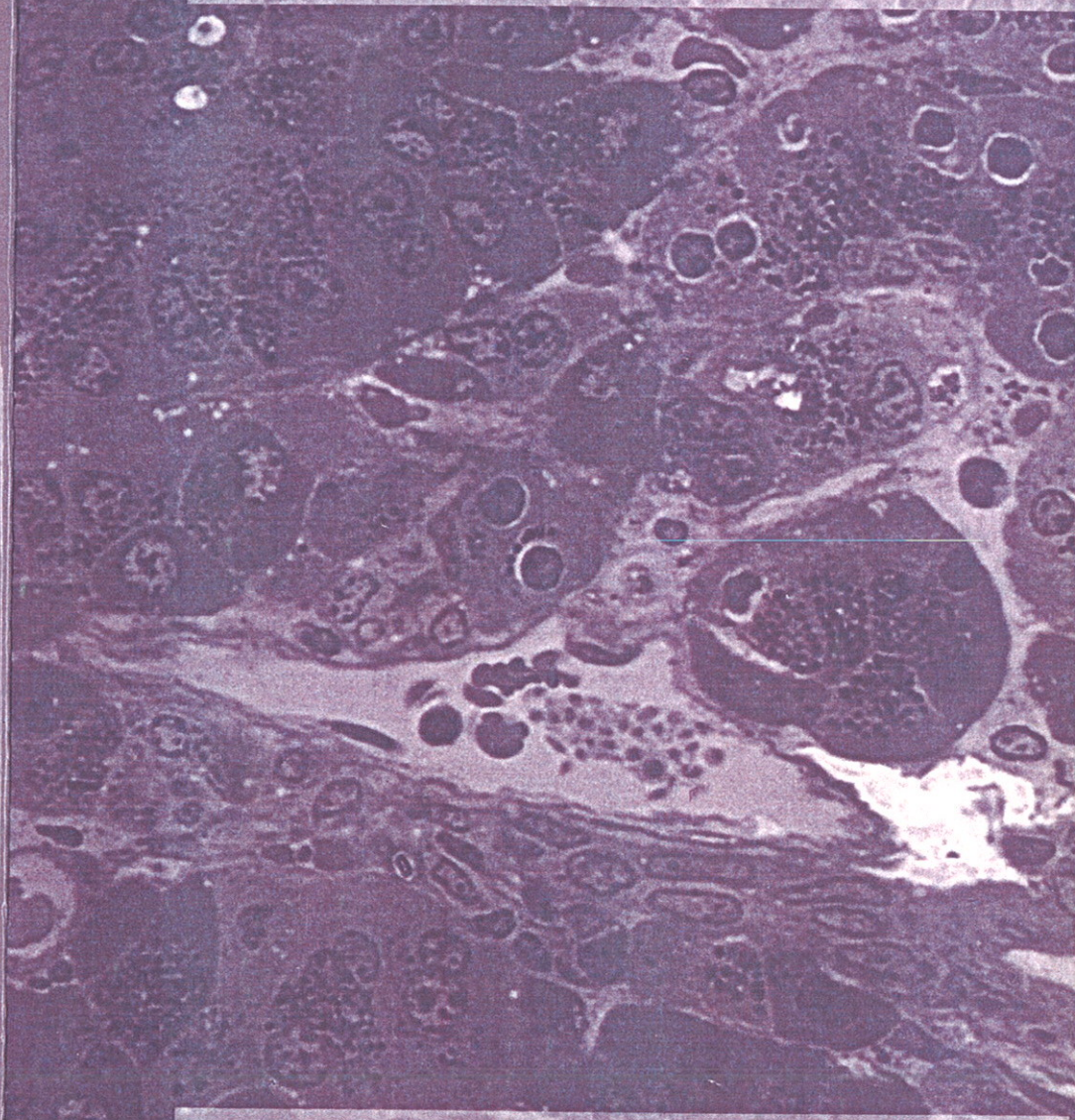
Hormones and Growth Factors in Experimental Exocrine Pancreatic Carcinogenesis



Corjan Visser

Hormones and Growth Factors in Experimental Exocrine Pancreatic Carcinogenesis

De betekenis van hormonen en groeifaktoren bij het ontstaan van kanker van de exocriene pancreas

(met een samenvatting in het Nederlands)

No reprints available.

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Voor pa en ma,

Aan Ingeborg

On the cover:

Photomicrograph of a semi-thin (1 μm) tissue section of a pancreas from an azaserine-treated rat. This tissue section was stained with polychrome in ethanol according the protocol of Sato Shamato, *Stain Technology* **48**, 223-227 (1973).

LIST OF ABBREVIATIONS

AACF	Atypical Acinar Cell Focus/Foci
AACN	Atypical Acinar Cell Nodule(s)
AGT	Aminoglutethimide
ATP	Adenosine Triphosphate
BOP	*N*-nitrosobis(2-oxopropyl)amine
BrdU	Bromo Deoxyuridine
BSA	Bovine Serum Albumine
CCK	Cholecystokinin
cDNA	Complementary DNA
CTP	Cytosine Triphosphate
DAB	Diaminobenzidine Hydrochloride
DNA	Deoxynucleic Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme Linked Immuno Sorbent Assay
GTP	Guanidine Triphosphate
H&E	Haematoxylin and Eosin
HPOP	*N*-nitroso(2-hydroxopropyl)(2-oxopropyl)amine
IGF	Insulin-like Growth Factor
IHC	Immunohistochemistry
ISH	*In-situ* Hybridization
kD	kilo Dalton
LHRH	Luteinizing Hormone-Releasing Hormone
mRNA	messenger RNA
OD	Optical Density
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PDGF	Platelet Derived Growth Factor
PDGFR	Platelet Derived Growth Factor Receptor
PVDF	Polyvinylidene Difluoride
RAShPO	Rabbit-anti-Sheep antibody, Peroxidase conjugated
RNA	Ribonucleic Acid
RNase	Ribonuclease
RIPA	Radio Immunoprecipitation Assay Buffer
RT	Room Temperature (±20°C)
SDS	Sandostatin
SMS	Somatostatin

SSC	Standard Sodium Citrate
SwARPO	Swine-anti-Rabbit antibody, Peroxidase conjugated
TGF-α	Transforming Growth Factor-α
Tm	melting Temperature (of a nucleic acid hybrid)
tRNA	transfer RNA
UTP	Uridine Triphosphate

CHAPTER ONE

PANCREATIC CANCER, HORMONES AND GROWTH FACTORS

1.0 Introduction

1.1 Epidemiology and etiology of human pancreatic cancer

1.2 Histology of human pancreatic cancer

1.3 Animal models of pancreatic cancer

1.4 Histogenesis of pancreatic cancer in experimental animals and its relevance for human cancer

1.5 Dietary modulation of (experimental) pancreatic carcinogenesis

1.6 Hormones

1.7 Growth factors

1.8 Receptors and signal transduction

1.9 Aim of the thesis

1.0 INTRODUCTION

This thesis deals with the role of hormones and growth factors in exocrine pancreatic carcinogenesis (**Figure 1.1**). Because of the large number of hormones and growth factors, it is virtually impossible to study all of them. Moreover, the experiments described, were not performed in humans, but in two animal models for exocrine pancreatic cancer, i.e. the azaserine-treated rat (leading to acinar adenocarcinomas) and the *N*-nitrosobis(2-oxopropyl)amine-treated hamster (leading to ductular adeno-carcinomas). In this chapter a short literature overview will be presented to explain the selection to study certain hormones and growth factors and to support the aims of this thesis.

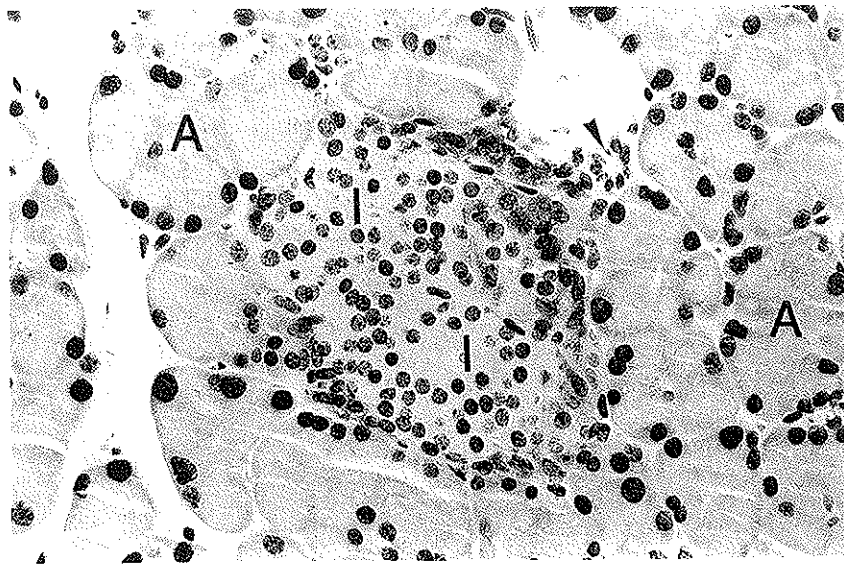

Figure 1.1 Exocrine and endocrine pancreas, H&E staining. I, islet of Langerhans (endocrine); A, acinar cells (exocrine); arrowhead point to ductule (exocrine).

1.1 EPIDEMIOLOGY AND ETIOLOGY OF HUMAN PANCREATIC CANCER

In most industrialized Western countries pancreatic cancer is relatively frequent in comparison with non-industrialized countries (1-3). In the United States of America adenocarcinoma of the pancreas is the second most common gastrointestinal malignancy

and has become the fifth leading cause of death due to cancer (1,2,4-6). In The Netherlands pancreatic cancer in males ranks third among gastrointestinal and fifth among all cancer deaths, whereas in females it ranks third and sixth, respectively (3). The mortality rate for pancreatic cancer is almost as high as its incidence because of its extremely poor prognosis. In the United States the average survival from diagnosis until death is approximately three months (2,4,7,8). This poor prognosis is mainly due to the lack of early symptoms. At the time of diagnosis most of the tumours have already extensively metastasized.

The prognosis of pancreatic cancer may be improved by a better understanding of its pathogenesis. However, epidemiological and toxicological studies have not identified factors unequivocally increasing the risk for pancreatic cancer (2,7,9). Nutrition seems to play an important role in the pathogenesis of pancreatic cancer, because the disease is more frequent in countries with a diet rich in fat and protein (2,9). For example, in Europe the incidence of pancreatic cancer is higher in North-Western countries such as The Netherlands, Denmark and Germany, than in the Southern countries such as France, Italy or Greece where carbohydrate-rich diets are common (10). In Japan, westernization of the diet is accompanied by a rapid increase in the incidence of pancreatic cancer (11). Both sex-hormones and gastrointestinal hormones are thought to play a role in the etiology of pancreatic cancer. This will be discussed in more detail in the next paragraph of this chapter.

A clear correlation has been found between excessive alcohol consumption and pancreatitis. Although pancreatitis is thought to be a predisposing factor for pancreatic cancer, no clear association was found between alcohol consumption and the occurrence of tumours of the exocrine pancreas (12,13). A number of other factors have been suggested to predispose for pancreatic cancer. Diabetes or impaired glucose tolerance occurs in about 80% of patients with pancreatic cancer at the time of diagnosis (14). However, recent evidence suggests that the impaired glucose tolerance, caused by peripheral insulin resistance, is a consequence of pancreatic cancer rather than a predisposing factor (15).

Since no single factor has been identified to be responsible for the development of pancreatic cancer, it is believed that factors promoting the effects of environmental carcinogens may play an important role in the pathogenesis of this tumour (16-18).

1.2 HISTOLOGY OF HUMAN PANCREATIC CANCER

Carcinoma of the pancreas usually refers to a neoplasm arising in the exocrine pancreas, whereas neoplasms of the endocrine pancreas are collectively named islet cell tumours or apudomas. Cubilla and Fitzgerald (19) and Morohoshi *et al.* (20), have classified the various histological types of human pancreatic exocrine tumours according to their presumed cell of origin and concluded that 89-95% were of ductal or ductular origin, while only 1-4% were of acinar origin. The remaining 1-10% were classified as being of uncertain histogenesis. The great majority of human pancreatic adenocarcinomas shows the presence of tubular structures, which is interpreted as evidence that pancreatic cancer arises from ducts or ductules. However, no conclusive evidence is yet available for this suggestion.

It is known that 'spontaneous' pancreatic neoplasms apparently originating from ductal or ductular epithelium are extremely rare in mammals, except man (21). In contrast, tumours apparently arising from acinar cells occur in several species (22-24). Interestingly, in rats prone to spontaneous acinar cell tumours, only this tumour type can be induced experimentally, and not the ductal carcinomas. On the other hand, in Syrian golden hamsters only ductular cell neoplasms can be induced; this type of neoplasm occurs spontaneously in this species (25). Acinar cell tumours in hamsters do neither occur spontaneously, nor under experimental conditions (21).

1.3 ANIMAL MODELS OF PANCREATIC CANCER

Animal models for exocrine pancreatic carcinogenesis can generally be divided into two separate groups: (a) those that result in predominantly ductal-type adenocarcinomas and (b) those that produce mainly acinar-cell tumours.

a. Pancreatic cancer model in hamsters (25-33).

Pancreatic ductal (ductular) tumours can be induced in Syrian golden hamsters by several specific propylnitroso compounds of which *N*-nitrosobis(2-oxopropyl)amine (BOP) has been found to have a great pancreatropic effect with a narrow tumour spectrum in other organs (lungs, liver, gall bladder and kidneys). Pancreatic neoplasms can be induced by weekly repeated subcutaneous injections of BOP in doses of 10 mg/kg body weight, as early as 8 weeks after the first BOP injection. After a single dose of BOP (20 mg/kg body weight) hyperplastic changes are seen within 17 weeks

consisting of multifocal cystic ductular complexes, tubular ductular complexes and intermediate ductular complexes. After a single dose of 20 mg/kg body weight, neoplastic lesions were found within 12 months.

b. Pancreatic cancer model in rats (34-43).

Induction of pancreatic hyperplastic nodules, adenomas and carcinomas in rats by intraperitoneal injection of azaserine was first reported by Longnecker *et al.* (34,35). Adenocarcinomas or poorly differentiated carcinomas developed in the pancreas of rats that were repeatedly treated with azaserine, as early as 11 months following initial azaserine treatment. Two months following azaserine treatment, the earliest manifestations are found. These local lesions have been described as atypical acinar cell foci or nodules. The number and size of these nodules/foci increases with time. Wistar and W/LEW rats were highly responsive to nodule induction. Virtually all azaserine-treated rats developed multiple cell foci four months after treatment. These putative preneoplastic atypical acinar cell foci can be easily quantified at 4-6 months after a single injection with 30 mg azaserine/kg body weight, which permits post-initiation modulation to be evaluated within a relatively short period of time. These primary foci and nodules can be classified as acidophilic (eosinophilic) and basophilic. The primary basophilic foci do not show detectable secondary phenotypic changes and, therefore, are not considered to be related to the development of cancer. The acinar adenocarcinomas seem to develop in a stepwise process from the eosinophilic pancreatic acinar foci and nodules (42). It has been demonstrated that even administration of a single dose of azaserine below the cytotoxic level induces a number of foci and nodules in the pancreas of Wistar rats (43).

1.4 HISTOGENESIS OF PANCREATIC CANCER IN EXPERIMENTAL ANIMALS AND ITS RELEVANCE FOR HUMAN CANCER

Because of the morphological similarity of the induced tumours to those occurring in humans, the BOP-hamster model has been suggested to provide a unique opportunity to study pancreatic carcinogenesis (31). The histogenesis of the ductal/ductular adenocarcinomas induced in hamsters, however, is still a topic of debate in the literature. In BOP-treated hamsters, many tumours are found to develop within or in the vicinity of islets, in the form of 'intra-insular ductules' associated with newly formed endocrine cells (nesidioblastosis). Therefore, Pour *et al.* (21,31) postulated that

ductular and islet precursor cells are the origin of the pseudoductular lesions and, hence, adenocarcinomas. More recently, the centroacinar cell (also a ductular cell type) has been emphasized as such (44-46). Electron microscopical examination of pancreatic tumours, induced in hamsters by nitrosamines, has led to the conclusion that the tumours arise from existing ducts without involvement of acinar cells (45,47,48). Other investigators, however, claim that pancreatic adenocarcinomas in hamsters develop from acinar cells by dedifferentiation (49-55).

In man, only a small percentage of tumours, namely those that have clearly recognizable acinar cell differentiation, is classified as being of acinar origin. Most other tumours contain duct-like structures and have been classified as from ductal origin. However, there is ultrastructural evidence of acinar cell characteristics in all human pancreatic tumours (56-58). Furthermore, acinar dysplasia appears to be common in patients with pancreatic cancer (37). Longnecker (39) proposed that early biochemical and perhaps even immunological markers may be different for neoplasms of acinar cell and ductal cell origin.

To collect more evidence about the cell type from which pancreatic adenocarcinomas originate, we recently have performed a twelve-months study in both hamsters and rats (59). In this study we placed osmotic pumps, that were subcutaneously secreting bromide-deoxyuridine (BrdU), three days before autopsy. Autopsy was performed on groups of rats and hamsters 2, 6, 12, 26 and 52 weeks after the last injection with carcinogen (or saline, in control animals). BrdU is incorporated in the DNA of dividing cells. Therefore, the immuno-histochemically determined BrdU labelling index reflects the number of cell divisions in a certain tissue. In this study we demonstrated that the pancreatic non-tumourous (centro)acinar and ductular cells in azaserine-treated rats did not show increased cell proliferation when compared with control rats. However, as expected, the acinar cells in the putative preneoplastic atypical acinar cell foci demonstrated a significantly increased cell proliferation (**Figure 1.2**). More surprising were the results in hamsters. Up to 26 weeks after the last BOP injection, the non-tumourous acinar cells in these animals demonstrated significantly increased labelling indices when compared to saline-treated controls, whereas the non-tumourous ductular cells only demonstrated significantly increased labelling indices two weeks after the last BOP-injection, but not at later time points.

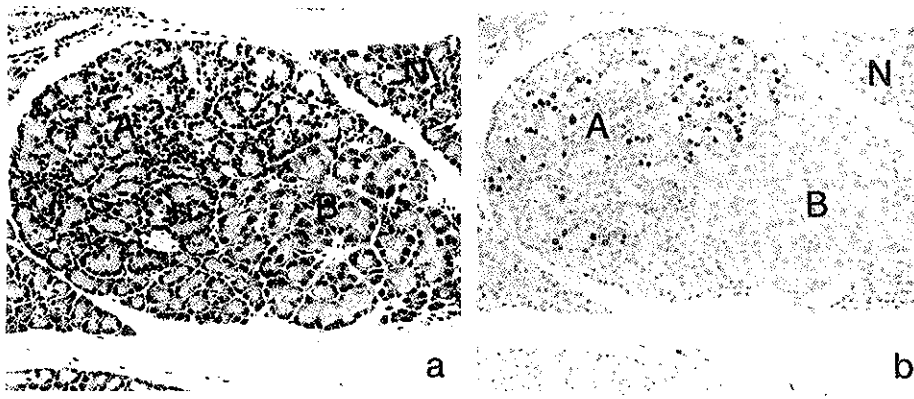

Figure 1.2 Serial sections of the pancreas of an azaserine-treated rat: **a.** H&E staining; **b.** BrdU incorporation, haematoxylin counterstaining. Note the extensive nuclear BrdU staining in the acidophilic atypical acinar cell focus (AACF). A, acidophilic AACF; B, basophilic AACF; N, normal acinar cells.

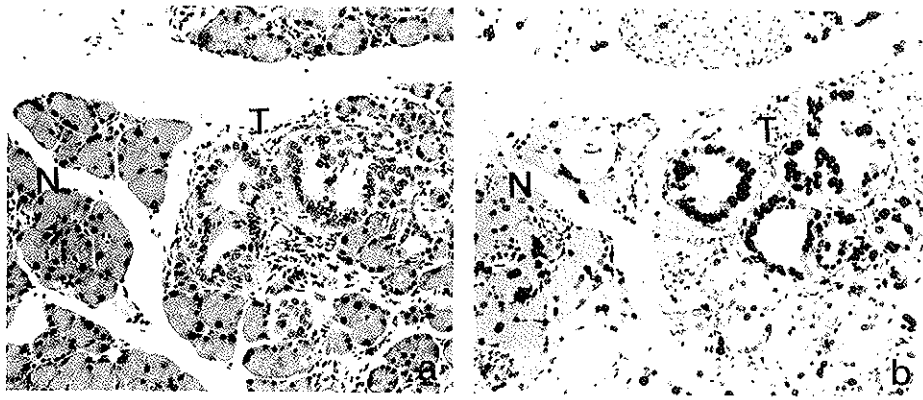

Figure 1.3 Serial sections of the pancreas of a BOP-treated hamster. **a.** H&E staining; **b.** BrdU incorporation, haematoxylin counterstaining. Note the extensive nuclear BrdU staining in the tubular ductular complex. N, normal acinar cells; T, tubular ductular complex.

These results suggest that a role for the acinar cells in ductular pancreatic carcinogenesis can not be excluded. As expected, in advanced tubular ductular complexes (putative preneoplastic lesions with high potency for malignant transformation, **Figure 1.3**) the labelling indices were significantly higher than in cystic ductular complexes (low potency for malignant transformation).

Labelling indices were highest in the putative preneoplastic lesions characterized by atypia, desmoplasia and inflammatory cells (borderline lesions; highest potency for malignant transformation). From this study we concluded, that determination of BrdU labelling indices provides a reliable parameter to discriminate between putative preneoplastic lesions with a high or a low growth potential, hence with a high or low potential to develop into ultimate carcinomas.

It seems likely that factors that promote or inhibit progression of early stages of carcinogenesis may be different in lesions originating from the two cell types. However, no conclusive data are available on the role of precursor lesions in pancreatic carcinogenesis. The above considerations indicate that not only the BOP-treated hamster, but also the azaserine-treated rat provides an animal model relevant for pancreatic cancer in man and that it is worthwhile to study the modulating effects of hormones such as cholecystokinin (CCK) and life-style factors such as dietary fat or cigarette-smoke on pancreatic carcinogenesis in both hamster and rat (60).

1.5 DIETARY MODULATION OF (EXPERIMENTAL) PANCREATIC CARCINOGENESIS

A high intake of dietary fat enhances azaserine-induced acinar cell adenomas and carcinomas in rat pancreas (61-64,68) as well as pancreatic ductular adenocarcinomas produced by *N*-nitrosobis(2-oxopropyl)amine in Syrian golden hamsters (65-69). Moreover, an increased intake of corn oil promotes the development of both the azaserine induced putative preneoplastic atypical acinar cell foci in rats (60-62) as well as the early putative preneoplastic ductular lesions in the hamster model (60). Therefore, these animal models create an unique opportunity to study the mechanism by which dietary fat enhances pancreatic carcinogenesis.

1.6 HORMONES

There are data indicating a possible role of cholecystokinin (CCK), a hormone produced in the gut, in the pathogenesis of pancreatic cancer. The positive correlation between diets high in protein and fat content and the occurrence of pancreatic cancer might be ascribed to an enhanced CCK release. Gastrectomy, which also has been found to give rise to an excessive release of CCK in response to ingested fat (70) has also been correlated with an increased risk for this disease (71,72). Other evidence for a role of CCK in the pathogenesis of pancreatic cancer has been derived from animal experiments. Raw soya flour as well as trypsin inhibitors, which cause CCK release in rats, have been shown to promote carcinogenesis in the azaserine-rat model (73-75). Exogeneously administered caerulein (a synthetic CCK analogue) or CCK itself has been shown to promote pancreatic carcinogenesis in rats (76,77).

The prevalence of pancreatic cancer in males suggests a role for sex hormones in this carcinogenic process (78-80). Indeed, receptors for oestrogen and testosterone have been demonstrated in pancreatic cells (81,82). Blood testosterone levels in pancreatic cancer patients are reduced, most probably due to binding of testosterone to the cancer cells (83). In addition, some hormones secreted by the hypothalamus have been identified in pancreatic tissue. Moreover, high activities of sex steroid biosynthetic enzymes have been measured in pancreatic tumours (84,85). The findings in these reports suggest that sex steroids are involved in the physiology of foetal, adult and malignant pancreatic tissue. In general, most studies are pointing to a protective effect of oestrogen in men.

Studies have been performed in animal models to investigate the effects of sex hormones on pancreatic carcinogenesis. Although there are some conflicting reports, generally in azaserine-treated rats, testosterone is found to promote and oestrogen to inhibit tumour growth (86-88). In contrast, in BOP-treated hamsters, oestrogen is thought to promote and testosterone to inhibit (89-91). Therefore, it has been suggested that agents such as aminoglutethimide, known to interfere with steroid metabolism, may be of therapeutic value for the treatment of pancreatic cancer.

Another hormone that has been mentioned in relation to pancreatic cancer is somatostatin. Somatostatin is a tetradecapeptide widely distributed throughout the body, being found in high concentrations in the brain, stomach, intestine and pancreas (92). The normal physiological role for somatostatin is the inhibition of secretion by-, and growth of various tissues. In the pancreas, somatostatin inhibits secretion of protein and bicarbonate (93). Inhibitory effects of somatostatin on endocrine pancreatic tumours have been reported (94), but specific somatostatin receptors could not be detected in

human exocrine pancreatic tumours (95) or in human pancreatic cancer cell lines (96). Experimental studies are not conclusive in reporting inhibition of pancreatic carcinogenesis due to somatostatin treatment (97, 98). However, Klijn (99) reported that most pancreas cancer patients experienced a subjective improvement due to a reduction of side-effects after treatment with the somatostatin-analogue Sandostatin. Therefore, it has been hypothesized that somatostatin might exert its effects by suppressing the secretion and/or action of gastrointestinal hormones such as cholecystokinin, secretin and gastrin (100). Recent studies in animal models for exocrine pancreatic cancer are suggesting that a combined treatment with testosterone inhibitors and somatostatin might be of therapeutic value for exocrine pancreatic tumours (101,102).

1.7 GROWTH FACTORS

A growing body of evidence points to an essential role of both cell associated and secreted polypeptide intercellular signalling molecules in the normal growth and development of multicellular organisms (103). Prominent among these molecules are growth factors that bind with high affinity to specific target cell surface receptors, that are, in turn, coupled to intracellular pathways of signal transduction.

Although chapters 5, 6 and 7 of this thesis will concentrate on growth factors in the most strict sense of the word, other factors stimulating cellular proliferation should also be mentioned. One group of such peptides is mentioned in chapters 2 and 3 of this thesis: hormones. Apart from their distinct biological function, some hormones also stimulate growth. The most apparent difference from 'real' growth factors is their way of targeting cells. Hormones typically are secreted into the blood-flow by endocrine organs. The blood-flow brings the hormones to their target organs, where they stimulate specific cell-membrane or nuclear-membrane bound receptors. In contrast, growth factors mostly act on the peptide producing cell itself (autocrine stimulation) or a neighbouring cell in the same organ (paracrine stimulation) (**Figure 1.4**).

In some cases, however, this distinction becomes unclear. For example, members of the Insulin-like Growth Factor (IGF) family have been found to work also in an endocrine fashion (104); IGF-I and IGF-II are present in the circulation and can be readily detected in plasma.

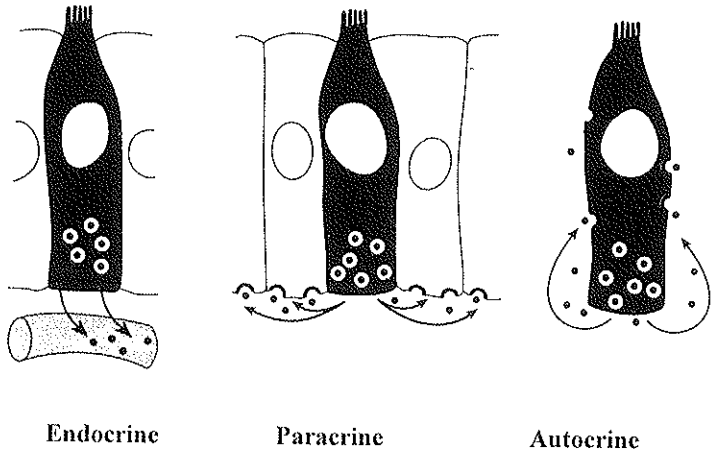

Figure 1.4 a. Endocrine stimulation; b. paracrine stimulation; c. autocrine stimulation (J.K. Heath, *In:* Growth Factors. D. Rickwood (Ed.), In Focus Series, 1993; by permission of Oxford University Press).

Another group of growth stimulating factors, are the small bioregulatory peptides such as bombesin, neuropeptide Y, or endothelin, indicating that growth regulation of cells is not a property preserved to a small set of molecules, but rather a complicated process that is the result of interactions between many different molecules with many different cell types. Furthermore, there is a specific group of agents involved in proliferative activities in the haematopoietic system (105). Haematopoiesis involves the coordinated proliferation of a variety of cell types originating from one common precursor cell. This is achieved by the subsequent action of different growth factors that act specifically on different developmental stages of the cell (and therefore different target cells) and only in a physiologically adequate environment for that specific growth factor (e.g. erythropoietin). The last group of growth factors that will not be discussed *in extenso* in this thesis, are the so called interleukins (cytokines). These peptides were originally found to play a role in the signalling between leucocytes and between leucocytes and other cells; they are involved in immunological processes. Especially in the hamster model for ductular pancreatic carcinogenesis, large lymphocytic infiltrates

can be observed around (pre)neoplastic lesions. Therefore, although not studied in the experiments described in this thesis, a role for interleukins may be of significance in this carcinogenic process.

The variety of the aforementioned molecules and processes stresses the hypothesis that each organ in the body is subject to complex and hierarchical control involving multiple growth factor species with restricted target-cell specificities. The broad ranging activities of many growth factors may be tightly constrained by control of their dissemination, availability, and delivery to responding cells (106).

Growth factors in the strict sense of the word involve also numerous different proteins. Based on their molecular structure and on their function, they can be categorized into five main families; the Epidermal Growth Factor family (up to 9 family members; a.o. EGF and TGF-α), the Platelet-Derived Growth Factor family (PDGF-AA, PDGF-AB and PDGF-BB), the Fibroblast Growth Factor family (a.o. basic FGF, acidic FGF), the Insulin-like Growth Factor Family (IGF-I and IGF-II) and the Transforming Growth Factor-ß family (a.o. TGF-ß1, -ß2, and -ß3).

The factors that regulate pancreatic cancer cell proliferation are not clearly defined. Chester *et al.* (107) injected EGF subcutaneously in Syrian hamsters together with weekly injections of the carcinogen BOP during 10 weeks, and observed almost a doubling in the incidence of pancreatic ductular adenocarcinomas compared to hamsters injected with BOP only. These results point to a cocarcinogenic effect of EGF in the BOP-hamster model but a promoting effect of EGF could not be excluded. PANC-1 and MIA PaCa-2 human pancreatic cancer cell lines have receptors for EGF (108-110). EGF stimulates the growth of MIA PaCa-2 pancreatic cancer cells in culture and may act as autocrine growth factor (109). It has also been found that pancreatic cancer cells express transforming growth factor-alpha (TGF-α; 111-113). Korc (111) determined that certain pancreatic carcinoma cells produce transforming growth factor-beta (TGF-ß), another growth factor that does not bind to the EGF receptor but to its own distinct receptors. They also found that TGF-ß inhibits the proliferation of some pancreatic cancer cell lines.

Apart from EGF and TGF-α and -ß, Mössner *et al.* (114) found that insulin-like growth factors (IGF-I and -II) had weak promoting effects on the growth of AR42J cells, a cell line that is derived from a transplantable acinar pancreatic tumour from a rat, but the role of these growth factors in the growth of normal exocrine pancreas or in the development of tumours of the exocrine pancreas is still unknown. Regulatory molecules with a distinct chemical structure generally act on distinct receptors because the receptor is specific for a unique part of the regulatory molecule. Regulatory

molecules such as cholecystokinin (CCK), somatostatin, gastrin, IGF, EGF, TGF-α or -ß, may act on unique receptors, related receptors, or identical receptors and this distinction is frequently difficult to make. Some of the regulatory molecules not only act on acinar cells, but also affect ductular cells where their biological response can be different but the mechanism of action may be similar.

This thesis focuses on two members of the epidermal growth factor family: the epidermal growth factor (EGF), the transforming growth factor-α (TGF-α) and their mutual receptor, the epidermal growth factor receptor (EGFR). The reason for this selection is that at the time the work underlying this thesis started, only this growth factor/receptor system was thought to play a significant role in pancreatic carcinogenesis in humans. Apart from that, there was also a more practical reason to concentrate on EGF, TGF-α and EGFR; these molecules were some of the few that were sequenced in more species than in humans only, which made starting up of the polymerase chain reaction (PCR) experiments more easy. In the course of this project, other growth factors were found that may also be involved in (human) pancreatic carcinogenesis. However, instead of studying more (a greater variety of) growth factors, we decided to focus on the signal transduction process that may be involved in the functioning of EGFR. Therefore, EGF and TGF-α will be described in more detail.

EGF and TGF-α are two closely related, highly conserved growth factors of the EGF-family sharing 33-40% homology. Both growth factors are made as a much larger amino-acid transmembrane precursor and the mature proteins are cleaved from the precursor by specific proteases (115).

EGF was the first growth factor isolated in pure form. When newborn mice were injected with homogenates of mouse submaxillary gland, accelerated maturation of various epithelia and premature eyelid opening could be observed. Especially submaxillary glands from male mice were found to be an extremely rich source of 6kDa polypeptide EGF, making this growth factor rather easy to isolate (in contrast to most other growth factors). Upon sequence analysis, the molecule urogastrone (isolated from the urine of pregnant women) appeared to be the human homologue of the murine EGF (116). Both EGF and urogastrone appeared to act as a mitogen on a wide variety of epithelial cells and also on 3T3 fibroblasts (117). EGF expression is restricted to a small number of organs, such as the submaxillary glands, the kidneys, and the Brunner's glands in the duodenum.

Fibroblasts infected with Moloney sarcoma virus (MSV) sometimes demonstrated sustained proliferation independent from the presence of EGF, whereas uninfected fibroblasts were dependent of EGF to proliferate. It was hypothesized, that MSV infection leads to EGF expression of these cells themselves. In that way, these

cells were able to stimulate their own proliferation, independent from exocrine factors (autocrine stimulation; **Figure 1.4**). An EGF-like bioactivity was found in media conditioned by MSV-infected cells which proved, upon purification, to be an EGF-related growth factor, later called TGF-α. TGF-α is a 6kDa polypeptide that acts as a potent mitogen for many epithelial and mesenchymal cells. Not only the mature TGF-α peptide, but also the membrane-bound TGF-α precursor is found to have bioactivity. In contrast to EGF, TGF-α is expressed in a wide variety of different fetal and adult tissues as well as in many tumours.

The discovery of TGF-α demonstrates three things. Firstly, EGF is not a unique entity, but rather a member of a large family of structurally and functionally closely related molecules. Secondly, EGF-like molecules are present in far more organs than the submaxillary glands only. Thirdly, the discovery of membrane-bound bioactive precursor molecules demonstrates a mechanism by which the action of growth factors could be highly restricted to autocrine or paracrine modes of action *in vivo* (106).

1.8 RECEPTORS AND SIGNAL TRANSDUCTION

Hormones and growth factors exert their activity on the cell by binding specific receptors with high affinity. Most of these receptors stick through the cell membrane, with an extracellular ligand binding domain, a small hydrophobic transmembrane region, and an intracellular domain with signal transduction activity. There are other localizations for receptors. For example, the oestrogen receptor is located on the nuclear membrane. However, in this thesis we will focus on the first type of transmembrane receptors, such as the Epidermal Growth Factor Receptor (EGFR) and the Cholecystokinin Receptor (CCKR).

Based on their molecular structure and their functional intracellular domains these receptors can be divided into four groups; those with tyrosine kinase activity (e.g. EGF receptors), those with serine-threonine kinase activity (e.g. TGF-ß receptors), those with G-protein binding capacity (e.g. CCK receptors, bombesin receptors), and the cytokine superfamily of receptors (e.g. GM-CSF receptors).

It has been found that cultured human pancreatic carcinoma cells overexpress the epidermal growth factor receptor (EGFR; 111,118). Although several observations suggest that overexpression of the EGFR is an important oncogenic stimulus *in vivo*, the mechanism(s) causing this overexpression, which results in enhanced growth of human pancreatic cancer cells, is not understood. Moreover, it still has to be elucidated how increased EGFR signalling causes oncogenic transformation.

The transfer of a phosphate group onto a protein (phosphorylation) is mediated by enzymes called kinases. Phosphorylation and dephosphorylation of proteins are thought to be the key-processes occurring in cells to activate or deactivate proteins respectively, in order to transduce signals from extracellular receptors towards the genes in the nucleus. The intracellular part of the EGFR contains a tyrosine kinase domain. Ligand binding to the extracellular domain of the receptor will result in dimerization of the receptor resulting in autophosphorylation. Phosphorylation of the intracellular receptor domain makes interaction with small cytoplasmic peptides possible, resulting in an cytoplasmic cascade of phosphorylation events on (amongst others) the following subsequent peptides: Grb2-Sos-ras-raf-MAPKK-MAPK. The latter peptide migrates into the nucleus where it phosphorylates transcription factors such as fos and jun. These transcription factors regulate DNA transcription leading to differentiation and proliferation.

Protein phosphorylation is a common event in eukaryotic intracellular signalling. Protein phosphorylation results in changing the activity of many proteins (119-122). Most proteins are phosphorylated on serine (about 90%) or threonine residues (about 9%). Only less than 0.1% of phosphate linked to protein is in the form of phosphotyrosine (123). The discrepancy between the abundance of protein tyrosine phosphorylation enzymes (more than 30 are known), and the level of phosphotyrosine points to strict negative control on normal cellular protein tyrosine phosphorylation. The ascribed functions of protein tyrosine kinases suggest that tyrosine phosphorylation is largely reserved for signal transduction and regulatory systems involved in cell growth, cell-cell interaction, and differentiation. Therefore, abrogation of the negative control of these processes may readily lead to cellular transformation caused by an oncogenic protein tyrosine kinase (124).

c-Src is the cellular homologue of the Rous sarcoma virus transforming protein v-src. It was the first human protein known to have protein tyrosine kinase (PTK) activity. Increase in c-src PTK-activity has been reported in a number of tumours, including tumours of the breast (125,126), the bladder (127), the head and neck, the colon (124,128) and neuroblastomas (129,130).

In several recent studies, Oude-Weernink *et al.* (131,132) demonstrated stimulation of the proto-oncogene c-src tyrosine kinase activity and overexpression on protein level after stimulation of various cancer cell lines with EGF or PDGF. From these studies and from those of other investigators, it was concluded that c-src might be involved in EGFR signalling.

In human pancreatic carcinogenesis little is known about the involvement of

cytoplasmic proto-oncogenes. Actually, point mutations in codon 12 of the Kirsten-ras (K-ras) proto-oncogene, which occurs in up to 80% of all human tumours, is the only known cytoplasmic proto-oncogene that is involved in pancreatic carcinogenesis (133-135). No studies have yet addressed the involvement of other cytoplasmic and nuclear oncogenes in human pancreatic cancer. It still has to be elucidated whether enhancement of overall PTK-activity in general, or c-src PTK-activity in particular, is involved in (the modulation of) pancreatic carcinogenesis.

1.9 AIM OF THE THESIS

The **general aim of the investigations described in this thesis** is to obtain more insight into the role of growth factors and hormones that are involved in pancreatic carcinogenesis, which includes the development of putative preneoplastic pancreatic cell lesions and ultimately, pancreatic adenocarcinomas. Further goals are to study the possibilities to modulate some of these factors thought to be involved in pancreatic cell proliferation, and to get more insight into the differences in the histogenesis of ductular and acinar pancreatic adenocarcinomas.

Using azaserine-treated rats (model for acinar adenocarcinomas) and *N*-nitrosobis(2-oxopropyl)amine (BOP)-treated hamsters (model for ductular adenocarcinomas), the following **questions** are addressed in this thesis in order to meet these aims:

1) **Does modulation of testosterone metabolism influence experimental pancreatic carcinogenesis?**

Several studies provided conflicting evidence on the role of testosterone in pancreatic carcinogenesis. The hypothesis has been postulated that the conversion of testosterone into oestrogen by the enzyme aromatase was a confounding factor in these studies. Therefore, in chapter two of this thesis, a twelve-months study is presented in which the effects of the aromatase inhibitor aminoglutethimide on exocrine pancreatic carcinogenesis induced in rats and hamsters, was investigated.

2) **Is it possible to enhance the inhibitory effects of somatostatin on experimental pancreatic carcinogenesis by surgical castration?**

Not only hormones involved in sex-differences were found to modulate pancreatic carcinogenesis, but also other hormones such as the gut-hormone cholecystokinin and bombesin, the amphibian analogue of the human gastrin-releasing peptide. In a previous

short-term (four months) study using the somatostatin analogue Sandostatin, growth of putative preneoplastic lesions was inhibited in hamsters, but not in rats. It was hypothesized, that more pronounced Sandostatin effects might be found in more advanced pancreatic lesions such as adenocarcinomas. This hypothesis was tested in a long-term (twelve months) study, using Sandostatin alone, and in combination with surgical castration, in azaserine-treated rats and BOP-treated hamsters (chapter three).

3) Are growth factors and/or their receptors involved in experimental pancreatic carcinogenesis?

In order to investigate whether growth factors and/or their receptors are differentially expressed either in normal, preneoplastic or neoplastic stages of pancreatic carcinogenesis, epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and epidermal growth factor receptor (EGFR) were studied in pancreatic tissues obtained from short- and long-term studies with azaserine-treated rats and BOP-treated hamsters. mRNA and protein expression and localization were determined using Northern blotting, semi-quantitative PCR, immunohistochemistry and *in-situ* hybridization (chapters four, five and six).

4) Are growth factors and/or their receptors involved in the modulation of experimental pancreatic carcinogenesis by hormones or dietary factors?

Enhancing or inhibitory effects on pancreatic carcinogenesis might be the result of multiple mechanisms involving growth factors. In this thesis, studies aimed to elucidate the role of EGF, TGF-α and EGFR in the modulating effects of dietary fat, caloric restriction and cholecystokinin on experimental pancreatic carcinogenesis are presented. For this purpose, the expression of these growth factors and their receptors was studied in azaserine-treated rats and BOP-treated hamsters, maintained on a standard low-fat diet, a high-fat diet, a caloric restricted diet, or injected with the cholecystokinin-analogue caerulein.

5) Is the proto-oncogene c-src involved in experimental pancreatic carcinogenesis?

The EGFR is known to be overexpressed in human pancreas adenocarcinomas. Stimulation of the EGFR results in a cascade of intracellular processes, ultimately resulting in modulation of several cellular processes, such as: motility, adhesion, differentiation and proliferation. In recent *in-vitro* experiments, the involvement of the proto-oncogene c-src in these EGFR-induced processes has been suggested. Therefore, a study has been performed to investigate whether c-src is also involved in pancreatic

carcinogenesis (chapter seven). In pancreata collected from azaserine-treated rats c-src expression and protein tyrosine kinase activity were investigated using immunohistochemistry, an immune-complex tyrosine kinase assay and a tyrosine kinase ELISA.

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CHAPTER TWO

EFFECTS OF AMINOGLUTETHIMIDE, ALONE AND IN COMBINATION WITH SURGICAL CASTRATION, ON PANCREATIC CARCINOGENESIS IN RATS AND HAMSTERS

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2.1 ABSTRACT

The present 12-month study was carried out to investigate the effects of the aromatase inhibitor aminoglutethimide, alone and in combination with orchiectomy, on pancreatic carcinogenesis in azaserine-treated rats and N-nitrosobis(2-oxopropyl)amine-treated hamsters. Treatment of the animals started four months after the last injection with the carcinogen. They were surgically castrated and/or treated with aminoglutethimide. Aminoglutethimide-treated rats developed less pancreatic tumours in comparison with untreated controls. Multiplicity of (pre)neoplastic acinar lesions was lower in orchiectomized rats in comparison with intact rats. Inhibition of pancreatic carcinogenesis was most pronounced in rats both orchiectomized and treated with aminoglutethimide. These effects were statistically significant after eight, but not after four months of treatment.

In hamsters, aminoglutethimide demonstrated an enhancing rather than an inhibitory effect on the formation of ductular pancreatic tumours. Castration did not show any effect on the development of N-nitrosobis(2-oxopropyl)amine-induced ductular lesions in the pancreas, neither alone, nor in combination with aminoglutethimide.

The present findings demonstrate that aminoglutethimide, alone and in combination with surgical castration, might be of therapeutic value for treatment of pancreatic acinar tumours, whereas the usefulness of aminoglutethimide for treatment of ductular adenocarcinomas of the pancreas is rather doubtful.

2.2 INTRODUCTION

In most countries, the age-adjusted incidence of pancreatic cancer is higher in men than in women (1). Apart from cigarette smoking, hormonal factors have been implicated as an important factor responsible for this difference. Intracellular receptors for oestrogen and androgen have been demonstrated in pancreatic cells (2,3). Moreover, in comparison with patients showing gastrointestinal tumours other than those of the pancreas and with patients without malignancies, pancreatic cancer patients exhibited significantly lower testosterone serum levels, which have been ascribed to interaction of testosterone with specific receptors on pancreatic tumour cells (4). Furthermore, significantly higher activities of aromatase and 5α-reductase have been found in pancreatic tumours in comparison with normal pancreas (5).

In azaserine-treated rats, atypical acinar cell lesions developed faster in male than in female animals (6-8). Furthermore, castration, ovariectomy and oestradiol- and testosterone-treatment have been shown to modulate number and growth of carcinogen-induced putative preneoplastic foci in the pancreas of rats treated with azaserine (6,7,9,10).

In BOP-treated hamsters, it was demonstrated that females developed more pancreatic tumours than male animals did (11). Furthermore, N-nitrosobis(2-oxopropyl)amine (BOP)-induced pancreatic adenocarcinoma cells (H2T) transplanted in hamster cheek pouches developed faster in females and castrated males than in ovariectomized females and intact males, respectively. In *N*-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (HPOP)-treated hamsters, incidence, tumour multiplicity, and size were generally greater in female as compared to male hamsters (12). In contrast, other investigators who used BOP-treated hamsters found that surgical castration or LH-RH-treatment (chemical castration) inhibited growth but not incidence of ductular adenocarcinomas (9,13).

Although the aforementioned observations are rather inconsistent, it has been suggested that agents known to interfere with steroid metabolism may be of therapeutic value for the treatment of pancreatic cancer. Since testosterone may act directly via the androgen receptor or indirectly via the oestrogen receptor after being metabolized to oestrogen by the enzyme aromatase, the mechanism by which orchiectomy may inhibit pancreatic carcinogenesis is unknown.

Apart from an effect on metabolism of cholesterol to pregnenolone (14,15), aminoglutethimide (AGT) is a well known aromatase inhibitor. Aromatase inhibitors have been found to induce regression of hormone-dependent breast tumours in rats (16) and humans (17). An inhibitory effect of AGT on human pancreatic adenocarcinomas has not been demonstrated (18). In a previously performed short-term study with azaserine-treated rats and BOP-treated hamsters, AGT, neither alone nor in combination with orchiectomy, inhibited the development of putative preneoplastic acinar or ductular pancreatic lesions, respectively (9).

In the present study, the long-term effects of AGT-treatment were investigated, either alone or in combination with surgical castration, on the development of pancreatic tumours induced in rats and hamsters by azaserine and BOP, respectively.

2.3 MATERIALS AND METHODS

Two hundred and twenty male weanling SPF albino Wistar Bor rats (WISW; Cpb) were obtained at 18 days of age from F. Winkelmann, FRG. All rats were injected intraperitoneally four times with 30 mg azaserine/kg body weight at 19, 26, 33 and 103 days of age. One hundred and sixty male Syrian golden hamsters, 4-5 weeks of age, obtained from Charles River Wiga (Sulzfeld; FRG) were injected s.c. once weekly with 20 mg BOP/kg body weight at 6, 7 and 8 weeks of age according to an injection protocol described previously (19). BOP (Ash Stevens, Inc., 5861 John C. Lodge Freeway, Detroit, MI 48202) and azaserine (Calbiochem-Behring Corp., LaJolla, CA) were dissolved freshly in 0.9% NaCl solution. Rats were kept in stainless steel cages, fitted with wire-mesh floors and fronts, the hamsters in macrolon cages on softwood bedding. All animals were housed under standard laboratory conditions, five per cage, and fed the Institute's stock diet. The Institute's basal diet is low in fat (5%) and compounded from natural feed ingredients. The percentage composition of the diet has been described previously (20). The animals were allocated to five different groups by a computerized randomization procedure. Four groups (A up to D) consisted of 50 or 35 animals (rats and hamsters respectively), whereas the fifth group (E), which was killed four months after the injection of carcinogen and prior to the start of treatment, consisted of 20 animals.

The animals were treated as follows: (A) saline (controls); (B) AGT (7x/wk, oral, 2 mg in 0.5 ml/animal); (C) orchiectomy; and (D) AGT + orchiectomy. In order to mimic the human situation, treatment started when pancreatic lesions were already present, i.e. four months after the last injection with the carcinogen.

Body weights were recorded weekly during the first three months, and once a month during the rest of the experiment. The general condition and behaviour of the animals were checked daily.

Autopsy of 20 rats and 20 hamsters (animals of group E) was performed four months after the last injection with carcinogen in order to establish the severity of the pancreatic lesions at the start of treatment. Interim autopsy of 20 rats of each group (A up to D) was performed four months after the start of the treatment-period. Terminal autopsy was performed on both rats and hamsters after eight months of treatment (= twelve months after the last carcinogen injection). The animals were anaesthetized by ether, exsanguinated by cannulating the abdominal aorta, autopsied and then examined for gross pathological changes. From each animal the pancreas, testes (if present), liver, adrenals and pituitary were excised, weighed and fixed in 10% buffered formalin. The

pancreata were completely processed for microscopy by conventional methods, step sectioned at 5 μm (about 5 per pancreas), stained with haematoxylin and eosin (H&E) and examined by light microscopy.

In rat pancreas azaserine-induced atypical acinar cell nodules (AACN), acinar cell adenomas, localized carcinomas (carcinoma *in-situ*; CIS) and invasive carcinomas were identified and classified according to the criteria of Longnecker (20). The area of the nodule transections was determined using a grid inside the ocular as described previously (21). In the hamster pancreas, major attention was paid to tubular ductal complexes showing dysplasia or anaplastic changes, desmoplasia and/or inflammation, with or without apoptosis, suggestive for progression to malignancy. Tubular ductal complexes exhibiting one or more of these characteristics were classified as 'borderline lesions' (22). The carcinomas were identified and classified according to Pour *et al.* (23). Carcinomas showing no invasion in the surrounding tissues were classified as carcinomas *in-situ*.

Body and organ weight data were statistically evaluated by two-way analysis of variance with initial body weight as covariable. The incidence of lesions was statistically evaluated by the Pearson chi-square test, whereas the number of lesions was statistically evaluated by a log linear model with a Poisson distribution. The relevant sources of variation were: treatment with AGT, surgical castration, combination of surgical castration and treatment with AGT, and their interaction.

2.4 RESULTS

Body weights

Growth was significantly decreased in orchiectomized animals (**Figures 2.1 and 2.3**). The effect on growth was less pronounced in hamsters ($P<0.05$) than in rats ($P<0.01$). In orchiectomized rats the reduced body weight gain was accompanied by a lower food intake (**Figure 2.2**). Orchiectomized hamsters treated with AGT showed a slight but not significant decrease in body weight gain in comparison with intact hamsters (**Table 2.1**). This effect may, at least partly, be ascribed to the surgical castration performed on day 120, which resulted in a decrease in body weight with the weight of the testes.

Figure 2.1

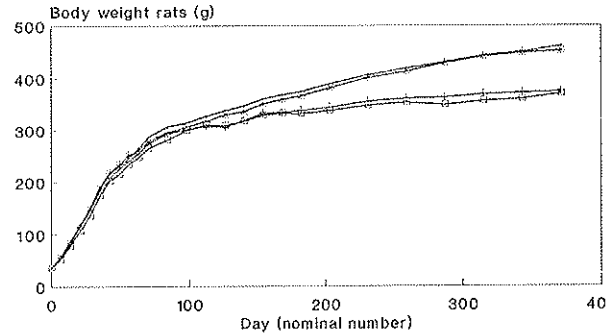
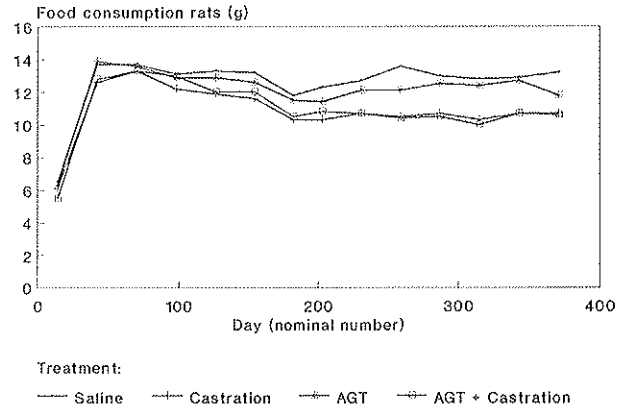


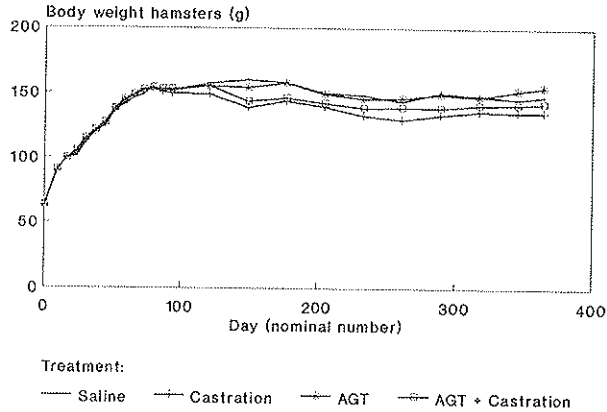
Figure 2.2



Organ weights

In castrated rats, absolute but not relative pancreas weight had significantly ($P<0.01$) decreased, independent of treatment with AGT (**Tables 2.1 and 2.2**). In castrated hamsters, on the contrary, relative but not absolute pancreas weight had significantly ($P<0.01$) increased independent of treatment with AGT. Relative and absolute pituitary weight had significantly increased in orchiectomized animals. Relative liver weight had significantly increased ($P<0.01$) in orchiectomized hamsters not treated with AGT, whereas absolute and relative liver weight had significantly ($P<0.01$) decreased in castrated rats independent of treatment with AGT. Neither adrenal nor testes weights were influenced by the treatments.

Figure 2.3



Microscopy

Rats killed four months after the last injection with azaserine (just before start of treatment) exhibited a large number of AACN in the pancreas with a transection area less than 0.5 mm^2, but no lesions were present in the larger size-categories (not shown). During treatment, most rats developed AACN with a transection area over 0.5 mm^2. Moreover, some rats developed pancreatic adenomas and carcinomas (**Table 2.3**). Orchiectomy caused a significant decrease in the number of both AACN and acinar adenocarcinomas after eight months (**Table 2.3**). AGT-treatment for eight months caused a significant decrease in the number of nodules with a transection area between 0.5 and 1.0 mm^2 ($P<0.01$), as well as in the number of nodules with a transection area larger than 1.0 mm^2, the number of carcinomas *in-situ* and in the total number of carcinomas ($P<0.05$; **Table 2.3**). The lowest numbers of pancreatic acinar lesions were found in orchiectomized rats treated with AGT for 8 months (**Table 2.3**). When the AGT-treated or orchiectomized groups were combined, the number of carcinoma-bearing rats was significantly lower ($P<0.05$) after eight months of treatment, in comparison with the groups not treated with AGT, or not orchiectomized, respectively (**Table 2.4**). Control hamsters autopsied after the eight months treatment-period showed a significant increase in multiplicity of borderline lesions ($P<0.001$) and adenocarcinomas ($P<0.01$) in comparison with those observed in hamsters autopsied four months after the last injection with BOP (t=0, just before treatment; **Table 2.5**). Moreover, the number of tumour-bearing animals had significantly ($P<0.05$) increased in controls killed after eight months treatment in comparison with those killed just before treatment (t=0; **Table 2.6**).

Table 2.1 Effects of the respective treatments on body weight and the absolute weight of different organs[a]

Treatment	N	Body weight at autopsy (g)	Absolute organ weights (g)				
			Pancreas	Testes	Pituitary	Adrenals	Liver
	Rat						
Control	27	460.7 ± 10.9	1.40 ± 0.05	3.55 ± 0.06	0.014 ± 0.000	0.054 ± 0.002	13.01 ± 0.37
AGT	30	452.0 ± 7.8	1.29 ± 0.04	3.60 ± 0.05	0.014 ± 0.000	0.055 ± 0.002	12.52 ± 0.28
Castration	24	373.7 ± 6.9**	1.14 ± 0.04**	-	0.018 ± 0.001**	0.047 ± 0.002	9.33 ± 0.25**
AGT + castration	24	369.8 ± 6.4**	1.12 ± 0.04**	-	0.016 ± 0.001*	0.048 ± 0.001	9.15 ± 0.24**
	Hamster						
Control	27	148.0 ± 3.6	0.517 ± 0.030	2.67 ± 0.14	0.007 ± 0.000	0.024 ± 0.001	8.19 ± 0.56
AGT	25	155.0 ± 3.2	0.554 ± 0.025	2.85 ± 0.08	0.007 ± 0.000	0.023 ± 0.001	8.68 ± 0.42
Castration	16	135.6 ± 3.9*	0.603 ± 0.033	-	0.009 ± 0.001**	0.019 ± 0.001	10.79 ± 1.33
AGT + castration	21	142.6 ± 3.4	0.626 ± 0.044	-	0.009 ± 0.000**	0.022 ± 0.002	9.01 ± 0.57

[a]Values are means ± SEM.

Statistics: Two-way analysis of variance (two-tailed) compared to controls: *P<0.05; **P<0.01.

Table 2.2 Effects of the respective treatments on relative weight of different organs[a]

Treatment	N	Relative organ weights (g/kg)				
		Pancreas	Testes	Pituitary	Adrenals	Liver
	Rat					
Control	27	3.06 ± 0.12	7.77 ± 0.15	0.031 ± 0.001	0.118 ± 0.005	28.3 ± 0.7
AGT	30	2.85 ± 0.07	8.01 ± 0.16	0.030 ± 0.001	0.124 ± 0.005	27.7 ± 0.5
Castration	24	3.07 ± 0.11	-	0.048 ± 0.001**	0.128 ± 0.007	25.1 ± 0.7**
AGT + castration	24	3.06 ± 0.11	-	0.043 ± 0.002**	0.129 ± 0.005	24.9 ± 0.7**
	Hamster					
Control	27	3.43 ± 0.20	18.06 ± 0.84	0.04 ± 0.00	0.16 ± 0.00	55.0 ± 3.3
AGT	25	3.55 ± 0.12	18.50 ± 0.55	0.04 ± 0.00	0.15 ± 0.00	56.0 ± 2.5
Castration	16	4.49 ± 0.25**	-	0.06 ± 0.01**	0.14 ± 0.01	78.0 ± 8.3**
AGT + castration	21	4.43 ± 0.26**	-	0.07 ± 0.00**	0.16 ± 0.02	63.4 ± 3.8

[a]Values are means ± SEM.

Statistics: Two-way analysis of variance (two-tailed) compared to controls: **P<0.01.

Table 2.3 Effects of the respective treatments on number of (pre)neoplastic lesions in the exocrine pancreas of azaserine-treated rats[a]

(Pre)neoplastic lesions observed	Number of lesions after a treatment period of:							
	Four months				Eight months			
	Control	AGT	Castr.	AGT + castr.	Control	AGT	Castr.	AGT + castr.
Effective number of rats	14	13	13	14	29	30	26	27
Nodules ($0.5 < TA^b < 1.0$ mm^2)	4	5	1	5	89	54**	27**	17**
Nodules (TA > 1.0 mm^2)	1	0	0	2	39	16*	6**	7**
Adenoma	1	0	0	1	3	7	4	1
Carcinoma *in-situ*	0	2	0	1	17	4*	5**	0**
Microcarcinoma	0	0	0	0	5	4	1*	1*
Adenocarcinoma	0	0	0	0	4	4	3	1
Total carcinoma	0	2	0	1	26	12*	9***	2***

[a]Values are totals per group; [b]TA: Transection area.

Statistics: log linear model with a Poisson distribution: *P<0.05; **P<0.01; ***P<0.001.

Table 2.4 Effects of the respective treatments on incidence of (pre)neoplastic lesions in the exocrine pancreas of azaserine-treated rats[a]

(Pre)neoplastic lesions observed	Incidence of lesions after a treatment period of:							
	Four months				Eight months			
	Control	AGT	Castr.	AGT + castr.	Control	AGT[c]	Castr.[d]	AGT + castr.[c,d]
Effective number of rats	14	13	13	14	29	30	26	27
Number of carcinoma-bearing rats	0	2	0	1	11	8	6	2
Nodules ($0.5 < TA^b < 1.0$ mm^2)	3	4	1	4	6	4	5	6
Nodules (TA > 1.0 mm^2)	0	0	0	0	4	4	2	5
Adenoma	1	0	0	1	1	4	2	1
Carcinoma *in-situ*	0	2	0	1	4	2	2	0
Microcarcinoma	0	0	0	0	4	2	1	1
Adenocarcinoma	0	0	0	0	3	4	3	1

[a]Incidences are based on scoring each animal once in the category of the most advanced lesion; [b]TA: Transection area.
Statistics: Pearson chi-square test.
[c]In the AGT-treated groups the number of carcinoma-bearing animals had significantly decreased after eight months ($P<0.05$).
[d]In the castrated groups the number of carcinoma-bearing animals had significantly decreased after eight months ($P<0.05$).

Table 2.5 Effects of the respective treatments on number of (pre)neoplastic lesions in the exocrine pancreas of BOP-treated hamsters after 8 months[a]

(Pre)neoplastic lesions observed	Number of lesions				
	t=0	Control	AGT	Castr.	AGT + castr.
Effective number of hamsters	17	27	30	23	28
Borderline lesions	9	81	65	66	66
Carcinoma *in-situ*	2	1	6[b]	1	3[b]
Adenocarcinoma	2	13	18	8	10
Total carcinoma	4	14	24	9	13

[a]Values are totals per group; [b]Number of carcinomas *in-situ* increased significantly in animals treated with AGT compared to animals not treated with AGT ($P<0.05$).
Statistics: Log linear model with a Poisson distribution.

Table 2.6 Effects of the respective treatments on incidence of (pre)neoplastic lesions in the exocrine pancreas of BOP-treated hamsters after 8 months[a]

(Pre)neoplastic lesions observed	Incidence of lesions				
	t=0	Control	AGT	Castr.	AGT + castr.
Effective number of hamsters	17	27	30	23	28
Number of tumour-bearing hamsters	3	12	17	8	12
Borderline lesions	4	13	11	14	13
Carcinoma *in-situ*	1	1	2	1	3
Adenocarcinoma	2	11	15	7	9

[a]Incidences are based on scoring each animal once in the category of the most advanced lesion.
Statistics: Pearson chi-square test; no statistically significant differences were observed.

In intact and orchiectomized hamsters, eight months AGT treatment caused a significant ($P<0.05$) increase in the number of carcinomas *in-situ* as compared to controls (**Table 2.5**). This increase was accompanied by a slight, but not significant, decrease in the number of borderline lesions. Orchiectomy did not cause any effect on the development of ductular tumours induced in the pancreas of hamsters by BOP.

2.5 DISCUSSION

The results of the present study indicate that AGT-treatment has an inhibitory effect on pancreatic carcinogenesis in azaserine-treated rats, provided the duration of treatment is longer than four months. The number of acinar tumours was significantly lower in orchiectomized rats in comparison with intact rats. Moreover, AGT treatment enhanced the inhibitory effect of orchiectomy on the development of pancreatic tumours in rats, but an interaction between these two treatments was not found, statistically.
Interestingly, in the BOP-hamster model, AGT-treatment caused an increase in multiplicity of carcinomas *in-situ*, pointing to an enhancing rather than an inhibitory effect on the formation of ductular pancreatic tumours, whereas castration did not have significant effects in this animal model.

The present findings with AGT on pancreatic carcinogenesis in rats are in accordance with those seen on oestrogen-sensitive breast tumours in rats and humans (15,16). The present finding that AGT-treatment for eight but not for four months is effective is in accordance with our previous observation that AGT has no effect on growth of early putative preneoplastic AACN when administered for 4 months (9). It has been demonstrated that oestrogen inhibits and testosterone promotes the development of putative preneoplastic acinar lesions in rat pancreas (6-8,10). The mechanism of action still remains obscure; testosterone may have a direct stimulatory effect via androgen receptors, or an indirect inhibitory effect via the oestrogen receptor after being metabolized by the enzyme aromatase. The presently observed inhibitory effect of AGT on pancreatic carcinogenesis in rats after eight months of treatment cannot easily be explained. The inhibition of the enzyme aromatase by AGT will result in a diminished metabolization of testosterone into oestrogen. It seems justifiable to assume that inhibition of this conversion may lead to increased testosterone levels. However, this effect of AGT did not seem to play an important role in the present study, since an elevated testosterone concentration would have led to an enhancing rather than an inhibitory effect on pancreatic carcinogenesis. It appeared, indeed, that the blood

testosterone levels were not elevated in AGT-treated rats (3.5 ± 1.2 nmol testosterone/L) when compared to blood testosterone levels in control rats (4.6 ± 0.6 nmol testosterone/L). The presence of normal testosterone levels in AGT-treated animals most probably can be ascribed to a feed-back mechanism through the pituitary gland.

The presently observed inhibitory effect of AGT on the development of advanced pancreatic lesions is most probably related to an inhibitory action on the conversion of cholesterol to pregnenolone, leading to a depletion of corticosteroids (13,14), rather than to an inhibitory effect on aromatase activity. The results of a previously performed 4-month study (9) with orchiectomized rats, however, indicated that the inhibitory effect on growth of putative preneoplastic acinar lesions may be due to a significant decrease in body weight gain of the orchiectomized rats rather than to a decrease in plasma testosterone concentration. The present findings with the orchiectomized rats support the hypothesis that testosterone does not seem to play an important role in the development of pancreatic cancer.

In the BOP-hamster model, AGT did not influence the development of ductular pancreatic tumours except for an enhancing effect on the development of carcinomas *in-situ*, accompanied by a (non-significant) decrease in number of borderline-lesions, pointing to a faster development from borderline-lesions into carcinomas in AGT-treated animals. This enhancing effect on pancreatic carcinogenesis in hamsters can hardly be ascribed to an inhibitory effect of AGT on the metabolism of testosterone to oestrogen by aromatase, since it has been found by other investigators that female hamsters treated with BOP developed more pancreatic tumours than male hamsters do (12) and that transplanted pancreatic ductal adenocarcinoma cells developed faster in female than in male hamsters. These observations point to an enhancing rather than an inhibitory effect of oestrogen on BOP-induced pancreatic carcinogenesis. Our observation that orchiectomy did not influence the development of ductular tumours in the pancreas of BOP-treated hamsters, is in agreement with the findings in a previously performed 4-month study (9) suggesting that testosterone is not involved in pancreatic carcinogenesis in hamsters. Castrated hamsters, similar to rats, exhibited lower body weights. In contrast to rats, however, in hamsters relative pancreas weight of orchiectomized hamsters was higher than that of intact controls, indicating that growth is not a complicating factor in the interpretation of the effects of castration on pancreatic carcinogenesis in hamsters.

Based on the observation that AGT inhibits pancreatic carcinogenesis in the azaserine-rat model (leading to acinar adenocarcinomas) but not in the BOP-hamster model (leading to ductular adenocarcinomas), it seems justifiable to conclude that AGT

may have a therapeutic potential as far as acinar pancreatic adenocarcinomas are concerned, whereas the therapeutic potential of AGT for treatment of ductular adenocarcinomas, which are most commonly found in humans, is rather doubtful.

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CHAPTER THREE

EFFECTS OF SANDOSTATIN, ALONE AND IN COMBINATION WITH SURGICAL CASTRATION, ON PANCREATIC CARCINOGENESIS IN RATS AND HAMSTERS

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3.1 ABSTRACT

Sandostatin, when administered prophylactically, inhibited growth of putative preneoplastic ductular lesions induced in hamster pancreas by N-nitrosobis(2-oxopropyl)amine, but not of acinar lesions induced in rat pancreas by azaserine. The present 12-month study was carried out to investigate the effects of Sandostatin (3μg/day), alone and in combination with orchiectomy, on pancreatic carcinogenesis in azaserine-treated rats and N-nitrosobis(2-oxopropyl)amine-treated hamsters. Treatment of the animals started four months after the last injection with the carcinogen. After treatment with Sandostatin for eight months rats developed fewer pancreatic atypical acinar cell nodules and tumours. Multiplicity of (pre)neoplastic acinar lesions was also lower in orchiectomized rats in comparison with intact rats. Sandostatin-treatment did not enhance the inhibitory effect of surgical castration on pancreatic carcinogenesis in rats.

In hamsters that were both orchiectomized and treated with Sandostatin, the development of borderline lesions was significantly inhibited, whereas such an effect was not present in hamsters that were either surgically castrated or treated with Sandostatin alone. In Sandostatin-treated hamsters a significantly lower number of microcarcinomas was found in comparison with hamsters not treated with Sandostatin.

The present findings demonstrate that Sandostatin, particularly in combination with surgical castration, might be of therapeutic value for treatment of human (mostly ductular) pancreatic tumours.

3.2 INTRODUCTION

Pancreatic cancer causes more deaths per year than most other malignant neoplasms, except those of the colorectal region, the lungs and breast. Early diagnosis of pancreatic cancer is difficult due to the lack of symptoms (1). The prognosis of patients with pancreatic cancer is poor. Usually no appropriate therapy is possible at the time of diagnosis (2,3). Pancreatic tumours appear to contain a large number of steroidal receptors and enzymes involved in steroid metabolism indicating possible hormone dependency. Hormonal therapy of pancreatic cancer might be promising since it has no, or only minor side effects. Recent papers demonstrated that pancreatic carcinogenesis can be modulated by oestrogen and testosterone in both azaserine-treated rats (4-9) and

BOP-treated hamsters (9-16). Furthermore, intracellular steroid receptors have been determined in pancreatic cells (17-20).

Apart from sex hormones, somatostatin is considered to be a growth modulating factor for the pancreas, based on the finding that caerulein-activated DNA-synthesis in rat pancreas could be inhibited by somatostatin and stimulated by somatostatin antiserum (21). In the same period, Redding and Schally (10) reported a reduced weight and volume of transplanted ductal pancreatic tumours (in hamsters) and acinar pancreatic tumours (in rats) after treatment with [L-5-Br-Trp^8] somatostatin-14, but not after treatment with cyclo(Pro-Phe-D-Trp-Lys-Thr-Phe) or somatostatin-28. Somatostatin may inhibit growth of human pancreatic cancers by suppressing the secretion and/or action of gastrointestinal hormones such as cholecystokinin, secretin and gastrin (22), or by dephosphorylation of an EGF-sensitive receptor (23). More recently, however, Reubi *et al.* (24) could not detect specific somatostatin-receptors in human exocrine pancreatic carcinomas, but did find somatostatin-receptors in rat transplantable acinar tumours (CA 20948) and in human islet cell carcinomas (insulinomas). These findings support data with the long acting somatostatin-analogue Sandostatin (SMS-201-995), which appeared to be effective in endocrine tumours (25-27), but not in exocrine tumours. Upp *et al.* (28) found inhibition of growth and volume of two human pancreatic ductal adenocarcinomas (SKI and CAV) transplanted in nude mice by treatment with Sandostatin. In a follow-up study, Poston *et al.* (29), however, demonstrated that human ductal pancreatic adenocarcinomas, transplanted in nude mice, do not all respond to Sandostatin. Most patients with pancreatic cancer, however, experienced subjective improvement in the absence of serious side effects after Sandostatin-therapy (30).

Zalatnai and Schally (12,13) reported inhibition of growth of experimentally induced ductal adenocarcinomas in exocrine pancreas of male and female Syrian golden hamsters after treatment with the somatostatin-analogue RC-160. In these animals survival was prolonged, body weight had increased and histopathological changes indicative for regression of the tumours were observed. Furthermore, growth of a poorly differentiated acinar pancreas tumour (CA 20948) transplanted in rats could be inhibited by the somatostatin-analogues Sandostatin and CGP15-425 (31). This inhibitory effect was more pronounced when treatment was combined with the LH-RH analogues Buserelin or Zoladex (chemical castration).

In a previous short-term study (four months), we found that Sandostatin, when administered prophylactically, inhibited growth of putative preneoplastic ductular lesions induced in hamster pancreas by BOP, but not of acinar lesions induced in rat pancreas by azaserine (32). Moreover, it has been demonstrated that pancreatic carcinogenesis can

be modulated by sex hormones in both rats (4-9) and hamsters (9-16). Therefore, the present long-term study (12 months) with rats and hamsters was carried out in order to investigate the effects of Sandostatin, alone and in combination with surgical castration, on the development of acinar and ductular pancreatic tumours.

3.3 MATERIALS AND METHODS

Two hundred and twenty male weanling (18 days of age) SPF albino Wistar rats (WISW; Cpb), were obtained from F. Winkelmann, FRG. All rats were injected intraperitoneally four times with 30 mg azaserine/kg body weight at 19, 26, 33 and 103 days of age. One hundred and sixty male Syrian golden hamsters (obtained at 4-5 weeks of age from Charles River Wiga; Sulzfeld; FRG) were injected s.c. once weekly with 20 mg BOP/kg body weight each at 5, 6 and 7 weeks of age according to an injection protocol described previously (33). BOP (Ash Stevens, Inc., Detroit, MI) and azaserine (Calbiochem-Behring Corp., LaJolla, CA) were dissolved freshly in 0.9% NaCl solution. Rats were kept in stainless steel cages, fitted with wire-mesh floors and fronts, the hamsters in macrolon cages on softwood bedding. All animals were housed under standard laboratory conditions, five per cage, and fed the Institute's stock diet. The Institute's basal diet is low in fat (5%) and compounded from natural feed ingredients. The percentage composition of this diet has been described previously (34). The animals were allocated to five different groups by a computerized randomization procedure. Four groups (A up to D) consisted of 50 or 35 animals (rats and hamsters, respectively), whereas the fifth group (group E), which was killed four months after the injection of carcinogen and prior to the start of treatment, consisted of 20. At that time, the remaining animals were treated as follows: (A) saline (controls); (B) Sandostatin (osmotic mini pumps, 3 µg/day); (C) surgical castration; and (D) Sandostatin + surgical castration.

Osmotic mini pumps (2 ml, model 2ML4; ALZA Corp., Palo Alto, CA) were filled with 100 µg Sandostatin (SMS 201-995) dissolved in saline (final concentration 0.05 µg/µl). The pumps were placed s.c. in the scapular region and exchanged once every month. The pumping rate was 2.5 µl/hour, resulting in a delivery of 3 µg Sandostatin/day. This dosage was chosen because it had highly significant antitumour effects in pancreatic acinar tumours transplanted in rats (35).

Body weights were recorded weekly during the first three months, and once a month during the rest of the experiment. Food consumption was measured on 7

consecutive days per month. In hamsters, food consumption could not be measured with high accuracy due to severe spill by the animals. The general condition and behaviour of the animals were checked daily.

Autopsy of 20 rats and 20 hamsters (animals of group E) was performed four months after the last injection with carcinogen in order to establish the severity of the pancreatic lesions at the start of treatment. Interim autopsy was performed on 20 rats of each group (A up to D) after four months of treatment. Terminal autopsy for both rats and hamsters was conducted after eight months of treatment. The animals were anaesthetized by ether, exsanguinated by cannulating the abdominal aorta, autopsied and then examined for gross pathological changes. From each animal the pancreas, testes (if present), liver, adrenals and pituitary were excised, weighed and fixed in 10% buffered formalin. The pancreata were completely processed for microscopy by conventional methods, step sectioned at 5 μm (about 5 per pancreas), stained with haematoxylin and eosin (H&E) and examined by light microscopy.

In rat pancreas azaserine-induced atypical acinar cell nodules (AACN), acinar cell adenomas, localized carcinomas (carcinoma *in-situ;* CIS) and invasive carcinomas were identified and classified according to the criteria of Longnecker (36). The area of the nodule transections was determined using a grid inside the ocular as described previously (34). In hamster pancreas, major attention was paid to tubular ductal complexes showing dysplasia or anaplastic changes, desmoplasia and/or inflammation, with or without apoptosis, suggestive for progression to malignancy. Tubular ductal complexes exhibiting one or more of these characteristics were classified as 'borderline lesions' (37). The carcinomas were identified and classified according to Pour and Wilson (38). Carcinomas showing no invasion in the surrounding tissue were classified as carcinomas *in-situ,* if local invasion was observed, the lesion was classified as (micro)carcinoma.

Body and organ weight data were statistically evaluated by two-way analysis of variance with initial body weight as covariable. The number of lesions was statistically evaluated by a log linear model with a Poisson distribution. The relevant sources of variation were: treatment with Sandostatin, surgical castration, combination of surgical castration and treatment with Sandostatin, and their interaction.

3.4 RESULTS

Body weights

In rats, body weight gain was significantly ($P<0.01$) inhibited in all treatment groups (**Figure 3.1, Table 3.1**). The decrease in body weight was accompanied by a significant reduction in food intake, particularly in orchiectomized rats, during almost the whole treatment period (**Figure 3.2**). In hamsters, body weight of the treated animals was lower than that of controls, but the difference reached the level of statistical significance ($P<0.05$) in orchiectomized hamsters only (**Figure 3.3**).

Organ weights

In rats treated with Sandostatin, both alone and in combination with orchiectomy, absolute but not relative pancreas and testes weights had significantly decreased (**Tables 3.1 and 3.2**). In orchiectomized rats, absolute and relative pituitary weights increased and absolute and relative liver weights decreased. Furthermore, absolute but not relative liver weight had decreased significantly in Sandostatin-treated rats. None of the treatments influenced adrenal weights.

Absolute and relative pancreas and pituitary weights had increased significantly in orchiectomized hamsters (**Tables 3.1 and 3.2**). Sandostatin caused a significant increase in relative weights of the pancreas and adrenals ($P<0.05$). Relative liver weight had increased significantly ($P<0.01$) in castrated hamsters not treated with Sandostatin.

Microscopy

Rats killed four months after the last injection of azaserine (just before start of treatment) exhibited a large number of AACN in the pancreas with a transection area less than 0.5 mm^2, but no lesions were present in the larger size-categories (not shown). The total number of (pre)neoplastic acinar lesions increased significantly in time in the pancreas of all rats (**Table 3.3**). Rats treated with Sandostatin for eight months showed a significantly ($P<0.001$) lower number of AACN with a transection area >1.0 mm^2 in comparison with rats not treated with Sandostatin. Moreover, the number of carcinomas *in-situ* was significantly ($P<0.05$) lower after eight months of treatment with Sandostatin (**Table 3.3**). Eight months after treatment, orchiectomized rats exhibited a significantly lower number of AACN with a transection area > 1.0 mm^2 ($P<0.001$), carcinomas *in-situ* ($P<0.05$) and microcarcinomas ($P<0.05$) in comparison with intact rats. Statistical evaluation of the number of AACN (0.5 mm^2 < transection area < 1.0 mm^2) and the total number of carcinomas revealed an antagonistic effect between surgical castration

and Sandostatin-treatment: the inhibitory effect of Sandostatin alone and that of orchiectomy alone on development of advanced pancreatic lesions was not present in the group of orchiectomized rats treated with Sandostatin. No significant effects of the respective treatments were noticed on the incidences of advanced pancreatic acinar lesions (**Table 3.4**).

Figure 3.1

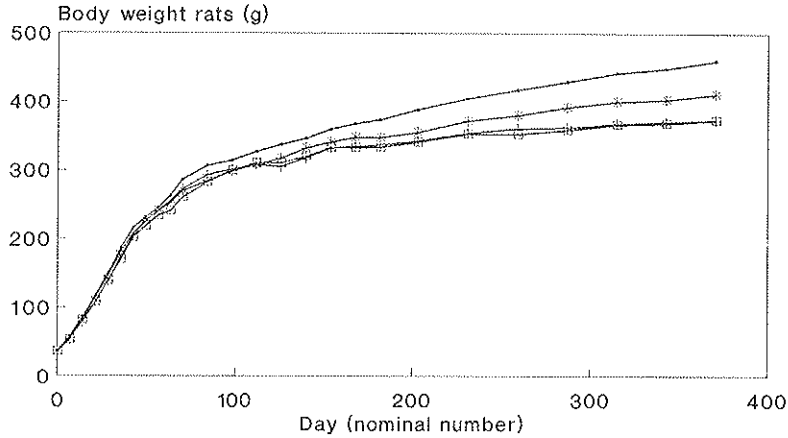


Figure 3.2

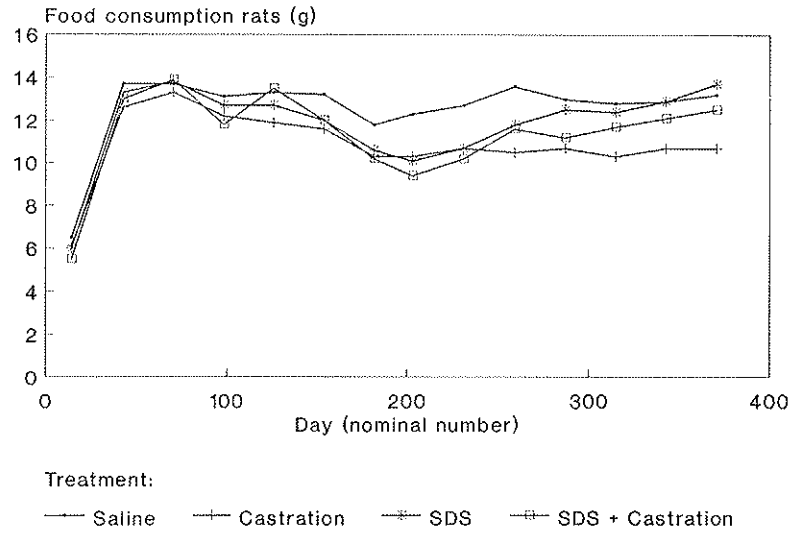
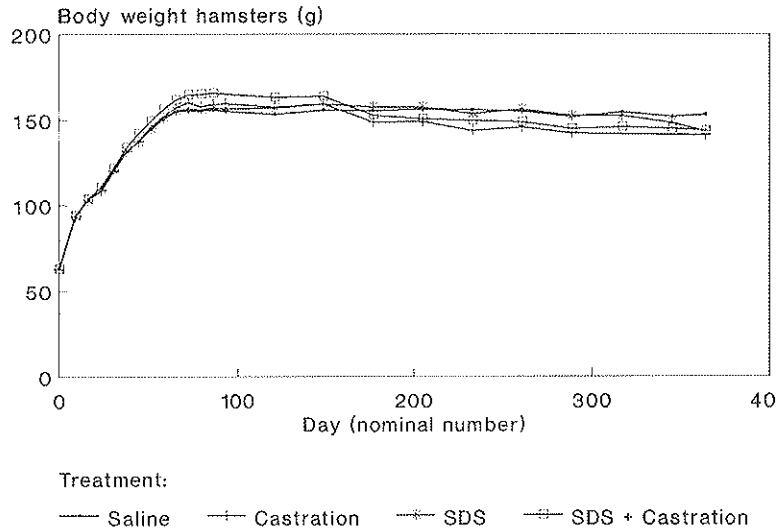


Figure 3.3



Four months after the last carcinogen injection (just before treatment, t=0) all hamsters showed small putative preneoplastic ductular lesions, 11 out of 20 demonstrated borderline lesions and one animal had developed a ductular adenocarcinoma (not shown). After eight months of Sandostatin-treatment, a significantly lower number of microcarcinomas ($P<0.05$) was found in comparison with hamsters not treated with Sandostatin (**Table 3.5**). Statistical evaluation of the number of borderline lesions revealed a significant interaction between orchiectomy and Sandostatin-treatment. Orchiectomized hamsters treated with Sandostatin exhibited a significant lower number of borderline lesions ($P<0.01$) in comparison with non-treated controls, whereas castration alone caused a significant increase ($P<0.05$) and Sandostatin-treatment alone did not cause any significant effect on development of borderline lesions, when the number of lesions was calculated per animal.

The number of tumour-bearing hamsters was not influenced by any of the respective treatments (**Table 3.6**).

Table 3.1 Effects of the respective treatments on body weight and absolute weight of different organs after 8 months[a]

Treatment	N	Body weight at autopsy (g)	Absolute organ weights (g)			
			Pancreas	Testes	Pituitary	Liver
	Rat					
Control	27	460.7 ± 10.9	1.40 ± 0.05	3.55 ± 0.06	0.014 ± 0.000	13.01 ± 0.37
SDS[b]	22	412.1 ± 8.9**	1.21 ± 0.04*	3.32 ± 0.08*	0.013 ± 0.001	11.01 ± 0.24**
Castration	24	373.7 ± 6.9**	1.14 ± 0.04**	-	0.018 ± 0.001**	9.33 ± 0.25**
SDS + castration	18	374.1 ± 9.7**	1.07 ± 0.03**	-	0.016 ± 0.001*	9.05 ± 0.21**
	Hamster					
Control	30	153.1 ± 3.6	0.511 ± 0.021	1.95 ± 0.17	0.007 ± 0.000	8.49 ± 0.46
SDS	27	143.6 ± 3.4	0.574 ± 0.021	0.87 ± 0.15	0.006 ± 0.000	8.34 ± 0.34
Castration	26	141.0 ± 3.0*	0.603 ± 0.021*	-	0.010 ± 0.000**	9.94 ± 0.67
SDS + Castration	24	143.9 ± 2.9	0.681 ± 0.035**	-	0.008 ± 0.000*	9.24 ± 0.52

[a]Values are means ± SEM; [b]SDS: Sandostatin.

Statistics: Two-way analysis of variance (two-tailed): *P<0.05; **P<0.01.

Table 3.2 Effects of the respective treatments on relative weight of different organs after 8 months[a]

Treatment	N	Relative organ weights (g/kg)			
		Pancreas	Testes	Pituitary	Liver
	Rat				
Control	27	3.06 ± 0.12	7.77 ± 0.15	0.031 ± 0.001	28.3 ± 0.7
SDS[b]	22	2.94 ± 0.08	8.15 ± 0.23	0.032 ± 0.001	26.8 ± 0.6
Castration	24	3.07 ± 0.11	-	0.048 ± 0.001**	25.1 ± 0.7**
SDS + castration	18	2.90 ± 0.14	-	0.044 ± 0.002**	24.3 ± 0.5**
	Hamster				
Control	30	3.38 ± 0.13	12.65 ± 1.07	0.05 ± 0.00	55.4 ± 2.71
SDS	27	4.04 ± 0.18*	5.90 ± 0.91	0.04 ± 0.00	58.6 ± 2.41
Castration	26	4.30 ± 0.15**	-	0.07 ± 0.00**	70.4 ± 4.39**
SDS + castration	24	4.88 ± 0.28**	-	0.06 ± 0.00**	64.3 ± 3.48

[a]Values are means ± SEM; [b]SDS: Sandostatin.

Statistics: Two-way analysis of variance (two-tailed): *P<0.05; **P<0.01.

Table 3.3 Effects of the respective treatments on number of (pre)neoplastic lesions in the exocrine pancreas of azaserine-treated rats[a]

(Pre)neoplastic lesions observed	Number of lesions after a treatment period of:							
	Four months				Eight months			
	Control	SDS[b]	Castr.	SDS + Castr.	Control	SDS	Castr.	SDS + Castr.
Number of rats	14	14	13	10	29	25	26	28
Nodules ($0.5 < TA^c < 1.0$ mm^2)	4	6	1**	2	89	18**	27**	22**
Nodules (TA > 1.0 mm^2)	1	1[d]	0[e]	0[d,e]	39	11[d]	6[e]	3[d,e]
Adenoma	1	0	0	0	3	3	4	1
Carcinoma *in-situ*	0	2	0[e]	1[e]	17	3[d]	5[e]	4[d,e]
Microcarcinoma	0	0	0[e]	0[e]	5	2	1[e]	0[e]
Adenocarcinoma	0	0	0	0	4	0	3	5
Total carcinoma	0	2	0	1	26	5*	9*	9

[a]Values are totals per group; [b]SDS: Sandostatin; Castr.: surgical castration; [c]TA: Transection area.
Statistics: log linear model with a Poisson distribution: *P<0.05; **P<0.01 compared to controls.
[d]Significant effect comparing SDS-treated animals with animals not treated with SDS.
[e]Significant effect comparing castrated animals with controls and animals treated with SDS.

Table 3.4 Effects of the respective treatments on incidence of (pre)neoplastic lesions in the exocrine pancreas of azaserine-treated rats[a]

(Pre)neoplastic lesions observed	Incidence of lesions after a treatment period of:							
	Four months				Eight months			
	Control	SDS[b]	Castr.	SDS + Castr.	Control	SDS	Castr.	SDS + Castr.
Number of rats	14	14	13	10	29	25	26	28
Number of carcinoma-bearing rats	0	2	0	1	11	4	6	8
No (pre)neoplastic lesions	10	11	12	8	7	11	11	11
Nodules (0.5 < TA[c] < 1.0 mm)	3	1	1	1	6	4	5	7
Nodules (TA > 1.0 mm)	0	0	0	0	4	3	2	1
Adenoma	1	0	0	0	1	3	2	1
Carcinoma *in-situ*	0	2	0	1	4	2	2	3
Microcarcinoma	0	0	0	0	4	2	1	0
Adenocarcinoma	0	0	0	0	3	0	3	5

[a]Incidences are based on scoring each animal once in the category of the most advanced lesion;
[b]SDS: Sandostatin; Castr.: surgical castration; [c]TA: Transection area.
Statistics: Pearson chi-square test.

Table 3.5 Effects of the respective treatments on number of (pre)neoplastic lesions in the exocrine pancreas of BOP-treated hamsters after eight months[a]

(Pre)neoplastic lesions observed	Number of lesions			
	Control	SDS[b]	Castr.	SDS + Castr.
Number of hamsters	40	28	26	24
Borderline lesions	292	201	231*	123**
Carcinoma *in-situ*	15	18	16	5
Microcarcinoma	5	1[c]	2	0[c]
Adenocarcinoma	14	11	7	14
Total carcinoma	34	30	25	19

[a]Values are totals per group, [b]SDS: Sandostatin; Castr.: surgical castration.
Statistics: Log linear model with a Poisson distribution: * $P<0.05$; ** $P<0.01$, compared to controls. [c]SDS-treated animals versus animals not treated with SDS (controls + castrated): $P<0.05$.

Table 3.6 Effects of the respective treatments on incidence of (pre)neoplastic lesions in the exocrine pancreas of BOP-treated hamsters after eight months[a]

(Pre)neoplastic lesions observed	Incidence of lesions			
	Control	SDS[b]	Castr.	SDS + Castr.
Number of hamsters	40	28	26	24
Number of tumour-bearing hamsters	22	20	16	13
Borderline lesions	18	8	10	11
Carcinoma *in-situ*	7	8	9	2
Microcarcinoma	4	1	0	0
Adenocarcinoma	11	11	7	11

[a]Incidences are based on scoring each animal once in the category of the most advanced lesion; [b]SDS: Sandostatin; Castr.: surgical castration.
Statistics: Pearson chi-square test.

3.5 DISCUSSION

The experiments reported demonstrate a significant inhibitory effect of Sandostatin on development of AACN and carcinomas induced in rat pancreas by azaserine. The development of AACN and carcinomas was also inhibited in orchiectomized animals not treated with Sandostatin. In orchiectomized hamsters treated with Sandostatin, the development of borderline lesions was significantly inhibited, whereas such an effect was not present in hamsters that were either orchiectomized or treated with Sandostatin. Sandostatin-treatment, but not surgical castration, has a slight inhibitory effect on pancreatic carcinogenesis (development of microcarcinomas) in hamsters.

In rats, Sandostatin-treatment did not enhance the inhibitory effect of surgical castration. In contrast, an antagonistic interaction between Sandostatin-treatment and castration occurred as far as the development of AACN (transection area < 1 mm^2) and total carcinomas are concerned. The inhibitory action of Sandostatin on azaserine-induced pancreatic carcinogenesis in rats is in agreement with the results obtained with transplantable acinar tumours (10,31). Sandostatin exerts its inhibitory effect on rat pancreatic carcinogenesis most probably through somatostatin receptors. Pancreatic acinar carcinoma cell lines (39) as well as transplantable pancreatic tumours (24) contain somatostatin receptors, indicating that a direct effect of Sandostatin on the cellular level is possible. Sandostatin can exert its effect also indirectly through somatostatin receptors present in the islets of Langerhans (24) or through inhibition of action and/or release of gastrointestinal hormones such as cholecystokinin (22,40,41) which significantly stimulate development of pancreatic carcinogenesis in azaserine-treated rats (42-44). The significant inhibitory effects of surgical castration observed on pancreatic carcinogenesis in azaserine-treated rats support the findings of other investigators who demonstrated that oestrogen inhibits and testosterone promotes the development of (pre)neoplastic acinar lesions in rat pancreas (6,7,9,42,45-49). The mechanism of action, however, still remains obscure. As in a previous 4-month study, conducted at our Institute (9), the presently observed inhibitory effect of orchiectomy was accompanied by a significant decrease in body weight gain and a substantially lower pancreas weight in comparison with intact control rats. This consistent finding supports our previous suggestion that the substantial decrease in growth rate as observed in orchiectomized or oestradiol-treated rats is a confounding factor which may lead to an overestimation of the direct role of testosterone in pancreatic carcinogenesis (9).

In the 4-month study an inhibitory effect of prophylactically administered Sandostatin was found on growth of putative pre-cancerous ductular lesions in hamster pancreas, but

not on growth of early pre-cancerous AACN in rat pancreas (9). The present results are fully in agreement with these findings since we also did not find an effect of Sandostatin on development of AACN in rat pancreas after a treatment period of four months, whereas a significant inhibition of pancreatic carcinogenesis was observed after a treatment period of eight months. Based on these observations it is not illogical to assume that AACN in contrast to advanced acinar cell lesions such as adenomas and carcinomas do not express somatostatin receptors and hence do not respond to Sandostatin treatment.

In BOP-treated hamsters, the administration of Sandostatin inhibited the development of microcarcinomas. This finding supports those of the group of Schally (12,13,50) who studied the therapeutic effect of the somatostatin analogue RC-160 on pancreatic adenocarcinomas induced in hamster pancreas by multiple injections of BOP and found histological evidence of regression (apoptosis) of the tumours in about 50% of the animals. These observations are in contrast with the finding that Sandostatin alone has no effective therapeutic effect in patients with advanced exocrine pancreatic carcinomas. It has been found that ductular adenocarcinomas of human pancreas do not contain somatostatin receptors (24), whereas Fekete *et al.* (51), demonstrated the presence of such receptors in ductular pancreatic tumours in hamsters. The presence of somatostatin receptors may explain the presently observed slight inhibitory effect of Sandostatin-treatment alone on development of ductular adenocarcinomas in BOP-treated hamsters. The absence of such receptors in human pancreatic adenocarcinomas offers an explanation for the absence of a therapeutic effect of Sandostatin in patients with advanced exocrine pancreatic carcinomas. At present, however, different subtypes of somatostatin receptors have presently been detected and characterized with different affinities for analogs of somatostatin (52). Detailed analysis of such receptor subtypes is important to find an effective therapy for pancreatic cancer.

The present observation that Sandostatin in combination with surgical castration can be more effective than either Sandostatin treatment or orchiectomy alone supports the findings of Szepeshazi *et al.* (50) who used the somatostatin analogue RC-160 in combination with chemical, instead of surgical, castration by the Luteinizing Hormone-Releasing Hormone antagonist SB-75 (Cetrorelix). Szepeshazi *et al.* (50) reported also an effect on tumour weight resulting in a significantly lower pancreas weight in animals treated with SB-75 and RC-160. We did not find an effect on either pancreas weight or incidence of pancreatic tumours suggesting that the doses of the somatostatin analogue as well as the administration schedule used by the group of Schally was more effective than we used in our experiment. These results support the conclusion of Szepeshazi *et*

al. (50) that further studies are warranted in order to find the most optimal doses and administration schedules of the various somatostatin analogues available in order to improve their therapeutic effects.

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CHAPTER FOUR

EPIDERMAL GROWTH FACTOR RECEPTOR EXPRESSION IN PANCREATIC LESIONS INDUCED IN THE RAT BY AZASERINE

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4.1 ABSTRACT

In the present study, the expression of the epidermal growth factor receptor (EGFR) was investigated in putative preneoplastic and neoplastic acinar cell lesions induced in the rat pancreas by azaserine, using Northern blotting, in-situ hybridization (ISH) and immunohistochemistry. EGFR protein levels were decreased in putative preneoplastic eosinophilic acinar cell lesions (atypical acinar cell nodules: AACN) in comparison with normal acinar cells of the pancreas. However, EGFR mRNA expression correlated positively with the volume of AACN in pancreatic homogenates and ISH showed equal or stronger EGFR mRNA expression in AACN than in the surrounding normal acinar cells. Neither EGFR protein nor EGFR mRNA was detected in more advanced lesions such as acinar adenocarcinomas (in-situ). Moreover, EGFR protein expression showed an inverse relationship with the mitotic rate of the acinar cells. These findings support a complex role for EGFR in the development of putative preneoplastic AACN induced in rat pancreas by azaserine. These findings suggest that down regulation of EGFR at the protein level may abrogate negative constraints on cell growth, which may stimulate the development of putative preneoplastic AACN to more advanced lesions, and ultimately, acinar adenocarcinomas.

4.2 INTRODUCTION

Despite an increasing number of advanced diagnostic techniques and new therapies, the five-year survival rate of patients with tumours of the exocrine pancreas is not more than three percent (1). This poor prognosis is mainly due to late diagnosis and the absence of effective therapeutic modalities. Therefore, there is a need for studies on pancreatic cancer that concentrate on the detection of modulating factors involved early in the carcinogenic process. As originally proposed by Temin (2), one of the causes for the uncontrolled proliferative character of tumours might be inappropriate autocrine or paracrine production of growth factor(receptor)s (3). The epidermal growth factor receptor (EGFR) is believed to be one of these factors (4). In non-transformed (normal) cells, EGFR expression generally appears to be regulated within a rather narrow range of 20,000-100,000 receptors per cell (5). In both normal and transformed keratinocytes, EGFR expression is regulated at several levels, including transcription, mRNA stability, translation, and post-translational modification (6). In certain tumour cell lines such as

the A431 epidermoid carcinoma cell line, EGFR mRNA and protein levels are markedly increased as a result of amplification of the EGFR gene (7). EGFR is also overexpressed in several human pancreatic cancer cell lines (8,9), as well as in the pancreas of patients with pancreatic cancer and chronic pancreatitis (10,11). Overexpression of EGFR in human pancreatic tumours has been associated with an autocrine cell growth stimulation cycle (10-12). Furthermore, the v-*erb*B proto-oncogene of the avian erythroblastosis virus encodes a protein resembling EGFR (13), supporting the oncogenic potential of EGFR.

In the present study, the expression of EGFR was characterized in putative preneoplastic atypical acinar cell lesions (AACN) and in acinar tumours induced in rat pancreas by azaserine (14,15). EGFR mRNA expression was determined and quantified by Northern blotting, whereas the localization of EGFR in the pancreas was determined by *in-situ* hybridization. Furthermore, EGFR distribution at the protein level was determined by immunohistochemistry, and was correlated with the proliferating cell nuclear antigen (PCNA = proliferative cell nuclear antigen) expression in the pancreas.

4.3 MATERIALS AND METHODS

Pancreas isolation

To induce pancreatic carcinogenesis, albino Wistar WU rats (Charles River Wiga GmBH, Sulzfeld, Germany) were injected intraperitoneally at 14 and 21 days of age with 30 mg azaserine (Calbiochem-Behring Corp., La Jolla, CA, USA) per kg body weight according to an injection protocol described previously (16). All animals were housed under similar standard conditions. The rats were killed 15 months after the last injection with azaserine. The animals were anaesthetized with ether, exsanguinated by cannulating the abdominal aorta, and then examined for gross pathological changes. The entire pancreas was excised. Grossly visible pancreatic tumours were separated from normal pancreas and portions of both tissues were frozen and stored in liquid nitrogen immediately after dissection, or fixed in 4% buffered formalin and routinely embedded in paraffin wax.

Histology

At three different levels, ten 5 μm serial sections were prepared from the paraffin embedded pancreas. One section from each series was stained with haematoxylin and eosin (H&E) and examined by light microscopy. Likewise, one part of the liquid

nitrogen frozen pancreas was used for preparation of cryostat sections, the adjacent part was used for the molecular biological techniques. The cryostat sections were fixed for 10 minutes in 4% buffered formalin, stained routinely with H&E and examined by light microscopy (15). Based on the results of the microscopical examinations, tissues with normal, preneoplastic, or tumourous histology were selected for further experiments.

Immunohistochemistry

Two different mouse monoclonal antibodies were used to detect EGFR immunoreactivity. One antibody was a generous gift from Dr. W.A. Dunn, University of Florida, diluted 1:100 and proved earlier to be suitable for immunohistochemical localization of the EGFR in rat tissue (17), the other EGFR antibody was purchased from SIGMA (St. Louis, MO; clone 29.1), diluted 1:2,000. Both antibodies were directed to the extracellular domain of EGFR (18). To demonstrate differences in mitotic rate, the proliferation marker PCNA (Santa Cruz Biotechnology, CA; dilution 0.2 μg/μl) was detected on parallel sections. Endogenous peroxidase activity was quenched by incubation in 0.6% hydrogen peroxide in methanol for 30 minutes. The sections were pretreated with 0.3% Triton X-100 in phosphate buffered saline (PBS, 0.14 mM NaCl; 8.93 mM Na_2HPO_4; 1.28 mM NaH_2PO_4; pH 7.4) for 15 minutes. The slides were incubated for 2 hours with the first antibody in 2% bovine serum albumin (BSA) in PBS in a humid slide chamber. To detect the monoclonal antibody a subsequent RAMPO/SWARPO (Dako A/S; Glostrup, Denmark, diluted 1:100) incubation was performed. Both RAMPO and SWARPO were diluted in PBS containing 10% normal rat serum and were incubated for 30 minutes. Between each incubation step the sections were washed three times for five minutes with PBS-Tween (0.05%), with the exception for the last washing step where Tween was omitted. Subsequently, a brown precipitate could be observed by light microscopy after the peroxidase reaction with 3,3'-diaminobenzidine tetrahydrochloride (SIGMA). The sections were counterstained with haematoxylin (after Mayer). Expression of the EGFR in this study is defined as staining of cell membranes, often accompanied by cytoplasmic staining.

RNA isolation

Total RNA was isolated by a modification of the procedures described by Chomczynski *et al.* (19) and Chirgwin *et al.* (20). Standard precautions were taken to prevent contamination of solutions and glasswork with RNases (21). RNA was isolated from -parts of- total pancreas, and from the grossly visible pancreatic adenocarcinomas isolated at final autopsy. Unfortunately, because of the extremely high RNase content in

rat pancreas, in combination with the relatively long time needed to dissect out the small, but macroscopically visible preneoplastic lesions, it appeared to be impossible to isolate good quality RNA from these small lesions. Therefore, effects on RNA levels in preneoplastic lesions, were only detected in total pancreas homogenates containing both 'normal' tissue and lesions. Frozen tissue samples (0.1-0.3 g) were homogenized in a high-speed homogenizer (Ultra Turrax; Janke & Kunkel-IKA, Staufen, Germany: 15-20 sec at 25,000 rpm) in 3-5 ml GuSCN (4 M guanidium thiocyanate; 1% ß-mercaptoethanol, 0.5% *N*-lauroylsarkosine; 25 mM sodium citrate). RNA was separated from DNA and proteins by ultra-centrifugation at 40,000 rpm overnight (o/n) through a 5.7 M cesium chloride cusion or by acid-phenol-chlorophorm extraction and subsequent precipitation at -20°C with 0.025 volume 1 M acetic acid and 0.5 volume ethanol. After removing the supernatant, the RNA pellet was redissolved in 800 µl GuSCN. RNA was precipitated again with 0.025 volume 1 M acetic acid and 0.6 volume ethanol. Finally, the RNA pellet was washed in 70% ethanol, dried for 5-10 minutes in a Speedvac (SVC100H; Savant, Farmingdale, NY), and redissolved in diethylpyrocarbonate (DEPC)-treated water. Quantity and quality of the RNA were monitored by spectrophotometry at 260 nm and 1% agarose-gel electrophoresis, respectively.

Northern blotting

25 µg total RNA was denatured in sample buffer (10x MOPS : 37% formaldehyde : formamide = 2:3:10) for 5 minutes at 65°C, and separated by size on a 1.2% denaturating gel for 4-5 hours. RNA was transferred to a nylon membrane and crosslinked by UV. Prehybridization and hybridization were performed over night at 65°C. Typically, 0.5-1x10^6 cpm/ml of the ^{32}P-labeled rat EGFR riboprobe was used for hybridization in the presence of hybmix (50% formamide; 0.4% SDS; 4x SSC; 4x Denhardt's; 0.2 mg/ml ssDNA; 0.04M $NaPO_4$ pH 7.4; 10% dextran sulfate). Blots were washed twice at low stringency (1x SSPE; 0.5% SDS; 15 min at 65°C) and 1-2 times at high stringency (0.1x SSPE; 0.5% SDS; 15 min at 65°C). Equality of RNA-loading was checked by hybridization with a 7S DNA-probe (22). The blots were exposed to a phosphor storage screen for 2 days and subsequently scanned by a phosphorimager (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA). EGFR mRNA levels were calculated as percentages of 7S RNA using the computer program ImageQuant (Molecular Dynamics), and subsequently statistically correlated with the number of lesions observed in parallel-sections.

In-situ hybridization

Serial five μm thick, formalin-fixed, paraffin-embedded tissue sections were placed on poly-L-lysine (1 mg/ml) coated glass slides, and subsequently deparaffinized, hydrated, permeabilized with 1 μg/ml proteinase K (Boehringer Mannheim) for 10 minutes at 37°C, and prehybridized in hybridization buffer (50% formamide, 20 mM Tris-HCl pH8.0, 5 mM EDTA, 1x Denhardt's, 5% dextran sulfate, 10 mM dithiothreitol, 0.3M NaCl) for 4 hours at 42°C. As a negative control for mRNA hybridization, the sections were treated with 200 μg/ml RNase A, 10 U/ml RNase T1 for one hour at 37°C, just before prehybridization. Hybridization was performed o/n at 50°C with $2.5x10^5$ cpm ^{33}P-labeled rat EGFR riboprobe and 1 μl tRNA (50 μg/μl) in 200 μl hybridization buffer per slide. After hybridization, non-bound probe was digested with RNase A (Boehringer Mannheim, 20 μg/ml) for 30 minutes at 37°C, and aspecific binding was washed with increasing stringency. The sections were coated with autoradiography emulsion (Eastman Kodak Co., Rochester, NY) and exposed for 5 days. The sections were developed and fixed, and subsequently counterstained with haematoxylin.

Preparation of the rat-EGFR riboprobe

The rat EGF receptor cDNA fragment, corresponding to nucleotide bases 249-951 (23) was amplified by PCR from reverse-transcribed rat liver RNA using PCR primers which contained unique BamH1 and Sph1 restriction sites. The PCR products were generated in 40 cycles (94°C, 1.5 min; 42°C, 1.5 min; and 72°C, 1.5 min), subsequently subcloned into a pGEM7Zf vector (Promega), and authenticity was confirmed by sequencing.

4.4 RESULTS

Immunohistochemistry

In the normal rat pancreas, immunohistochemical studies with monoclonal antibodies directed to the EGFR demonstrated expression in the cytoplasm of acinar cells, sometimes with accentuation of the cell membranes (**Figure 4.1**). Both anti-EGFR monoclonal antibodies gave similar staining patterns in all tissue sections that were investigated. Pancreatic cells of rats not treated with azaserine showed similar staining patterns as histologically normal pancreatic cells of rats which were treated with azaserine. No differences in EGFR staining intensities were observed in non-pancreatic

cells (such as vascular endothelial cells, macrophages or nerve fibres) when comparing azaserine-treated with untreated rats. Ductular and endocrine cells were negative. Surprisingly, normal acinar cells stained more intensely for EGFR than the eosinophilic acinar cells of putative preneoplastic atypical acinar cell lesions (AACN; **Figures 4.1 and 4.2**).

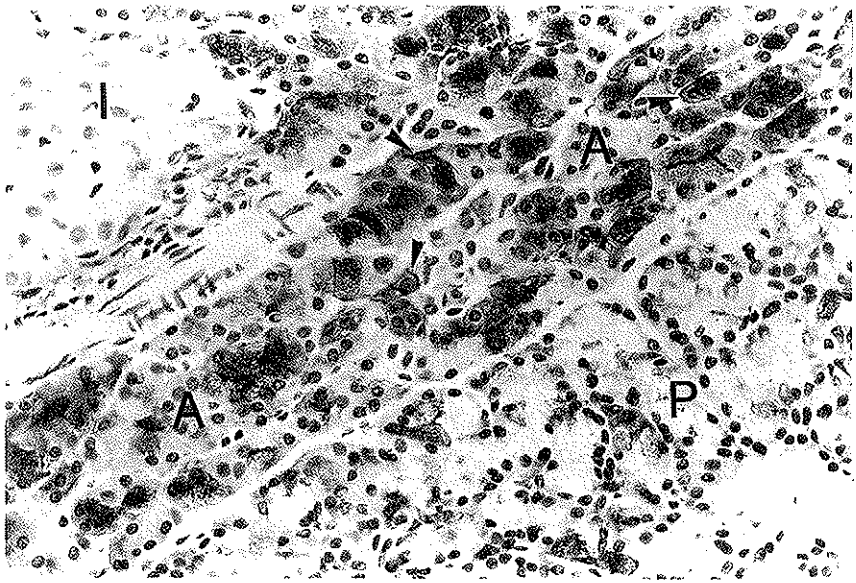

Figure 4.1 Immunohistochemical localization of EGFR in azaserine-treated rat pancreas. I=islet of Langerhans cells; A=normal pancreas acinar cells; P=putative preneoplastic atypical acinar cell nodule; arrowheads point to membranous staining.

More advanced lesions such as nodules-in-nodules (**Figure 4.3a**), carcinomas *in-situ* and acinar adenocarcinomas were negative. The AACN exhibited a large variation in their intensity of EGFR immunoreactivity (**Figure 4.2**), which was found predominantly in the cytoplasm in these lesions since hardly any immunoreactivity to EGFR was present on the cellular membranes. The AACN also varied in number of cells with immunoreactivity to EGFR. In some lesions only a few dispersed cells were positive, whereas other lesions showed an almost similar immunoreactivity to EGFR as the surrounding normal acinar cells.

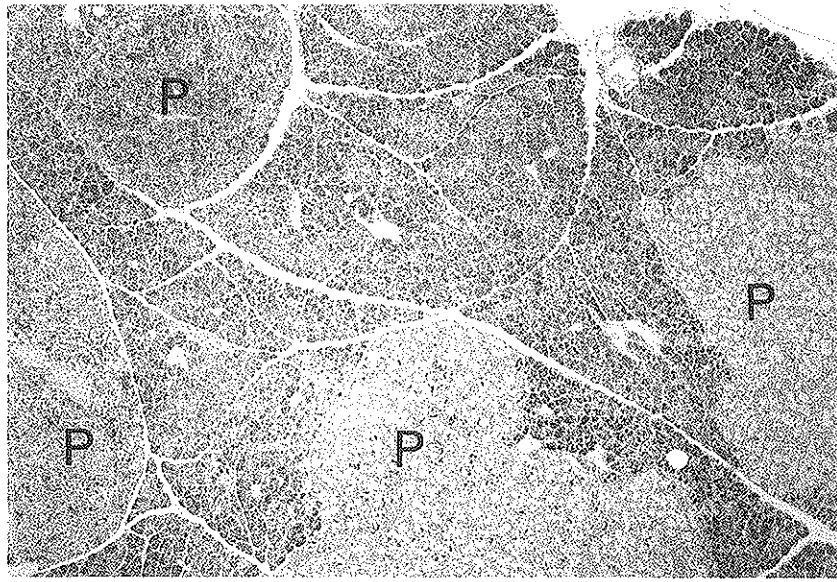

Figure 4.2 Immunohistochemical localization of EGFR in azaserine-treated rat pancreas. Note differences in intensities of EGFR in the putative preneoplastic lesions. P= putative preneoplastic atypical acinar cell nodule.

Immunohistochemical detection of the proliferation marker PCNA in parallel sections clearly demonstrated an inverse relationship between mitotic rate and immunoreactivity to EGFR (**Figure 4.3b**). In contrast with the putative preneoplastic eosinophilic atypical acinar cell foci, the basophilic atypical acinar cell foci (which are not considered to be preneoplastic) could not be distinguished from normal acinar cells by incubation with either the anti-EGFR antibodies or the PCNA antibody (not shown). Sections of the rat submaxillary and sublingual salivary glands were used as controls for the specificity of the anti-EGFR antibodies. As expected, strong immunoreactivity was apparent only on the serous cells. Omitting the anti-EGFR or anti-PCNA first antibodies resulted in complete absence of staining.

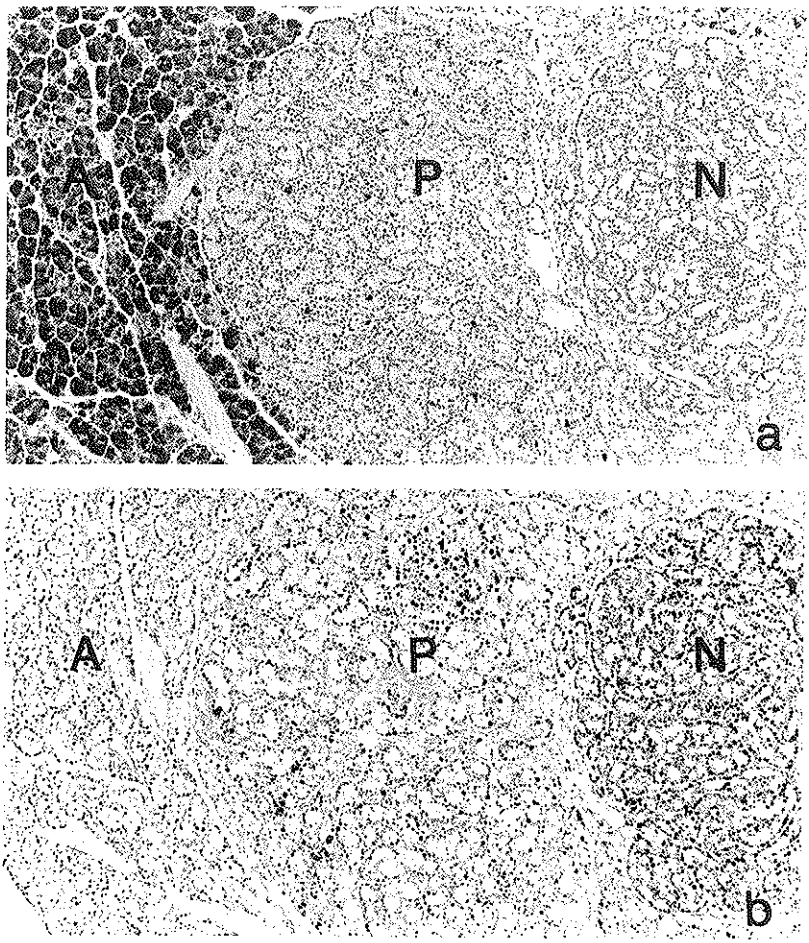

Figure 4.3 Immunohistochemical localization of a. EGFR, and b. PCNA, in azaserine-treated rat pancreas. A=normal pancreas acinar cells, P=putative preneoplastic atypical acinar cell nodule (primary lesion); N=nodule-in-nodule (secondary lesion).

Northern Blotting

Seventeen rat pancreatic tissues comprising normal and putative preneoplastic acinar cells, and five macroscopically isolated acinar adenocarcinomas were analyzed by Northern blotting (**Figure 4.4**). A significantly positive correlation was found for the EGFR mRNA levels with the number of atypical acinar cell lesions found in

homogenates from azaserine-treated rat pancreas (four independent Northern blots: r=0.9950, P<0.05; r=0.9993, P<0.01; r=0.85263, P<0.05; r=0.9953, P<0.05).However, no EGFR mRNA expression was detected by Northern blotting on total RNA from the isolated acinar adenocarcinomas, while the 7S control was equally detectable (**Figure 4.4**).

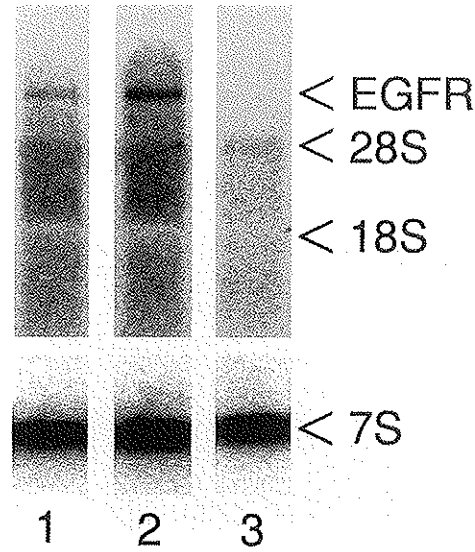

Figure 4.4 EGFR mRNA levels detected by Northern blotting in pancreatic homogenates from azaserine-treated rats. The blots were rehybridized with a 7S cDNA probe to control for RNA loading. Lane 1, normal pancreas; lane 2, preneoplastic pancreas; lane 3, acinar adenocarcinoma. 28S, 18S = location of the ribosomal subunits.

In-situ hybridization

Hybridization of rat pancreas sections with the ^{33}P labeled rat-EGFR riboprobe revealed a positive signal in the acinar cells of normal pancreas, whereas no grains could be observed in the ductular (**Figure 4.5a**) and endocrine cells (not shown). Furthermore, putative preneoplastic atypical acinar cell nodules exhibited a similar number or even more grains than the normal acinar cells (**Figure 4.5b**). However, in more advanced lesions (e.g. carcinoma *in-situ*), EGFR mRNA was only faintly detectable (**Figure 4.5c**) or undetectable. When the sections were pretreated with RNase, no (differences in)

signals were observed; the few remaining grains were considered to represent background (**Figure 4.5d**).

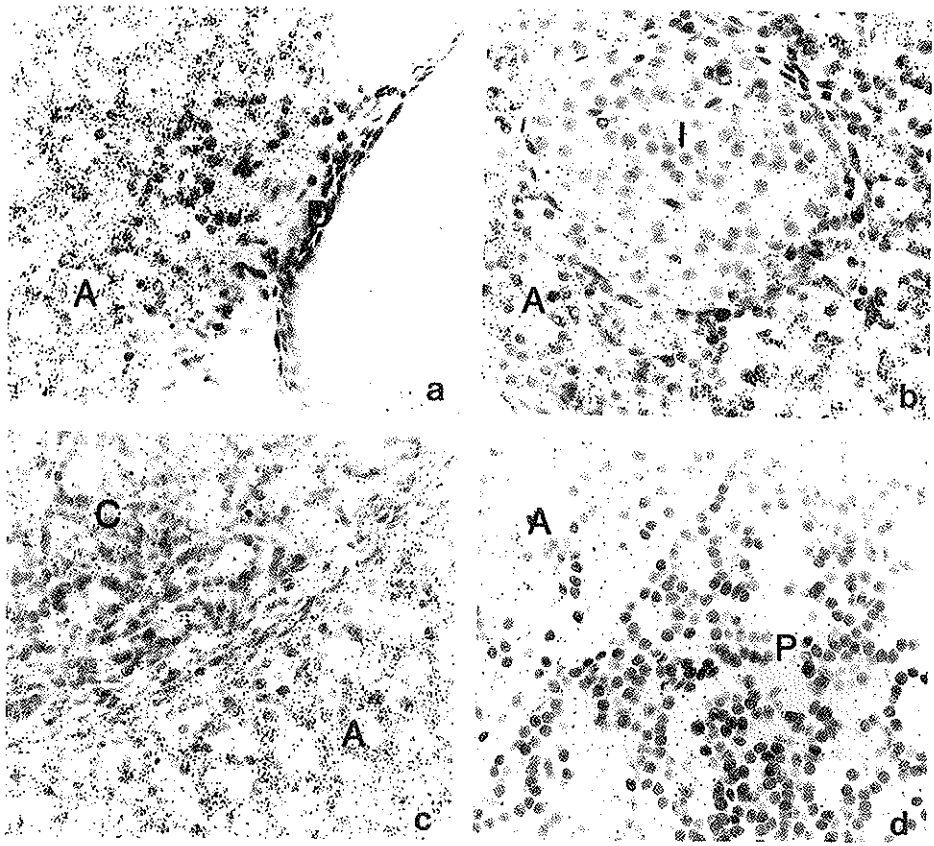

Figure 4.5 EGFR mRNA detection by *in-situ* hybridization in azaserine-treated rat pancreas. **a.** A=acinar cells; D=ductular cells, **b.** A=acinar cells; I=islet of Langerhans, **c.** A=normal pancreas acinar cells; C=adenocarcinoma, **d.** after RNase treatment, A=normal pancreas acinar cells; P=putative preneoplastic atypical acinar cell nodule. Differences in labelling-intensities in corresponding cell-types between the various photomicrographs, are due to differences in autoradiographic conditions in the various tissue slides.

4.5 DISCUSSION

Using immunohistochemistry, Northern blotting, and *in-situ* hybridization, the present study clearly demonstrates that epidermal growth factor receptors (EGFR) are detectable on pancreatic acinar cells of normal rats and of rats treated with azaserine, as summarized in **Table 4.1**. By Northern blotting, the presence of a high number of putative preneoplastic atypical acinar cell nodules (AACN) was associated with increased expression of EGFR mRNA compared to pancreatic tissues containing no or a low number of AACN. In contrast, by immunohistochemistry, most AACN exhibited decreased EGFR immunoreactivity, indicating a decrease in the amount of EGFR protein. The discordance between EGFR mRNA and protein levels could be due to inhibition of EGFR at the translational level, increased EGFR protein degradation, or shedding of EGFR by the AACN cells.

Table 4.1 Summary of the results obtained from pancreatic acinar cells of azaserine-treated rats[a]

Phenotype:	EGFR protein (IHC)	EGFR mRNA (ISH, North.)	PCNA (IHC)
Normal	+	+	±
Putative preneoplastic	±	+/++	±/+/++
Neoplastic	-	-	++

[a]The values are based on microscopical examinations of pancreatic acinar cells or densitometrical data as indicated in the text. Values are relative levels in the different tissue types: -, not present; ±, moderately present; +, clearly present; ++, strongly present; IHC, immunohistochemistry; ISH, *in-situ* hybridization; North., Northern blotting.

EGFR was not detectable in acinar adenocarcinomas by either immunohistochemistry, *in-situ* hybridization or Northern blotting, indicating that in advanced acinar lesions, transcription of DNA coding for EGFR either does not take place, or EGFR mRNA is markedly and rapidly degraded. These findings suggest that EGFR may not play an essential role in the pancreatic carcinogenic process in azaserine-treated rats. Alternatively, EGFR may be involved in the regulation of differentiated functions in the rat pancreatic acinar cell, and may exert growth-suppressive effects on this cell type.

It cannot be excluded that diminished EGFR immunoreactivity as observed in the present study is the result of a change of the epitope instead of actual decrease in the number of EGFR present. However, this explanation does not seem to be very likely, since we found similar results with two different monoclonal antibodies directed to the EGFR. Moreover, it has been established that the antibodies used are reactive in rats since they demonstrate a positive reaction with salivary glands collected from the same animals.

It is conceivable that the findings presented in this paper are typical for acinar pancreatic cells only, and may very well not apply for ductular pancreatic cells or ductular adenocarcinomas of the pancreas. To our knowledge, human acinar adenocarcinomas have not been investigated for the presence of EGFR. Similar experiments as have been described in this paper will be performed in our Institute, with *N*-nitrosobis(2-oxopropyl)amine-treated hamsters (model for ductular adenocarcinomas; (24)), in order to investigate whether EGFR might play a role in the development of pancreatic ductular carcinomas.

Decreased EGFR-mediated signaling may thus lead to loss of differentiated functions and increased propensity toward neoplastic transformation. Indeed, several lines of evidence suggest an anti-mitogenic role for EGFR in the rat exocrine pancreas. First, the rat pancreatic acinar cell has specific high-affinity EGF receptors (25), and EGF is necessary for the maintainance of this cell type in serum-free culture (26). Second, EGF enhances rat acinar cell survival and pancreatic protein synthesis at concentrations as low as 42 pM (26-28). Third, this action of EGF is relatively specific, since a comparable effect occurs only at 2.7 nM IGF-1 and does not occur with insulin (28). Fourth, EGF decreases thymidine incorporation into pancreatic DNA in male Sprague-Dawley rats (29) and increases pancreatic content of amylase and chymotrypsinogen while preventing caerulein-mediated desensitization of the acinar cell secretory responsiveness (29). Fifth, EGF binding is decreased in the regenerating rat pancreas following 90% pancreatectomy, in parallel with an increase in acinar cell mitotic activity (30). Thus, a decrease in EGFR-mediated signaling in the rat pancreatic acinar cell may lead to enhanced carcinogenesis. Another explanation could be, that acinar pancreatic cells of rats which are altered by azaserine treatment do not need EGFR for their survival anymore, since the receptor signaling has been taken over by other members of the EGFR-family, such as c-erbB2 (HER-2, erbB2, neu (31-33)) or erbB3 (34,35).

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CHAPTER FIVE

TRANSFORMING GROWTH FACTOR-α AND EPIDERMAL GROWTH FACTOR EXPRESSION IN THE EXOCRINE PANCREAS OF AZASERINE-TREATED RATS; MODULATION BY CHOLECYSTOKININ OR A LOW FAT-HIGH FIBER (CALORIC RESTRICTED) DIET

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5.1 ABSTRACT

The expression of transforming growth factor-α (TGF-α) and the epidermal growth factor (EGF) was studied in normal pancreas tissue and in (pre)neoplastic pancreatic lesions of azaserine-treated rats. They were either given a low fat-high fiber (low caloric) diet to inhibit carcinogenesis, or a low fat diet combined with injections of the cholecystokinin-analogue caerulein to enhance carcinogenesis. The control groups, maintained on a low fat diet, were injected with azaserine, or were not treated at all. Autopsy was performed at 6 and 15 months after the last azaserine injection.

Both after 6 and 15 months, immunohistochemistry revealed a weak expression of EGF and TGF-α peptides in the acinar cells, and a stronger expression in the ductular and centro-acinar cells. TGF-α peptide expression was reduced in both putative preneoplastic and neoplastic acinar cell lesions, but no differences in EGF peptide expression were observed between the various stages of exocrine pancreatic carcinogenesis. After 16 months, an increase in TGF-α mRNA due to treatment with azaserine was detected by semi-quantitative polymerase chain reaction (PCR) in total pancreatic homogenates, whereas EGF mRNA expression had decreased. TGF-α mRNA levels in macroscopically isolated tumours were significantly lower, but EGF mRNA levels were significantly higher, than in total pancreatic homogenates from azaserine-treated rats. Furthermore, EGF and TGF-α mRNA levels in isolated tumours did not differ significantly from mRNA levels in non-carcinogen treated rats. Neither with immunohistochemistry, nor with PCR, differences in EGF or TGF-α expression were observed due to either inhibition or stimulation of carcinogenesis.

It is concluded that putative preneoplastic acinar cell lesions induced in rat pancreas by azaserine may develop into acinar adenocarcinomas independently of TGF-α and EGF. The results suggest involvement of these growth factors at the early stage of the carcinogenic process, during the initiation of normal acinar cells into putative preneoplastic cells. However, modulation of azaserine-induced pancreatic carcinogenesis by cholecystokinin or a low fat-high fiber (caloric restricted) diet appeared not to be regulated by EGF or TGF-α.

5.2 INTRODUCTION

It is believed that dietary and hormonal factors such as fat and cholecystokinin, which may promote the effects of environmental carcinogens, play an important role in the pathogenesis of exocrine pancreatic cancer (1,2). However, little is known about the cellular mechanisms involved in the pathogenesis of this highly fatal form of cancer (3). In general, growth factors are thought to play a major role in carcinogenesis (4-6), and they have also been found to be of significance for the development of pancreatic cancer. Epidermal growth factor (EGF) injected simultaneously with the pancreatic carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP) in Syrian golden hamsters significantly enhanced pancreatic carcinogenesis compared to injection of BOP alone (7,8). The epidermal growth factor receptor (EGFR), known to be bound and activated by EGF, transforming growth factor-α (TGF-α) and at least six other members of the EGF-family (9), was overexpressed in several cultured human pancreatic cancer cell lines (10,11). Furthermore, it has been found that human pancreatic cancer cell-lines express TGF-α (12), and overexpression of the EGFR has been associated with a concomitant increase in the levels of EGF and TGF-α in human pancreatic cancer (13,14). Also, ductular cells of the human pancreas appeared to secrete substantial amounts of EGF, which could be enhanced by cholecystokinin treatment (15).

Up to now there is only very little knowledge about the role that growth factors play in dietary or hormonal *in-vivo* modulation of the carcinogenic process. In the present study we have investigated whether EGF and/or TGF-α are involved in the promotion phase of pancreatic carcinogenesis in azaserine-treated rats (model for acinar adenocarcinomas; 16-18). After initiation with azaserine, the rats were either treated with the cholecystokinin analogue caerulein to enhance the carcinogenic process, or were given a low fat-high fiber (caloric restricted) diet to inhibit carcinogenesis. The effects of these treatments on pancreatic tumour development are analyzed after 6 and 15 months. At the same time-points, using a semi-quantitative PCR technique, we investigated whether differences in EGF or TGF-α mRNA expression levels occurred between the various groups. Furthermore, the distribution and expression of EGF and TGF-α proteins in the rat pancreas were investigated by immunohistochemistry.

5.3 MATERIALS AND METHODS

Induction of carcinogenesis

To induce pancreatic carcinogenesis, three groups (A,B,C) of 35 male albino Wistar WU rats (Charles River Wiga GmBH, Sulzfeld, Germany) were injected i.p. at 14 and 21 days of age with 30 mg azaserine (Calbiochem-Behring Corp., La Jolla, CA) per kg body weight, according to an injection protocol described previously (19). Ten extra rats, which were injected with saline, served as non-carcinogen controls; group D. During the initiation phase, all rats were fed a standard laboratory chow. One week after the last azaserine injection, the rats were maintained on an AIN-76 based diet and were assigned randomly to one of three experimental groups: A) low fat diet (5% corn oil); B) low fat diet + s.c. injections with 2.5 µg caerulein/kg body weight, three times a week; and C) 20% caloric restricted low fat diet. 20% caloric restriction was achieved by substituting cellulose for wheat starch (**Table 5.1**).

Table 5.1 Weight percentage composition of the AIN76-based diets[a]

	Groups A, B, D Low fat	Group C 20% Caloric restriction
Casein	25.0	25.0
DL-Methionine	0.38	0.38
Wheat starch	54.47	36.37
Cellulose	7.5	25.6
Choline bitartrate	0.25	0.25
AIN-76-AM minerals	4.38	4.38
AIN-76-AM vitamins	1.25	1.25
CaH_2PO_4	1.77	1.77
Corn oil	5.0	5.0
Total	100.0	100.0
Calories (MJ/kg)	15.5	12.4

[a]The diets were prepared freshly every 2 months. All diets were stored at -20°C until use.

All animals were kept in stainless steel cages, fitted with wiremesh floors and fronts, housed under similar standard laboratory conditions, 5 rats per cage. Body weights and food intake were recorded weekly during the first three months, and once a month during the rest of the experiment. The general condition and behaviour of the animals were checked daily. An interim kill (groups A-C, 15 rats; group D, 5 rats) was performed after 6 months and terminal autopsy (all remaining rats) was performed 15 months after the last injection with azaserine. Therefore, the animals were anaesthetized with ether, exsanguinated by cannulating the abdominal aorta, and then examined for gross pathological changes. The entire pancreas and all gross lesions suspected of being tumours were excised. Pancreata and livers were weighed. Grossly visible pancreatic tumours were separated from normal pancreas. One part of all collected tissues was snap-frozen in liquid nitrogen to be used for molecular biological techniques. The adjacent part was processed for microscopy by conventional methods, step-sectioned at 5 μm (at least three tissue sections per pancreas), stained with hematoxylin and eosin (H&E), and examined by light microscopy.

Histology and immunohistochemistry

A total area of 100-200 mm^2 of pancreatic tissue was examined per rat. Azaserine-induced atypical acinar cell nodules (AACN), acinar cell adenomas, localized carcinomas (carcinoma *in-situ*; CIS) and invasive adenocarcinomas were identified and classified according to the criteria of Longnecker (16) and Rao *et al.* (20). The area of nodule transections was determined using a grid inside the ocular as described previously (21).

The remaining parallel sections were used to detect TGF-α or EGF peptides. TGF-α was detected with a mouse anti-rat monoclonal antibody (dilution 1:15, AB-2, Oncogene Science, Uniondale, N.Y.). To detect EGF we performed incubations with three different polyclonal antibodies, a rabbit anti-rat (a generous gift from Ryoichi Oyasu, Northwestern University Medical School, Chicago, IL (22)); a rabbit anti-recombinant human EGF (Ab-3, Oncogene Science); and a rabbit anti-rat (Biomedical Technologies Inc., Stoughton, MA). Specificity was checked on rat salivary glands and all antibodies proved to be immunoreactive (23). Two monoclonal anti-mouse EGF antibodies (MON8001 and MON8002, Monosan, Uden, The Netherlands) did not show immunoreactivity with our tissues. Antigen retrieval was achieved by boiling the tissue sections in citrate buffer (sodium citrate, 2.94 g/L aqua dest; pH 6.0) for 10 minutes. Immunoreactivity of the antibodies was detected by subsequent incubations with peroxidase conjugated rabbit-anti-mouse antibodies (RAMPO; Dako a/s, Glostrup, Denmark; dilution 1:100 in 10% normal rat serum) and/or peroxidase conjugated swine-

anti-rabbit antibodies (SWARPO; Dako a/s; dilution 1:100 in 10% normal rat serum). A brown precipitate was obtained after the peroxidase reaction of H_2O_2 in the presence of 4,4-diaminobenzidine (DAB; Sigma). To demonstrate weak immunohistochemical differences, a visually stronger, black precipitate was obtained by performing the DAB reaction in the presence of nickel. Sections were counterstained with hematoxylin or nuclear-fast-red. The slides were observed by light microscopy, and relative staining intensities (no staining; weak staining; strong staining) in the tissues were determined by blind scoring of the sections by an independent person.

RNA isolation

Total RNA was isolated by a modification of the procedure described by Chirgwin (24) from -parts of- total pancreas (further on referred to as 'total pancreas'), and from the grossly visible pancreatic adenocarcinomas isolated at final autopsy. Unfortunately, because of the extremely high RNase content in rat pancreas, in combination with the relatively long time needed to take out the small, but macroscopically visible preneoplastic lesions, it appeared to be impossible to isolate good quality RNA from these small lesions. Therefore, effects on RNA levels in preneoplastic lesions, were detected in total pancreas homogenates containing both 'normal' tissue and lesions. Frozen tissue (0.1-0.3 g) was homogenized by a high-speed homogenizer (Ultra Turrax; Janke & Kunkel-IKA, Staufen, Germany: 15-20 sec at 25,000 rpm) in 3 ml GTC (4 M guanidium thiocyanate, 1% ß-mercaptoethanol, 0.1 M Tris-HCl, and 0.01 M EDTA; Ph 7.5). After homogenization sarkosyl (N-lauroylsarkosine, 0.5%) was added. The homogenate was carefully poured on a CsCl-cushion (1.3 ml: 5.7 M cesiumchloride and 0.1 M EDTA; pH 7.5) in a SW50.1 polyallomer ultra-centrifugation tube (Nalgene, Nalge Company/Sybron Corp., Rochester, NY). RNA was separated from DNA and proteins by ultracentrifugation overnight, at 40,000 rpm, 18°C. The RNA-pellet was redissolved in 3 ml GND-HCl (6 M guanidine-HCl, 0.075% ß-mercaptoethanol, 25 mM EDTA; pH 7.5). To get rid of remaining ribonucleases and to concentrate, the RNA was precipitated repeatedly. Quantity and quality of the RNA were checked by spectrophotometry at 260 nm and 1% agarose-gel electrophoresis, respectively.

Semi-quantitative Polymerase Chain Reaction (PCR)

Because EGF and TGF-α mRNA levels appeared too low to be detected quantitatively by Northern blotting, a semi-quantitative PCR was performed in this study. Every group statistically analyzed comprised at least 5 independent observations (*in duplo*). Fifteen µg total RNA was used to synthesize complementary DNA (cDNA).

cDNA-synthesis was performed with a kit from Promega (Medison, WI, USA) based on a method described by Gubler and Hoffman (25). mRNA was reverse transcribed at 42°C during one hour using 1 µl AMV-reverse transcriptase (22 U/µl; Promega)/15 µg RNA and annealed with a mixture of 0.5 µg oligo-dT_{15} and 0.5 µg oligohexamers (Boehringer, Mannheim, Germany)/µg mRNA. cDNA was stored at -20°C in 20 µl autoclaved milli-Q water. Specific TGF-α (P1 & P2; **Table 5.2**), EGF (P4 & P5) and glyceraldehyde phosphate dehydrogenase (GAPDH; P7 & P8) complementary oligonucleotides were synthesized by cyanoethyl phosphoramide chemistry using an automated DNA-synthesizer (ABI 318A, Foster City, CA). PCR (26,27) was performed with 0.5 µl cDNA from the final cDNA solution in a mixture containing 50 mM KCl, 10 mM Tris-HCl; pH 8.0, 4x5 µl deoxynucleotides (10 mM), 1.5 mM $MgCl_2$, 0.1 µl Taq-polymerase (5U/µl; Perkin Elmer-Cetus, Norwalk, CT, USA), 2x0.5 µl primers (0.5 µg/µl) in a total volume of 50 µl. For each animal, PCR was performed *in duplo* in one session in one PCR machine (Perkin Elmer-Cetus Thermocycler 480) for GAPDH, TGF-α and EGF, simultaneously. The temperature was varied as follows: 5 min at 95°C, then 25-40 times the following cycle (file #3): 1 min to reach 95°C, two min to reach 60°C, one min to reach 72°C, and another 30 sec at 72°C to elongate. The last cycle was followed by 10 min at 72°C.

Table 5.2 Polymerase chain reaction primers and oligoprobes.

Primer fragment	5' Sequence 3'	Length PCR
P1 TGF-α 5'	ACCTGCAGGTTTTTGGTGCAG	266 bp
P2 TGF-α 3'	GGAGGGCGCTGGGCTTCTCG	
P3 TGF-α probe	TGCTTCTTCTGGCTGGCAGC	
P4 EGF 5'	GTCGTACGATGGGTACTGCCTC	136 bp
P5 EGF 3'	GCGCAGCTTCCACCAACGTAAG	
P6 EGF probe	CAATATAGCCAATGACACAGTTGC	
P7 GAPDH 5'	GCATCCTGGGCTACACTGAG	162 bp
P8 GAPDH 3'	CACCACCCTGTTGCTGTAGC	
P9 GAPDH probe	GCTCATTTCCTGGTATGTGGCTGG	

GAPDH (a housekeeping enzyme) was used as standard to control for variations in RNA-isolation, cDNA synthesis and PCR performance. The PCR products were taken from the machine after 25, 30, 35 and 40 cycles, respectively. In this way it was established where PCR-reactions still were in the exponential (quantifiable) phase. GAPDH, TGF-α and EGF PCR-products from one animal were visualized simultaneously by 2% agarose-gel electrophoresis (**Figure 5.1**). This gel was photographed on an instant-film with photo-negative (Kodak, Rochester, N.Y.). Subsequently, the negative was scanned by a laser densitometer (Personal Densitometer, Molecular Dynamics, Sunnyvale, CA), quantified by the computer program ImageQuant (Molecular Dynamics) and relative optical densities of TGF-α and EGF PCR products were calculated, expressed as percentages of GAPDH.

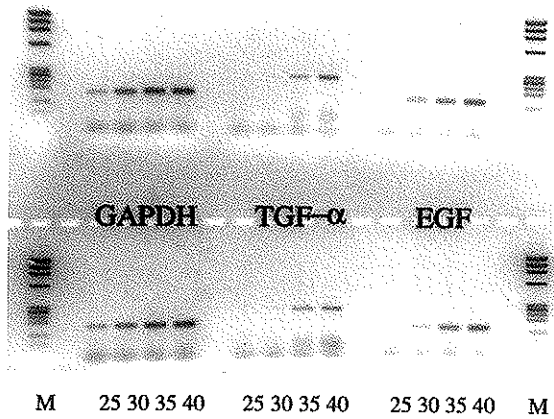

Figure 5.1 Negative of the photograph of an ethidium bromide stained 1% agarose gel, GAPDH (165 bp), TGF-α (266 bp) and EGF (136 bp), after 25, 30, 35 and 40 cycles of a semi-quantitative PCR experiment performed in duplicate (top & bottom). Molecular weight marker is phage φX-DNA digested with HaeIII.

Southern blotting PCR products

To check specificity, PCR products were transferred to a nylon membrane

(Hybond-N+, Amersham, Amersham, England) and subsequently hybridized with specific internal oligoprobes (P3, P6 and P9, for TGF-α, EGF and GAPDH, respectively; Table II). 15 ng oligo-probe was kinated in a total volume of 10 µl containing 1 µl PNK-buffer (10x: 0.5 M Tris-HCl; pH 7.6, 0.1 M $MgCl_2$, 50 mM DTT, 1 mM spermidine-HCl and 1 mM EDTA), 3 µl H_2O, 1 µl T_4 polynucleotide kinase (T_4PNKase, 10 U/µl, Promega) and 3 µl [γ^{32}P]dATP (10 µCi/µl, Amersham) for one hour at 37°C. These probes were added to the blot and (pre)hybridization solution (blots were prehybridized at 55°C for 4 hours in 5x SSC; 20 mM NaPi, pH 7.0; 7% SDS; 10x Denhardt's) and hybridization was performed overnight, at 55°C. Aspecific binding was removed by washing three times 15 minutes with 3x SSC, 1% SDS at 55°C. Subsequently, the blots were exposed to autoradiography film (X-Omat AR, Kodak) for about 30 minutes.

Statistics

Body weight data were statistically evaluated by analysis of variance with initial body weight as covariable, followed by Dunnett's tests (two-sided). Organ weight and food intake data were statistically evaluated by analysis of variance followed by two-sided Dunnett's tests or L.S.D. tests, respectively. Atypical acinar cell foci data were evaluated by a log-linear regression analysis. The semi-quantitative PCR data and the number of lesions larger than 1.0 mm^2 were evaluated by 2-sample *t*-tests, whereas the incidence (the number of tumour-bearing animals) was evaluated by Pearson χ^2-test.

5.4 RESULTS

Body weights

Rats given a low fat-high fiber (caloric restricted) diet showed a lower growth rate than rats of the other groups (**Figure 5.2**). Mean body weight of the former group was substantially, but not statistically significant, lower at interim kill, and statistically significant lower ($P<0.01$) at final autopsy (**Table 5.3**). The reduced body weight gain in the caloric restricted group was accompanied by a significantly higher food intake ($P<0.001$, interim kill; $P<0.05$, final autopsy; **Table 5.4** and **Figure 5.2**). Mean body weights and mean food intake were neither influenced by azaserine treatment, nor by treatment with caerulein (**Table 5.3**).

Table 5.3 Body and organ weights of azaserine-treated and untreated rats at interim kill and final autopsy[a]

Postinitiation treatment group	N	Body wt (g)	Pancreatic wt (g)	Liver wt (g)	Relative pancreatic wt (g/kg BW)	Relative liver wt (g/kg BW)
			Interim kill			
Low fat	15	483.2 ± 9.8	1.14 ± 0.04	12.75 ± 0.60	2.36 ± 0.08	26.3 ± 0.9
Low fat + caerulein	15	494.4 ± 10.9	2.20**± 0.13	12.98 ± 0.55	4.45**± 0.25	26.2 ± 0.8
Caloric restriction	15	474.2 ± 9.5	1.29 ± 0.06	13.47 ± 0.45	2.70 ± 0.09	28.3 ± 0.5
No carcinogen	5	495.2 ± 28.1	1.24 ± 0.11	13.63 ± 1.66	2.56 ± 0.35	27.2 ± 2.3
			Final autopsy			
Low fat	19	571.8 ± 12.2	1.66 ± 0.16	16.57 ± 0.72	2.91 ± 0.27	28.9 ± 1.0
Low fat + caerulein	19	561.5 ± 10.2	3.76**± 0.26	16.20 ± 0.32	6.66**± 0.41	28.7 ± 0.3
Caloric restriction	19	518.9**± 8.6	1.40 ± 0.05	14.57* ± 0.50	2.72 ± 0.11	28.0 ± 0.6
No carcinogen	5	554.5 ± 27.8	1.32 ± 0.14	13.94 ± 1.56	2.43 ± 0.34	24.8* ± 1.9

[a]Values are means ± SEM.

Statistics: organ weights, analysis of variance plus Dunnett's tests (two-sided; $^{*}P<0.05$, $^{**}P<0.01$); body weights (BW), analysis of variance with initial body weight as covariable plus Dunnett's test (two sided; $^{**}P<0.01$).

Table 5.4 Food intake of azaserine-treated and untreated rats at interim kill and final autopsy[a]

Postinitiation treatment group	Interim kill		Final autopsy	
	No. of rats	Food intake (g)	No. of rats	Food intake (g)
Low fat	35	16.8 ± 0.2	20	17.8 ± 0.3
Low fat + caerulein	35	16.6 ± 0.2	20	18.1 ± 0.5
Caloric restriction	35	19.5*** ± 0.3	20	19.7* ± 0.9
No carcinogen	10	16.6 ± 0.7	5	17.2

[a]Values are means per cage (5 rats) ± SEM.
Statistics: analysis of variance plus L.S.D. tests (two-sided; * P<0.05; *** P<0.001); experimental unit: cage.

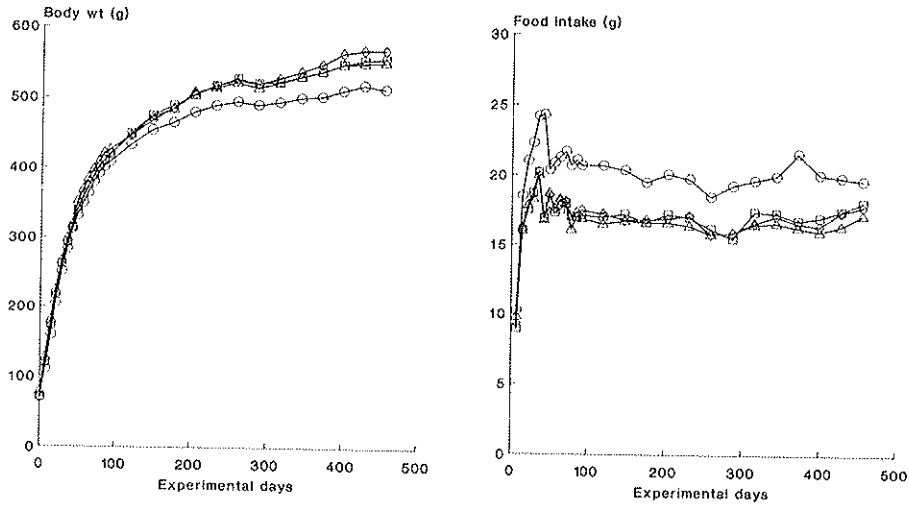

Figure 5.2 Body weight gain (individual means) and food intake (group means) of azaserine-treated rats maintained on the following post-initiation treatments; ◊, Low fat; □, low fat + caerulein; ○, caloric restriction, and Δ, non-carcinogen treated rats maintained on a low fat diet.

Organ weights

In caerulein-treated rats, mean absolute and relative pancreatic weights were significantly increased at interim and final autopsy ($P<0.01$, **Table 5.3**). Absolute, but not relative mean liver weight of the low fat-high fiber group was reduced significantly ($P<0.05$, **Table 5.3**) at final autopsy, but not at interim autopsy. Relative liver weight was significantly lower at final autopsy in rats not injected with carcinogen ($P<0.05$, **Table 5.3**).

Histology

Both the observed transection data of acidophilic atypical acinar cell foci and the calculated volumetric data of foci were significantly increased (**Table 5.5**) in the groups treated with the CCK-analogue caerulein, when compared to the azaserine-treated low fat group. Although all the observed and calculated data of foci were decreased due to the caloric restricted diet, the values never reached the $P<0.05$ level of statistical significance (**Table 5.5**). The nodules with a diameter over 1 mm showed the same phenomena (**Table 5.6**). Treatment with caerulein however, resulted in a significant increase in the number of these lesions. Moreover, caerulein treatment caused a significant increase in tumour incidence at final autopsy (**Table 5.6**; Pearson χ^2 test, $P=0.0184$).

Immunohistochemistry

Immunohistochemistry with the monoclonal antibody directed against TGF-α revealed a strong expression in the cytoplasm of ductular cells in normal rat pancreas, whereas acinar cells showed a weak expression of the TGF-α peptide in the cytoplasm (**Figure 5.3a**). Surprisingly, in azaserine-treated rat pancreas, normal acinar cells stained more intense than those of acidophilic atypical acinar cells nodules (AACN; **Figure 5.3b**), acinar adenomas, or carcinomas (*in situ*). Comparison of tissue sections from azaserine-treated with non-treated control rats revealed a slightly weaker TGF-α immunoreactivity in the non-carcinogen treated pancreata.

Because EGF peptide expression appeared to be very weak in acinar pancreatic cells, three different antibodies directed against EGF were tested. All antibodies showed the same staining pattern, with only some variation in 'background staining' of fat, blood vessels and fibrous material.

Table 5.5 Effects of six months postinitiation treatments on development of acidophilic atypical acinar cell foci induced in rat pancreas by azaserine[a]

Postinitiation treatment group	Observed transection data of foci		Calculated volumetric data of foci							
	Total no./cm^2	Transection area (mm^2)	no./cm^3 with mean diameter (μm)					Total no/cm^3	Mean diameter (μm)	Area as % of pancreas
			272.5	385	545	770	1090			
Low fat	5.21	0.094	203	54	5	< 1	< 1	312.6	319.2	0.49
Low fat + caerulein	61.56***	0.273***	416*	471***	364***	103***	113***	1558.7***	486.3***	16.78***
Caloric restriction	3.22	0.089	151	29	4	< 1	< 1	227.4	314.4	0.29

[a]Values are means; standard errors are taken into account in the statistical calculations, but are omitted from the table for reasons of clarity. Data are based on hematoxylin and eosin (H&E)-stained paraffin sections. The untreated controls showed no histological abnormalities in the pancreas.

Statistics: regression analysis; values with superscripts (*) are significantly different from the low fat group (*P<0.05, ***P<0.001).

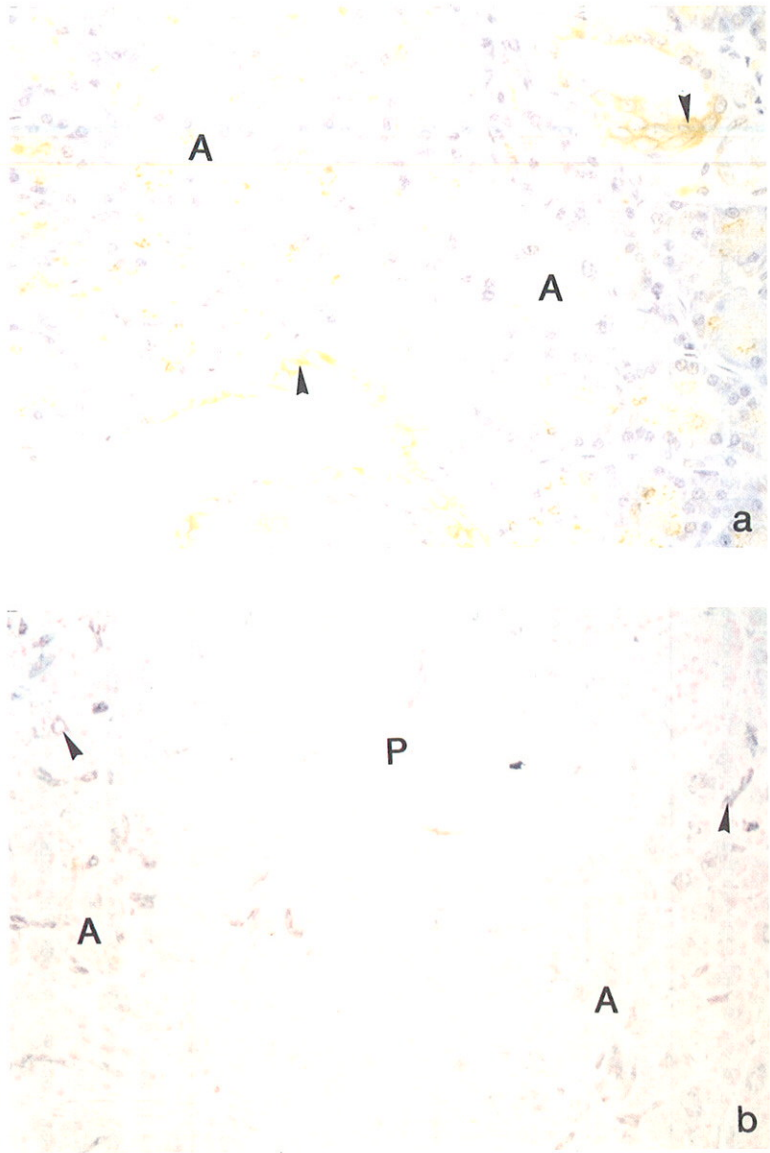

Figure 5.3 Immunohistochemical localization of TGF-α in rat pancreas. **a.** Weak expression in acinar cells (A), stronger expression in ductular cells. **b.** Weaker expression in putative preneoplastic atypical acinar cell nodule than in surrounding 'normal' acinar cells. A=normal pancreas acinar cells; P=putative preneoplastic atypical acinar cell nodule, arrowheads point to strong TGF-α expression in ductular cells.

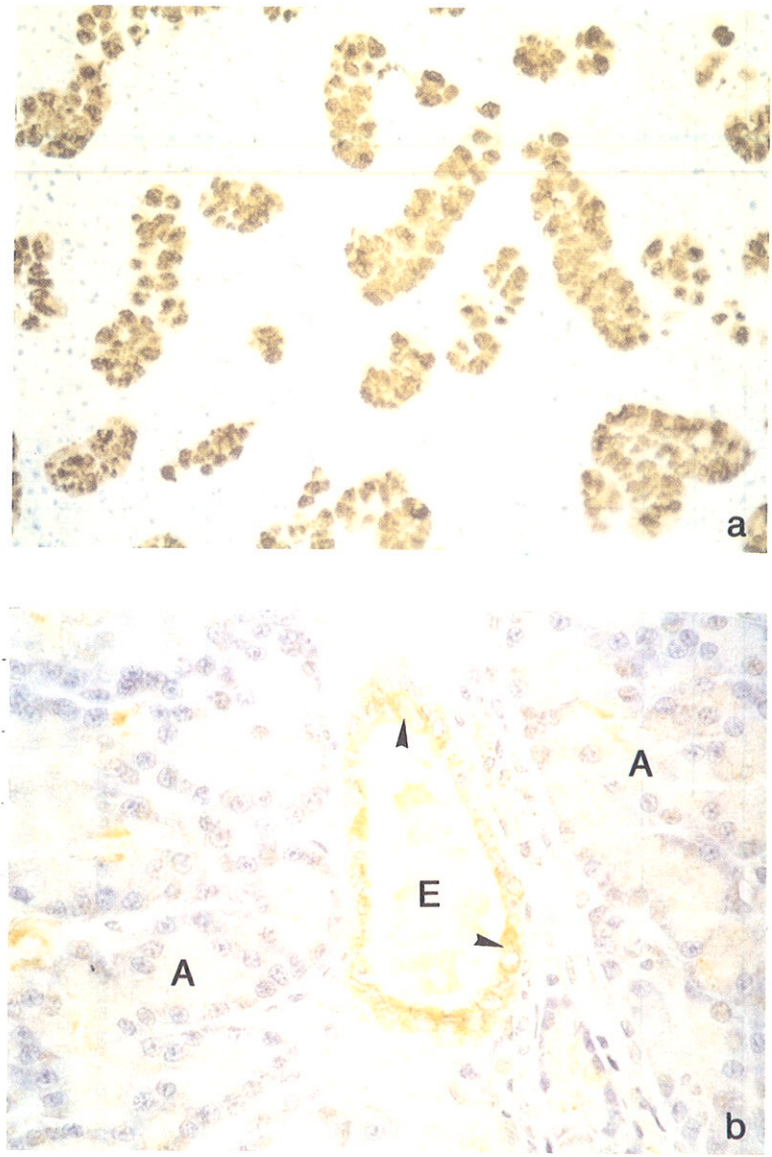

Figure 5.4 Immunohistochemical localization of EGF. **a.** in rat salivary gland. **b.** in rat pancreas. Arrowheads point to ductular cells; A=acinar cells; E=EGF immunoreactivity in ductal lumen.

Table 5.6 Number of pancreatic lesions in azaserine-treated rats after 6 or 15 months post-initiation treatments[a]

(Pre)neoplastic lesions observed	No. of lesions					
	Low fat		Low fat + caerulein		Caloric restriction	
Autopsy (I=interim, F=final)	I	F	I	F	I	F
No. of rats	15	19	15	19	15	16
No. of tumour-bearing animals (%)[b]	-	6 (32)	-	13 (68)[d]	-	4 (25)
AACN (Ø1-3 mm)[c]	1	15	90**	274***	2	3
Adenoma	0	3	3	50**	0	1
Carcinoma *in-situ*	0	5	0	25*	0	2
Adenocarcinoma	0	6	1	12	0	2
Total no. of carcinomas	0	11	1	37*	0	4

[a]Values are totals per group. Data are based on H&E-stained paraffin sections. The untreated controls showed no histological abnormalities in the pancreas based on H&E-stained sections. [b]Tumour-bearing animal, animal that bears one or more carcinoma (*in situ*) in the pancreas at final autopsy. [c]AACN, Atypical Acinar Cell Nodule.

Statistics: no. of tumour-bearing animals, χ^2-test, [d]P=0.0184; no. of lesions, 2-sample *t* test, values with superscripts (*) are significantly different from the low fat group (*P<0.05, **P<0.01, ***P<0.001).

Moreover, all EGF antibodies demonstrated strong immunoreactivity towards the EGF producing cells in rat salivary gland (**Figure 5.4a**). More pronounced EGF peptide staining was observed in the cytoplasm of ductular cells when compared to the acinar cells (**Figure 5.4b**). Moreover, EGF immunoreactivity was detected in the lumen of large intralobular ducts (**Figure 5.4b**). In contrast to TGF-α, no differences in EGF expression were observed when comparing normal and (pre)neoplastic acinar pancreatic cells. Comparison of tissue sections from azaserine-treated with non-treated rats demonstrated a slightly stronger EGF immunoreactivity in non-carcinogen treated pancreata.

Semi-quantitative PCR

EGF and TGF-α mRNA levels were detected using a semi-quantitative PCR technique. Specificity of the PCR products was demonstrated by comparing the products with the molecular size marker phage φX-DNA digested with HaeIII (TGF-α, 265 bp; EGF 135 bp, **Figure 5.1**), and by hybridization with a radiolabeled specific internal oligoprobe, which revealed specific bands on the autoradiogram (**Figure 5.5**).

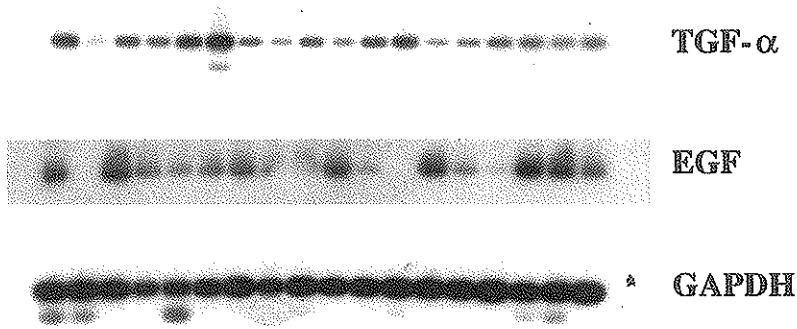

Figure 5.5 Southern blotting to check specificity of the TGF-α, EGF and GAPDH PCR-products. Hybridizations were performed with specific [^{32}P]-labelled oligoprobes.

EGF mRNA expression was significantly decreased by azaserine treatment, at final autopsy ($P<0.05$; **Table 5.7**), but not at interim kill. Comparison of the caerulein-treated

group or the low fat-high fiber group with the azaserine-treated low-fat group did not elucidate any statistically significant differences in EGF mRNA expression, either at interim kill or at final autopsy (**Table 5.7**).

Comparison of the azaserine-treated low-fat group with the saline-treated low-fat group revealed a substantially, but statistically not significantly, increased TGF-α mRNA expression due to carcinogen treatment at final autopsy. Moreover, azaserine-treated rats either fed a low fat-high fiber diet or injected with caerulein showed a significantly higher TGF-α mRNA level ($P<0.05$, **Table 5.7**) than control rats. Furthermore, comparing all azaserine-treated animals (relative TGF-α mRNA level = 70.91 ± 9.85 (mean ± SEM)), with the control rats (relative TGF-α mRNA level = 23.35 ± 5.53) revealed a significant increase ($P<0.001$) of TGF-α mRNA level in the carcinogen-treated group at final autopsy. At interim kill, azaserine-treated rats injected with caerulein showed a significantly lower TGF-α mRNA expression ($P<0.05$) than carcinogen-treated rats maintained on a low fat diet.

Relative EGF and TGF-α mRNA levels in grossly visible tumours (acinar adenocarcinomas) isolated at final autopsy, were also determined by semi-quantitative PCR. The mean relative EGF mRNA level in the tumours (131.61 ± 8.90) was significantly higher ($P<0.05$) than the mean EGF mRNA level in total pancreas homogenates collected from carcinogen-treated rats (81.63 ± 11.67). No significant difference was observed between the EGF mRNA level in tumours and the EGF mRNA level in total pancreas homogenates collected from non-carcinogen rats at final autopsy (114.3 ± 15.4). The mean TGF-α mRNA level in tumours (23.49 ± 3.18) was significantly lower ($P<0.001$) than the mean TGF-α mRNA level in total pancreas homogenates collected from azaserine-injected rats (70.91 ± 9.86). Also, no significant difference was observed between the TGF-α mRNA level detected in isolated tumours, when compared with the TGF-α mRNA level detected in non-carcinogen rats at final autopsy (23.3 ± 3.2).

Finally, the number of preneoplastic- and neoplastic lesions of the individual rats were compared with their individual growth factor expression levels as determined by PCR; no correlation was observed, either at interim kill, or at final autopsy data (not shown).

Table 5.7 EGF and TGF-α mRNA expression in azaserine-treated and untreated rats[a]

Postinitiation treatment group	Interim kill		Final autopsy	
	EGF	TGF-α	EGF	TGF-α
Low fat	169.8 ± 43.7	36.0 ± 2.8[1]	46.8 ± 15.3[2]	77.1 ± 23.5
Low fat + caerulein	117.0 ± 14.9	22.4 ± 3.3[1]	105.8 ± 18.9	75.5 ± 10.9[3]
Caloric restriction	150.2 ± 14.1	51.5 ± 16.7	92.3 ± 18.9	60.6 ± 11.4[4]
No carcinogen	99.6 ± 27.3	34.2 ± 8.1	114.3 ± 15.4[2]	23.3 ± 3.2[3,4]

[a]Values are mean optical densities obtained by laser-densitometry as percentages of GAPDH, representing the relative amounts of EGF- or TGF-α mRNA expressed ± standard error of mean (SEM).

Statistics; 2-sample *t*-test, [1,2,3,4]values with identical superscripts are significantly different, $P<0.05$.

5.5 DISCUSSION

In general, growth factors are defined as polypeptides regulating cell proliferation and/or differentiation by binding to a specific cell-membrane receptor. In contrast to the endocrine activity of hormones, they mostly act in a paracrine or autocrine way. Loss of requirement for multiple specific growth factors is a common finding in many types of cancer cells (28, 29). In the present long-term study, EGF and TGF-α expression was detected in normal rat pancreas and in the pancreas of rats injected with azaserine. In the exocrine pancreas the strongest EGF and TGF-α peptide signals were found in ductular cells, whereas acinar cells expressed the peptides only at a low level. Microscopically we demonstrated a lower level of TGF-α peptide in preneoplastic and neoplastic acinar cell lesions, induced in rat pancreas by azaserine, than in the surrounding 'normal' acinar cells. The apparently reduced TGF-α peptide levels in (pre)neoplastic lesions can be explained by an increased expression of TGF-α in the surrounding 'normal' acinar cells, because the TGF-α immunoreactivity appeared to be stronger in normal acinar cells in azaserine-treated rat pancreas than in acinar cells of non-carcinogen treated rat pancreas. EGF peptide levels were not significantly changed in (pre)neoplastic acinar cell lesions compared to the surrounding normal acinar cells, although there was stronger EGF immunoreactivity observed in pancreata from non-carcinogen treated rats.

EGF and TGF-α mRNA levels determined by PCR in the pancreata of azaserine-treated rats at 15 months, demonstrated a significant decrease in EGF and a significant increase in TGF-α expression, when compared to the non-carcinogen treated rats. At 6 months, however, no significant differences were observed between these groups. An explanation for this phenomenon might be that at interim kill the rats were still growing. It has been described that growth factor expression alters during ageing (30), which may have interfered with our comparison between both groups. Both TGF-α and EGF mRNA levels in isolated pancreatic acinar adenocarcinomas from azaserine-treated rats did not differ significantly from the levels in the pancreata of non-carcinogen treated animals.

Azaserine treatment results in a reduction of EGF mRNA and an increase of TGF-α mRNA, which becomes detectable only after 15 months. Furthermore, the lower EGF and higher TGF-α mRNA levels were not detected in acinar adenocarcinomas, and TGF-α peptide level was increased only in acinar cells with normal morphology. Therefore, it can be concluded that the changes took place early in the carcinogenic process, in these still normal, acinar cells. TGF-α levels increased, whereas EGF (mRNA) level decreased due to carcinogen treatment. Because these growth factors are acting via binding the same receptor (the EGFR), these results might suggest that TGF-α

replaces the action of EGF in the initiation phase of pancreatic acinar carcinogenesis in the rat.

In the present study we also examined the role that EGF or TGF-α might play during post-initiation modulation of pancreatic carcinogenesis. Therefore, we either enhanced or inhibited the carcinogenic process by caerulein (a cholecystokinin analogue) injections or a low fat-high fiber (caloric restricted) diet, respectively. Caerulein caused a significant increase in the number of both putative preneoplastic atypical acinar cell nodules (AACN) and acinar adenocarcinomas, indicating a stimulation of the carcinogenic process. Previously, Meijers *et al.* (31) reported an enhancing effect of cholecystokinin (CCK) on the development and growth of AACN, but not on the development of acinar adenocarcinomas in azaserine-treated rats. Other authors also reported (32,33) a stimulatory action of CCK on the development of putative preneoplastic acinar pancreatic lesions in rats, but they did not report effects of CCK on the development of acinar adenocarcinomas. However, Appel *et al.* (34) reported an increased number of acinar adenocarcinomas in rats treated with CCK, and high affinity CCK receptors were detected on rat pancreatic adenocarcinoma cells (35,36). The present findings support the latter findings and suggest that CCK stimulates the development of both putative preneoplastic and neoplastic lesions induced in rat pancreas by azaserine. Decrease in energy intake (37) or increase in energy expenditure can inhibit cancer development (38-40). In the present study, pancreatic carcinogenesis was only slightly inhibited by the low fat-high fiber (caloric restricted) diet. This rather weak effect of caloric restriction can be explained by the significantly higher food intake of the rats maintained on this diet. However, the rats still showed reduced body, liver and pancreatic weights at final autopsy. Moreover, the low fat-high fiber diet inhibited pancreatic carcinogenesis as demonstrated by a decrease in the number of (pre)neoplastic lesions present in the animals of this group. The caloric restriction, however, was very moderate. Therefore, it can be concluded that these inhibitory effects were caused mainly by the significantly higher cellulose (fiber) intake of the rats maintained on the low fat-high fiber diet, when compared to rats maintained on the normal low fat diet. This conclusion is supported by numerous studies reporting inhibition of carcinogenesis by high fiber diets (41,42).

Comparison of the effects of postinitiation treatments on the number of (pre)neoplastic pancreatic lesions and on EGF and TGF-α mRNA expression data determined by semi-quantitative PCR did not demonstrate any correlation. The caerulein-stimulated group, for example, showed significantly more pancreatic lesions

than the azaserine-treated control group, whereas EGF or TGF-α mRNA levels were not significantly different. Therefore, it can be concluded that the effects of azaserine-treatment on the EGF and TGF-α mRNA expression levels determined by PCR in total pancreas homogenates, were not caused by differences in EGF or TGF-α mRNA levels in the putative preneoplastic atypical acinar cell lesions. Moreover, effects on EGF or TGF-α mRNA expression detected between the various post-initiation treatment groups were not convincing. The few differences detectable at interim kill, were not detectable anymore at final autopsy. No differences in EGF or TGF-α expression in the various post-initiation treatment groups were detected by immunohistochemistry experiments.

Based on the aforementioned observations it seems justified to conclude that although the post-initiation treatments modulate pancreatic carcinogenesis, the EGF and TGF-α levels were not influenced. Moreover, the observation that TGF-α peptide levels had decreased in putative (pre)neoplastic acinar cell lesions, and the observation that EGF and TGF-α mRNA levels in tumours were comparable with the levels in homogenates of non-carcinogen treated rat pancreata, suggests that AACN may develop into acinar adenocarcinomas independently of EGF and TGF-α. These conclusions are supported by our finding, that (pre)neoplastic acinar cell lesions induced in rat pancreas by azaserine showed a decrease in EGFR expression (Chapter four of this thesis). A disturbance in the equilibrium between these growth factors, however, may have played a role in the initiation of AACN since pancreata of azaserine-treated rats showed increased TGF-α and decreased EGF mRNA levels in comparison with pancreata of non-carcinogen treated rats. Therefore, it can be concluded that although EGF and TGF-α may be involved in the induction of focal hyperplasia, the development of these putative preneoplastic atypical acinar cell lesions into acinar adenocarcinomas almost certainly involves other, (independent) molecular perturbations.

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CHAPTER SIX

OVEREXPRESSION OF TRANSFORMING GROWTH FACTOR-α AND EPIDERMAL GROWTH FACTOR RECEPTOR, BUT NOT EPIDERMAL GROWTH FACTOR IN EXOCRINE PANCREATIC TUMOURS IN HAMSTERS

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6.1 ABSTRACT

Using immunohistochemistry, Northern blotting and a semi-quantitative PCR technique, epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and epidermal growth factor receptor (EGFR) expression were studied in the pancreas of N-nitrosobis(2-oxopropyl)amine (BOP)-treated hamsters. After initiation, pancreatic carcinogenesis was modulated by a high fat diet, a low fat/high fibre (caloric restricted) diet, or injections with the cholecystokinin-analogue caerulein. Autopsies were performed six and twelve months after the last injection with BOP.

Immunohistochemistry revealed a weak expression of TGF-α in normal acinar cells, and a stronger expression in ductular and centro-acinar cells. Overexpression of TGF-α was observed in advanced putative preneoplastic lesions (classified as borderline lesions) and in ductular adenocarcinomas. EGFR immunoreactivity was present only in ductular adenocarcinomas. EGF peptide expression was observed both in acinar and ductular normal and tumour cells and the level of expression did not change significantly during carcinogenesis. Moreover, the post-initiation treatments did not cause differences in EGF, TGF-α or EGFR peptide or mRNA levels, except for a significantly lower expression of TGF-α mRNA in hamsters fed a high fat diet when compared to those fed a low fat diet. TGF-α mRNA levels had increased, whereas EGF mRNA levels had decreased significantly in total pancreatic homogenates of BOP-treated hamsters in comparison with untreated controls. Also in ductular adenocarcinomas TGF-α and EGFR (but not EGF) mRNA levels were significantly higher than in normal pancreatic homogenates. In pancreatic homogenates obtained six months after the last BOP injection, these differences were less pronounced in comparison with those obtained after 12 months.

It is concluded that overexpression of TGF-α (but not EGF) might have a paracrine or autocrine stimulatory effect on pancreatic tumour development in BOP-treated hamsters via simultaneously overexpressed EGFR. However, TGF-α, EGF and EGFR do not seem to be involved in the modulating effects of a high fat diet or caerulein treatments on pancreatic carcinogenesis in BOP-treated hamsters.

6.2 INTRODUCTION

Exocrine pancreatic cancer is associated with a poor prognosis because of the propensity to metastasize by the time the diagnosis is established (1). This feature makes it the fourth or fifth leading cause of death due to cancer in Western society. Most molecular biological studies dealing with the process of pancreatic carcinogenesis, concentrated on the detection of abnormally expressed genes in these tumours. These investigations revealed the presence of mutations in the p53 tumour suppressor gene (2,3), in K-ras (3,4) and in MTS1, the gene coding for the p16 inhibitor of cyclinD/Cdk-4 complexes (5) in human pancreatic tumours. Mutations in K-ras were also found in early lesions and ductular adenocarcinomas in a hamster model for pancreatic carcinogenesis (6).

A number of growth factors has also been described to play a role in the promotion of abnormal pancreatic growth. Simultaneous overexpression of the transforming growth factor-α (TGF-α), the epidermal growth factor (EGF) and their receptor (the epidermal growth factor receptor; EGFR, c-erbB, HER-1), in human pancreatic adenocarcinomas suggested an autocrine or paracrine growth stimulatory mechanism (7). These stimulatory processes were also suggested after the finding of concomitant overexpression of the hepatocyte growth factor (HGF) and its receptor MET in human pancreatic tumours (8). Overexpression of other growth factor receptors such as c-erbB2 (HER-2, neu), c-erbB3 (HER-3) (9), transforming growth factor-ß2 (TGF-ß2 Type II) (10), and the fibroblast growth factor type I (11) has also been reported in human pancreatic tumours.

Apart from these 'genetic' changes, environmental factors have been reported to be involved in pancreatic carcinogenesis. Although a single factor could not be identified to cause pancreatic cancer, the male gender (12), smoking (13), and high fat-high protein diets (14) have been correlated with pancreatic cancer prevalence. In animal models, pancreatic carcinogenesis could be enhanced by dietary factors such as fat (15-18), or hormonal factors such as the gut hormone cholecystokinin (CCK) (19,20). The mechanisms by which fat or CCK modulates pancreatic cancer have not yet been elucidated.

It can be argued that these factors may exert their effects on pancreatic cancer development by modulating the expression of growth factor(receptor)s. To investigate this hypothesis, a long-term experiment (12 months) was performed in the well described *N*-nitrosobis(2-oxopropyl)amine (BOP)-hamster model for ductular pancreatic carcinogenesis (21).

Previously we performed a similar study in azaserine-treated rats, another well-

known model for acinar pancreatic carcinogenesis (22). In that study pancreatic carcinogenesis was stimulated by a high fat diet and by the cholecystokinin-analogue caerulein. Some investigators reported no or inhibitory effects of CCK/caerulein on the development of ductular pancreatic lesions induced in hamsters by BOP (23,24), while in contrast, other investigators reported stimulation of the carcinogenic process (25). We included a caerulein-stimulated group in the present hamster experiment, in order to compare the results of this study with those previously obtained with the rat study. Furthermore, pancreatic carcinogenesis was enhanced by a high fat diet, whereas another group of hamsters was maintained on a caloric-restricted/high fibre diet in order to inhibit tumour development. At autopsies after six and twelve months, expression of the epidermal growth factor and transforming growth factor-α was investigated in the pancreas, using both semi-quantitative PCR, and immuno-histochemistry. In the same tissues, expression of the EGFR was investigated by Northern blotting and immunohistochemistry.

6.3 MATERIALS AND METHODS

Tumour induction

To initiate pancreatic carcinogenesis, 140 Syrian golden hamsters (obtained from Harlan-CPB, Austerlitz, The Netherlands) were injected s.c. at 5, 6 and 7 weeks of age with 20 mg *N*-nitrosobis(2-oxopropyl)amine (BOP; Ash Stevens, Detroit, MI, USA) per kg body weight according to an injection protocol described previously (18). Twenty extra hamsters, injected with saline instead of BOP, served as non-carcinogen controls (Group E, see **Tables 6.1** & **6.2**). The 140 animals were equally divided in four groups and treated as follows: group A: low fat diet; 5% corn oil, group B: low fat diet with s.c. injections of 2.5 μg caerulein/kg body weight three times a week, group C: high fat diet; 20% corn oil, and group D: 20% caloric restriction/high fibre diet. The composition of the diets is described in **Table 6.1**.

All animals were housed in similar standard conditions (five animals per cage). Body weights and food intake were recorded weekly during the first three months and once a month during the rest of the experiment. The general condition and behaviour of the animals were checked daily. The animals were killed and investigated at six months and at 12 months after the last BOP injection. The animals were anaesthetized with ether, exsanguinated by cannulating the abdominal aorta, and then examined for gross pathological changes. The entire pancreas and all gross lesions suspected of being tumourous were excised. Grossly visible pancreatic tumours were separated from normal

pancreas. One part of all pancreatic tissues was routinely fixed in 4% buffered formalin and subsequently embedded in paraffin wax to cut tissue sections, and the adjacent part was snap frozen in liquid nitrogen to be used for molecular biological techniques.

Table 6.1 Weight percentage composition of the AIN76-based diets[a]

Group	Postinitiation treatment group	No. of hamsters	Body wt (g)
	Groups A, B, E Low fat	Group C High fat	Group D 20% Caloric restriction
Casein	20.0	25.0	20.0
DL-Methionine	0.3	0.35	0.3
Wheat starch	63.5	40.79	44.49
Cellulose	5.0	6.62	25.25
Choline bitartrate	0.2	0.23	0.16
AIN-76-AM minerals	3.5	4.09	2.8
AIN-76-AM vitamins	1.0	1.17	0.8
CaH_2PO_4	1.5	1.75	1.2
Corn oil	5.0	20.0	5.0
Total	100.0	100.0	100.0
Calories (MJ/kg)	16.2	16.7	12.6

[a]The diets were prepared freshly every 2 months. All diets were stored at -20°C until use.

Histology

At least three formalin-fixed, paraffin-embedded tissue sections, cut at different levels, were stained routinely with haematoxylin and eosin (H&E) and examined by light microscopy. Pancreatic lesions were classified as borderline lesions, carcinomas *in-situ*, or ductular adenocarcinomas, according to the criteria of Meijers *et al.* (24) and Pour and Wilson (26). In the remaining parallel sections TGF-α, EGF and EGFR peptides were detected by immunohistochemistry. TGF-α was detected with a mouse anti-rat monoclonal antibody (dilution 1:15, AB-2, Oncogene Science, Uniondale, N.Y.). To detect EGF we performed incubations with four different polyclonal antibodies, a

rabbit-anti-mouse polyclonal found to be immunoreactive in hamster ovaries (a generous gift of Dr. Shyamal K. Roy, University of Nebraska Medical Center, Omaha, Nebraska) (27), a rabbit anti-rat (a generous gift from Ryoichi Oyasu, Northwestern University Medical School, Chicago, IL) (28); a rabbit anti-recombinant human EGF (Ab-3, Oncogene Science); and a rabbit anti-rat (Biomedical Technologies Inc., Stoughton, MA). Specificity was checked on salivary glands and all antibodies proved to be immunoreactive (29). Two monoclonal anti-mouse EGF antibodies (MON8001 and MON8002, Monosan, Uden, The Netherlands) did not show immunoreactivity with hamster tissues. EGFR antibodies were a generous gift from Dr. W.A. Dunn, University of Florida, or were obtained from SIGMA (St. Louis, MO; clone 29.1). EGF and TGF-α peptides were detected using an antigen retrieval method described previously (22). EGFR peptides were detected using a triton-pretreatment method as described previously (30).

RNA isolation

Based on the results of the microscopical examinations, the frozen tissues were divided into normal, preneoplastic and tumour tissue. Total RNA from pancreas homogenates and from separated ductular adenocarcinomas was isolated using the guanidium-isothiocyanate/acid phenol-chloroform isolation method, described in detail previously (22,30). From the hamster pancreas it appears impossible to collect putative preneoplastic lesions separately. Therefore, preneoplastic pancreas actually is a homogenate of both normal pancreatic tissue and preneoplastic lesions containing pancreatic tissue.

Northern Blotting

EGFR mRNA levels were detected in pancreatic homogenates by Northern blotting as described in detail previously (30). Shortly, a hamster EGFR cDNA fragment was PCR-cloned into a pGEM7ZF vector (Promega) and authenticity was controlled by nucleotide-sequencing. Hybridisations were performed with riboprobes, synthesized and labelled with ^{32}P by *in-vitro* transcription. Ribosomal 7S mRNA was detected on the same blots to control for RNA loading, using a ^{32}P-labelled cDNA probe. Blots were washed under high-stringency conditions and EGFR autoradiography signals were detected using a phosphorimager (Molecular Dynamics, Sunnyvale, CA) and subsequently quantified using the software program ImageQuant (Molecular Dynamics).

Semi-Quantitative Polymerase Chain Reaction (PCR)

Because Northern blotting did not appear to be sensitive enough to detect EGF and

TGF-α mRNA in pancreatic homogenates, a semi-quantitative PCR was performed as described in detail previously (22). Shortly, the cDNA sample from one animal, synthesized using oligo-dT primers, was amplified in diploid with both EGF, TGF-α and GAPDH primers. Specificity of the PCR-products was checked by hybridisation with radioactive labelled internal oligoprobes and by nucleotide sequencing. The amount of the PCR products was determined using a laser densitometer (Molecular Dynamics, Sunnyvale, CA). Quantification of the PCR products was achieved by calculating the relative amounts compared to the expression of the housekeeping gene GAPDH.

Statistics

Body weight data were statistically evaluated by analysis of variance with initial body weight as co-variable, followed by Dunnett's tests (two-sided). Pancreatic weight data were statistically evaluated by analysis of variance followed by two-sided Dunnett's tests. Food intake data were statistically evaluated by a Wilcoxon matched pairs test. The number of pancreatic lesions was evaluated by a 2-sample Student's *t*-test. The Northern blotting and semi-quantitative PCR data were statistically evaluated by the Wilcoxon/Mann Whitney U-test. The incidence of tumours (the number of tumour-bearing animals) was evaluated by Pearson χ^2-test.

6.4 RESULTS

Body and pancreatic weights

Body weights were significantly ($P<0.05$) higher at 12 months in the high fat group when compared with the other groups. Pancreatic weights, however, were not significantly influenced (**Table 6.2**). The cholecystokinin analogue caerulein caused a substantial increase in absolute pancreatic weights, but when using the analysis of variance, these effects did not reach the $P<0.05$ level of statistical significance. On the other hand, a high fibre/low fat (caloric restricted) diet caused a substantial, but also not statistically significant decrease in absolute pancreatic weight.

Food intake

Mean daily food intake is presented in **Figure 6.1**. Food intake was not significantly influenced by injecting BOP-treated hamsters with caerulein. However, BOP-treated hamsters given a high fat diet demonstrated a significantly ($P<0.001$) reduced food intake when compared with the BOP-treated hamsters given a low fat diet. Moreover, BOP-treated hamsters given a caloric restricted diet demonstrated

significantly ($P<0.001$) increased food intake when compared to BOP-treated hamsters given a low fat diet. Comparing BOP-treated hamsters given a low fat diet, with saline-injected hamsters given a low fat diet, did not reveal differences in food intake.

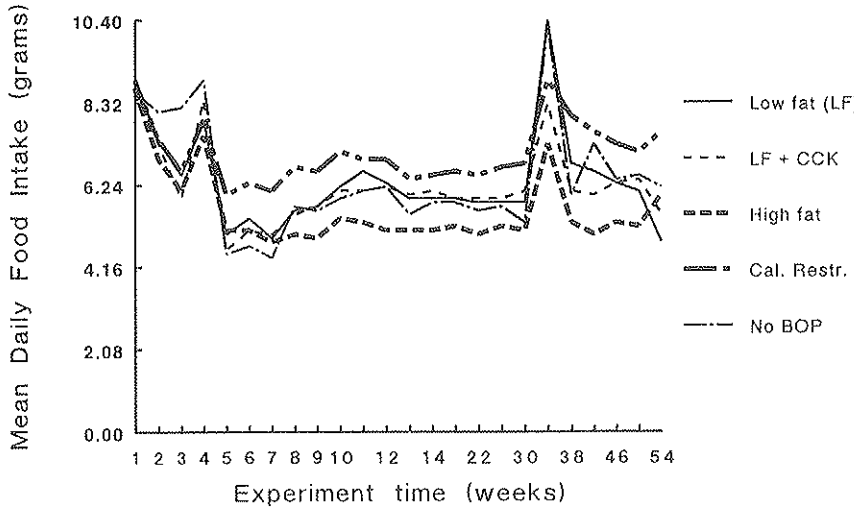


Figure 6.1 Food intake (group means) of BOP-treated hamsters maintained on various treatments.

Histology

The number of pancreatic lesions present in hamsters after six months and after 12 months is summarized in **Table 6.3**. Hamsters treated with caerulein demonstrated a significant ($P<0.05$) increase in number of borderline lesions (BLL) after six, but not after twelve months. BOP-treated hamster maintained on a high fat diet demonstrated a significantly ($P<0.01$) increased number of BLL at six months, but not at 12 months, when compared to hamsters fed a low fat diet. The caloric restricted diet did not have an effect on the number of pancreatic lesions, either at six or at 12 months. The number of ductular adenocarcinomas and carcinomas *in-situ* was higher in the groups treated either with caerulein or maintained on a high fat diet, when compared to hamsters given a low fat diet, but the numbers were too low to allow statistical analyses. Hamsters injected with saline instead of BOP did not show any pancreatic abnormalities.

Table 6.2 Body and pancreatic weights of BOP-treated and untreated hamsters at 6 and 12 months[a]

Group	Postinitiation treatment group	No. of hamsters	Body wt (g)	Pancreatic wt (g)	Relative pancreatic wt (g/kg BW)
6 months					
A	Low fat	12	127.3 ± 5.9	0.323 ± 0.022	2.55 ± 0.16
B	Low fat + caerulein	14	127.6 ± 3.4	0.384 ± 0.029	3.04 ± 0.24
C	High fat	10	133.0 ± 6.4	0.341 ± 0.050	2.57 ± 0.32
D	Caloric restriction	14	140.3 ± 5.1	0.282 ± 0.017	2.02 ± 0.11
E	No carcinogen	9	136.4 ± 8.0	0.316 ± 0.031	2.33 ± 0.18
12 months					
A	Low fat	10	153.1 ± 3.9	0.447 ± 0.064	2.93 ± 0.42
B	Low fat + caerulein	9	161.6 ± 7.1	0.573 ± 0.083	3.06 ± 0.31
C	High fat	10	175.0 ± 4.2*	0.384 ± 0.036	2.22 ± 0.24
D	Caloric restriction	11	162.5 ± 7.1	0.389 ± 0.039	2.43 ± 0.27
E	No carcinogen	6	145.4 ± 4.1	0.438 ± 0.040	3.03 ± 0.31

[a]Values are means ± SEM.

Statistics: organ weights, analysis of variance plus Dunnett's tests (two-sided); body weights (BW), analysis of variance with initial body weight as covariable plus Dunnett's test (two sided, and compared to low fat group; *$P<0.05$).

Table 6.3 Number of pancreatic lesions in BOP-treated hamsters after 6 or 12 months post-initiation treatments[a]

(Pre)neoplastic lesions observed	No. of lesions							
	Low fat		Low fat + caerulein		High fat		Caloric restriction	
	Interim	Final	Interim	Final	Interim	Final	Interim	Final
No. of hamsters	12	10	14	9	10	10	14	10
No. of tumour-bearing animals (%)[b]	8 (67)	8 (80)	12 (86)	9 (100)	10 (100)	10 (100)	12 (86)	10 (100)
Borderline lesion	15	23	38*	33	26**	30	25	24
Carcinoma *in-situ*	0	0	1	2	1	2	0	0
Ductular adenocarcinoma	1	0	0	1	1	1	0	1
Total no. of carcinomas	1	0	1	3	2	3	0	1

[a]Values are totals per group. Data are based on H&E-stained paraffin sections. The untreated controls (n=9 at interim kill; n=6 at final autopsy) showed no histological abnormalities in the pancreas based on H&E-stained sections. [b]Tumour-bearing animal, animal that bears one or more carcinoma (*in-situ*) in the pancreas at final autopsy.

Statistics: no. of lesions, 2-sample Student's *t*-test, values with asterisks are significantly different from the low fat group (*$P<0.05$, **$P<0.01$).

Table 6.4 EGF and TGF-α mRNA expression in pancreatic tissues of BOP-treated and untreated hamsters[a]

Post-initiation treatment group		EGF		TGF-α	
		Interim	Final	Interim	Final
A	Low fat	42.7 ± 11.5	28.8 ± 6.6	13.2 ± 4.2	40.7 ± 3.6[1]
B	Low fat + caerulein	38.3 ± 3.5	45.0 ± 11.9	22.2 ± 7.9	52.0 ± 13.7
C	High fat	61.7 ± 6.2	25.9 ± 5.0	14.1 ± 1.9	25.3 ± 3.6[1]
D	Caloric restriction	28.3 ± 4.0	43.8 ± 11.0	12.9 ± 5.4	80.6 ± 26.3
E	No carcinogen	57.1 ± 7.4*	169.9 ± 51.5***	10.2 ± 1.5	5.0 ± 1.7[2,***]
A,B,C,D	Adenocarcinoma	ND	88.0 ± 8.2	ND	20.7 ± 8.5[2]

[a]Values are mean optical densities obtained by laser-densitometry as percentages of GAPDH, representing the relative amounts of EGF- or TGF-α mRNA expressed ± standard error of mean (SEM). ND, not done.
Statistics; Wilcoxon/Mann-Whitney U-test, [1,2]values with identical superscripts are significantly different, $P<0.05$; saline-treated hamsters are significantly different from BOP-treated hamsters (taken together as one group) *$P<0.05$; ***$P<0.001$.

Immunohistochemistry

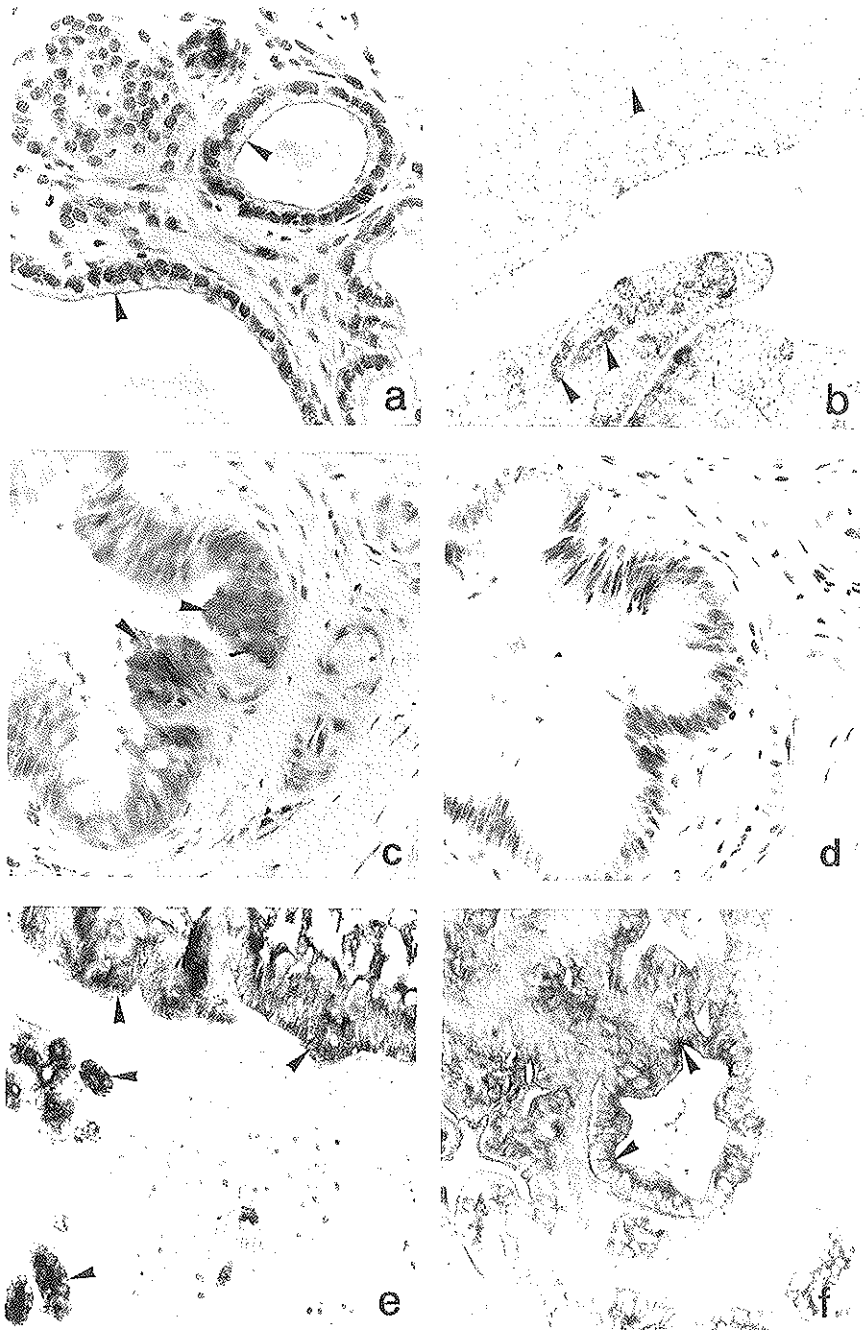
Immunohistochemistry, using the various antibodies directed against EGF did not reveal distinct EGF expression patterns. Acinar, ductular and endocrine cells stained faintly and equally weak for EGF, but a slightly stronger signal was observed at the apical site of the ductular cells (**Figure 6.2a**). Using the same incubation conditions, specific EGF staining was determined in the ductular cells of hamster salivary glands as a control (**Figure 6.2b**). Comparison of pancreata from BOP-treated hamsters with pancreata from saline-treated hamsters did not reveal significant differences in EGF staining patterns or intensities.

Using an anti-rat monoclonal antibody directed against TGF-α (AB-1, Oncogene Science), weak staining was found in acinar cells and a slightly stronger staining in ductular and centro-acinar cells of normal hamster pancreas. Significantly stronger TGF-α staining was observed in the ductular cells of borderline lesions and tubular ductular complexes, whereas only weak staining was observed in lesions that are thought to have a low potency to develop to ductular adenocarcinomas, such as cystic and intermediate ductular complexes (30). Ductular adenocarcinomas demonstrated strong TGF-α immunoreactivity (**Figures 6.2c & 6.2d**).

The two antibodies directed against EGFR demonstrated identical staining patterns. In normal hamster pancreas, strong cytoplasmic, apical and lateral staining for EGFR was only found in ducts with high epithelium, but not in ducts with low epithelium, or in acinar cells (**Figure 6.2e**). No staining was found for EGFR in putative preneoplastic tubular ductular complexes. However, ductular adenocarcinomas exhibited a strong EGFR immunoreactivity (**Figure 6.2f**).

On page 123:

Figure 6.2 Arrowheads point to immunohistochemical staining in BOP-treated hamsters. DAB-staining, haematoxylin nuclear counterstaining. **a.** apical EGF immunoreactivity in ductular cells of hamster pancreas; **b.** EGF immunoreactivity in ductular cells of hamster salivary glands; **c.** TGF-α immunoreactivity in ductular cells of a hamster pancreas adenocarcinoma; **d.** negative control of c.; **e.** EGFR immunoreactivity in the ductular cells of normal hamster pancreas (not treated with BOP); **f.** EGFR immunoreactivity in ductular cells of a hamster pancreas adenocarcinoma.


a
b
c
d
e
f

No differences in EGF, TGF-α or EGFR immunoreactivity (localization and intensity) were observed amongst the various post-initiation treatment groups.

Strong TGF-α, but not EGF or EGFR, immunoreactivity was observed in peripheral endocrine cells of the islets of Langerhans (**Figure 6.3**). Double staining experiments revealed that the localization of these TGF-α positive cells correlates partly with the localization of glucagon or somatostatin positive cells. Glucagon and somatostatin producing cells were also localized in the islet of Langerhans periphery, but double staining for TGF-α and glucagon or somatostatin only occurred in about 5% of the TGF-α positive cells. TGF-α staining could not be blocked by pre-incubation with antibodies directed against glucagon, somatostatin, or insulin. Moreover, in these endocrine cells no differences in cell staining intensities were observed between pancreata of normal and BOP-treated hamsters.

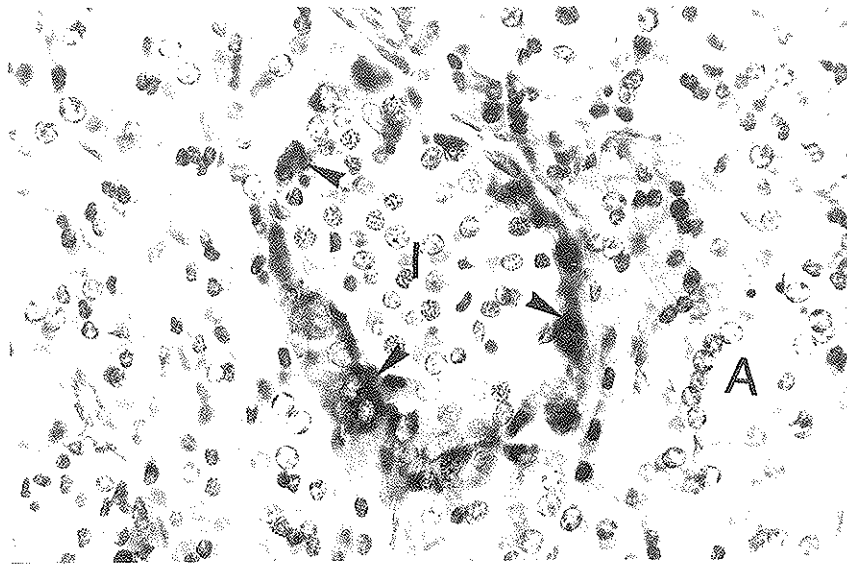


Figure 6.3 TGF-α immunoreactivity (arrowheads) in peripheral cells of an islet of Langerhans in the pancreas of a Syrian golden hamster. I, islet of Langerhans; A, acinar cells.

Northern blotting

EGFR mRNA levels were detected by Northern blotting in homogenates from normal hamster pancreas, pancreas from BOP-treated hamsters containing putative preneoplastic lesions (indicated as "preneoplastic pancreas") and homogenates from

ductular adenocarcinomas. Analyzing more than 50 samples, no significant differences in EGFR mRNA expression levels were observed comparing preneoplastic pancreas with normal pancreas. However, ductular adenocarcinomas demonstrated a significantly (3-5 fold) increase in mRNA expression when compared to total pancreas homogenates (both normal and preneoplastic) (**Figure 6.4**).

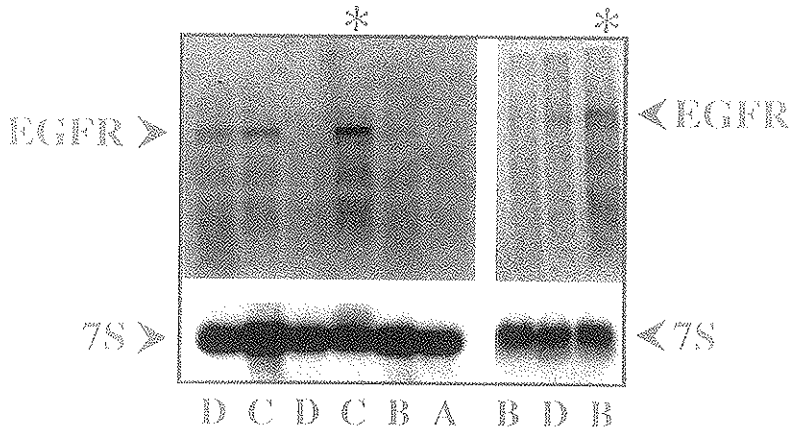


Figure 6.4 Detection of EGFR mRNA in pancreas homogenates of BOP-treated hamsters by Northern blotting. Note the inconsistent differences in hybridization signals in the various lanes comprising RNA obtained from hamsters from the various treatment groups (A,B,C,D); * indicates RNA isolated from ductular adenocarcinomas.

Semi-quantitative PCR

Pancreatic tissues obtained from carcinogen-treated and untreated hamsters were used for PCR. The data summarized in **Table 6.4**, which represent duplicate experiments with material obtained from at least five different animals, demonstrate TGF-α and EGF mRNA expression in normal hamster pancreas. TGF-α and EGF mRNA expression was also demonstrated using the PCR technique on preneoplastic pancreas and on pancreatic ductular adenocarcinomas.

The most striking effects were found when comparing EGF and TGF-α mRNA levels in carcinogen-injected hamsters with those in saline-injected hamsters:

1) BOP-treatment increases TGF-α mRNA expression.
2) BOP-treatment decreases EGF mRNA expression.

Ad.1) After 12 months, but not after six months, BOP-treated hamsters showed significantly ($P<0.001$) higher TGF-α mRNA levels than saline-treated hamsters. Ductular adenocarcinomas also demonstrated significantly ($P<0.05$) increased TGF-α levels when compared to pancreatic homogenates from saline-treated hamsters.

Ad.2) In contrast, at final autopsy, EGF mRNA levels in BOP-treated hamsters were significantly ($P<0.001$) lower than in saline-treated controls. Ductular adenocarcinomas collected at final autopsy did not demonstrate significant differences in EGF mRNA expression in comparison with total pancreatic homogenates (both normal and preneoplastic). At interim kill, only the BOP-treated hamsters injected with caerulein or given the caloric restricted diet demonstrated significantly ($P<0.01$) decreased pancreatic EGF mRNA levels in comparison with hamsters not injected with BOP. However, when combining all carcinogen-treated hamsters, this also revealed a significantly ($P<0.05$) lower EGF mRNA expression in the pancreas of BOP-treated hamsters than in saline-treated hamsters at interim kill.

Postinitiation treatments did not modulate pancreatic TGF-α or EGF mRNA levels, except for a significantly ($P<0.05$) lower TGF-α mRNA expression at final autopsy in the pancreas of BOP-treated hamsters given a high fat diet, in comparison with BOP-treated hamsters given a low fat diet.

6.5 DISCUSSION

The data presented in this paper demonstrated reduced epidermal growth factor (EGF) and increased transforming growth factor (TGF-α) mRNA synthesis in pancreatic ductular adenocarcinomas experimentally induced in hamsters by the carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP), in comparison with EGF and TGF-α mRNA synthesis in normal pancreas. We also demonstrated overexpression of the Epidermal Growth Factor Receptor (EGFR) in pancreatic ductular adenocarcinomas.

Overexpression of EGFR, a tyrosine kinase transmembrane receptor known to be activated by binding of -amongst others- EGF and TGF-α (32), has also been reported in ductular adenocarcinomas of human pancreas (33,34). A concomitant (over)expression of EGF, TGF-α and the EGFR in human pancreatic adenocarcinomas has been reported, pointing to an autocrine or paracrine cell-growth or cell-division stimulatory cycle (7,35). The findings in the present paper suggest a similar mechanism in BOP-induced pancreatic cancer in Syrian golden hamsters. Moreover, in the present study, a shift was

observed from EGF towards TGF-α (over)expression during the carcinogenic process, which might imply an even stronger growth stimulatory effect, since the biological activity of TGF-α is often found to be higher than the activity of EGF (36,37).

Because of the similarity between the present observations in BOP-treated hamsters and those in human pancreatic tumours, the role of growth factor (receptors) in the development of ductular adenocarcinomas induced in hamster pancreas by BOP may be comparable to the role of these growth factors in human exocrine pancreas carcinogenesis. This observation supports the conclusion of several other investigators, that the BOP-hamster model is highly relevant to mimic pancreatic carcinogenesis in men and therefore, the putative preneoplastic ductular lesions induced in hamsters might mimic early stage neoplasia in men.

In addition to the role of EGF and TGF-α in pancreatic carcinogenesis we studied whether these growth factors are involved in the modulating effects of the post-initiation treatments by caerulein, dietary fat or caloric restriction on pancreatic carcinogenesis in hamsters. Unfortunately, the 20% caloric restriction did not cause an inhibition of pancreatic carcinogenesis, because the hamsters appeared to compensate for the reduced caloric content of the diet by eating significantly more than controls. In contrast, caerulein treatment as well as a high fat diet enhanced pancreatic carcinogenesis. However, when comparing the various post-initiation treatment groups with controls, no consistent statistically significant effects on EGF or TGF-α mRNA expression levels were detected. Moreover, the modulating effects of caerulein and fat on pancreatic carcinogenesis were most pronounced after six months, whereas the differences in EGF and TGF-α mRNA levels were most pronounced at 12 months. The number of preneoplastic and neoplastic lesions per animal was highest at 12 months. Therefore, it is concluded, that the effects of caerulein or a high fat diet on tumour formation were most probably not caused by modulation of these growth factors. Thus, it is most likely that the effects on EGF and TGF-α expression were detected only at 12 months, because the differences between saline-treated hamsters -showing no (pre)neoplastic pancreatic lesions-, and BOP-treated hamsters -showing several (pre)neoplastic pancreatic ductular lesions-, were most pronounced 12 months after the last injection with BOP.

TGF-α peptide expression was also demonstrated by immunohistochemistry in the peripheral endocrine cells of the islets of Langerhans. This strong TGF-α expression most probably did interfere with the mRNA determinations presented in this paper. Because isolated ductular adenocarcinomas did not contain endocrine cells, the mRNA levels in these tissues were found to be lower than in total pancreatic homogenates from BOP-treated hamsters, which did contain endocrine cells. However, notwithstanding this confounding factor, TGF-α mRNA expression was significantly increased in ductular

adenocarcinomas in comparison with homogenates of normal pancreas from control hamsters. Moreover, immunohistochemistry demonstrated a distinctly increased TGF-α expression in ductular adenocarcinoma cells when compared to exocrine ductular cells of normal pancreas. These observations confirm that the observed overexpression of TGF-α mRNA in (pre)neoplastic pancreas is caused by overexpression in the ductular cells due to BOP-treatment.

Double staining experiments revealed concomitant expression of TGF-α and somatostatin, or TGF-α and glucagon in some, but not all of the endocrine cells. No differences were observed in localization or intensity, when comparing the staining in pancreatic endocrine cells of BOP-treated hamsters with these of saline-treated controls. Using immunohistochemistry, the islets of Langerhans, either from BOP-treated hamsters or saline-treated controls demonstrated only a weak and diffuse EGF staining. Therefore, it can be concluded, that BOP-treatment, in contrast to the observations in the exocrine pancreas, did not affect TGF-α or EGF expression in the endocrine Syrian golden hamster pancreas.

Finally it is concluded, that -similarly to the observations in human pancreatic tumours- the concomitant overexpression of TGF-α and EGFR might point to an autocrine or paracrine growth stimulatory cycle in hamster pancreas carcinogenesis. However, unlike in human pancreatic carcinogenesis, EGF overexpression does not appear to play a role in hamster pancreatic carcinogenesis. In fact, in BOP-treated hamsters overexpression of TGF-α is accompanied by a reduction in expression of EGF, which might suggest that a change in the equilibrium between EGF and TGF-α somehow causes a growth advantage in these pancreatic ductular tumours.

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CHAPTER SEVEN

INCREASED IMMUNOREACTIVITY AND PROTEIN TYROSINE KINASE ACTIVITY OF THE PROTO-ONCOGENE PP60$^{C\text{-}SRC}$ IN PRENEOPLASTIC LESIONS IN RAT PANCREAS

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7.1 ABSTRACT

Expression of the proto-oncogene $pp60^{c\text{-}src}$ (c-src) was investigated in acinar pancreatic (pre)neoplastic lesions induced in rats by azaserine, and compared with the expression in normal rat pancreas. The data indicated extremely low or absent c-src protein level and c-src tyrosine kinase activity in the pancreas of untreated control rats. However, compared to these controls, c-src protein immunoreactivity was increased in 'normal' acinar cells and in putative preneoplastic atypical acinar cell nodules (AACN) in rats treated with azaserine. More advanced lesions, such as secondary transformed acinar cells (nodules-in-nodules) demonstrated no c-src immunoreactivity. Rats treated with azaserine showed a 7-fold higher c-src tyrosine kinase activity in their pancreas, and the level of c-src tyrosine kinase activity correlated positively with the number of lesions in the pancreas. Promotion of azaserine-initiated pancreatic carcinogenesis in rats by the cholecystokinin analogue caerulein resulted in a more than 10-fold increase in the number of pancreatic acinar cell lesions, which was accompanied by a 6-fold increase in c-src tyrosine kinase activity, when compared to azaserine treatment alone. c-Src tyrosine kinase activity was predominantly found in the cytoskeletal subcellular fraction, which appeared to be responsible for on average 40% of the total tyrosine kinase activity in the pancreatic homogenates. Furthermore, the transformation from normal to preneoplastic pancreatic tissue in azaserine-treated rats was accompanied by a change in the localization of the c-src protein. Using immunohistochemistry and confocal laser scanning microscopy, in morphologically normal pancreatic acini the protein was detected in the cytoplasm, whereas in AACN it was detected both in the cytoplasm and in the nuclei. It is concluded that c-src might be involved early in pancreatic carcinogenesis. c-Src probably plays a minor role in pancreatic acinar cells after transformation to malignancy.

7.2 INTRODUCTION

Tyrosine phosphorylation is recognized as an important regulatory mechanism in response to a number of processes including the action of growth factors and oncogenes (1-4). Recently, many studies have been focussed on the role that growth factors play in exocrine pancreatic carcinogenesis. Several studies report overexpression of the epidermal growth factor (EGF), transforming growth factor-α (TGF-α) and their

receptor (the epidermal growth factor receptor (EGFR)), in human pancreatic tumours and cell lines (5-8). However, little is known about the intra-cellular processes induced by these growth factors in pancreatic tumours, or the relationship between growth factor expression, signal transduction, and growth rate.

The proto-oncogene c-src, the cellular homologue of the transforming protein encoded by the Rous-sarcoma virus gene v-src, has been reported to contribute significantly to the elevated protein tyrosine kinase activity in human tumours including breast (9,10), colon (11), bladder (12) and neuroblasts (13). c-Src is thought to be involved in signal transduction processes after stimulation by (amongst others) platelet derived growth factor receptor (PDGFR; 14, 15, 16) and EGFR (17-20), which are mediating cell proliferation (15, 17), cell differentiation (21) and mitosis (22,23). Untill now, no studies have been published on the role of c-src in exocrine pancreatic carcinogenesis.

The v-src protein is mainly located on the inner surface of the plasma membrane, which position was reported to be indispensable for its transforming capacity (24,25). More recently, it was demonstrated that v-src is associated with the cytoskeleton (26). Moreover, it was shown that the degree of association with the cytoskeleton correlated with cell transformation, suggesting that association of v-src with the cytoskeleton is indispensable for morphological transformation (26,27). However, c-src was demonstrated on endosomal membranes (28), but recent studies also demonstrated association of c-src with the cytoskeleton after stimulation of glioblastoma cells with PDGF or EGF (29). In the present study we analyzed both the expression and localization of the c-src protein by immunohistochemistry and by biochemical methods in azaserine-treated rats, a well-known model for acinar adenocarcinomas in exocrine pancreas (30,31). Moreover, c-src protein tyrosine kinase activities were determined in normal and (pre)neoplastic pancreatic tissues, using an immuno complex kinase assay. Finally, the contribution of c-src to overall protein tyrosine kinase activity in pancreatic tissues was evaluated by an enzyme linked immuno sorbent assay (ELISA).

7.3 MATERIALS AND METHODS

Animals and collection of tissues

To induce pancreatic carcinogenesis, albino Wistar WU rats (Charles River Wiga GmBH, Sulzfeld, Germany) were injected intraperitoneally at 14 and 21 days of age with 30 mg azaserine (Calbiochem-Behring Corp., La Jolla, CA, USA) per kg body

weight according to an injection protocol described previously (32). All animals were housed under similar standard conditions and were maintained on an AIN-76 based low-fat diet. To stimulate pancreatic carcinogenesis, one group of azaserine-treated rats was injected with caerulein, an analogue of the gut-hormone cholecystokinin (s.c. injections with 2.5 µg caerulein/kg body weight on 3 consecutive days per week for the whole experimental period).

The rats were killed 15 months after the last injection with azaserine. Therefore, the animals were anaesthetized with ether, exsanguinated by cannulating the abdominal aorta, and then examined for gross pathological changes. The entire pancreas was excised, partly frozen and stored in liquid nitrogen immediately after dissection, and partly fixed in 4% buffered formalin and embedded in paraffin wax.

Histology

At three separate levels, ten 5 µm serial sections were cut from the paraffin embedded pancreas. Of each series one section was stained with haematoxylin and eosin (H&E) and examined by light microscopy. Likewise, one part of the liquid nitrogen frozen pancreas was used for preparation of cryostat sections, the adjacent part was used for biochemical analysis. These cryostat sections were fixed for 10 minutes in 4% buffered formalin to be stained routinely with H&E and examined by light microscopy. Based on the results of the microscopical examinations, we selected tissues with no (from rats not injected with azaserine), with a few (0-10; from rats injected with azaserine), and with many (>75; from rats injected with azaserine and subsequently treated with caerulein) putative preneoplastic atypical acinar cell foci. In this last group, the pancreas almost totally consisted of (pre)neoplastic tissue.

Immunohistochemistry

A sheep-anti c-src polyclonal antibody (Affinity; Nottingham, UK, 1:150 diluted) was used to detect c-src immunoreactivity in paraffin embedded sections. Endogenous peroxidase activity was quenched by incubation in 0.6% hydrogen peroxide in methanol for 30 minutes. For antigen retrieval, the sections were boiled in citrate buffer (sodium-citrate, 2.94g/L H_2O, pH 6.0) for 15 minutes and subsequently, cooled down very slowly to room temperature. The slides were incubated for 2 hours with the primary antibody in 4% bovine serum albumin (BSA) in phosphate buffered saline (PBS, 0.14 mM NaCl; 8.93 mM Na_2HPO_4; 1.28 mM NaH_2PO_4; pH 7.4) in a humid slide chamber at room temperature (RT). To detect the polyclonal antibody, the slides were subsequently incubated with a peroxidase-conjugated rabbit-anti-sheep antibody (RAShPO, DAKO

a/s, Glostrup, Denmark; dilution 1:100; 30 min at RT) and a peroxidase-conjugated swine-anti-rabbit antibody (SwARPO, DAKO, dilution 1:100; 30 min at RT). Both antibodies were diluted in PBS containing 10% normal rat serum. In between every incubation step the sections were washed three times for five minutes with PBS-Tween (0.05% Tween-20 in PBS, pH 7.4), with exception of the last washing step where Tween was omitted. Subsequently, a brown precipitate was observed by light microscopy after the peroxidase reaction with 60 mg 3,3'-diaminobenzidine tetrahydrochloride (DAB; SIGMA, St.Louis, MO, USA) and 10 µl 37% H_2O_2 in 100 ml H_2O. The sections were counterstained with hematoxylin (after Mayer), and covered with DEPEX. For negative controls, the primary antibody was omitted; all negative controls did not show any staining.

To examine the sections with a confocal laser scanning microscope (CLSM MRC1000, Bio-Rad, Hercules, California) the antibody directed against c-src was detected by changing the third incubation step for a TRITC-labeled swine-anti-rabbit polyclonal antibody (SwAR-TRITC, Nordic Immunological Laboratory, Tilburg, The Netherlands, dilution 1:40). These sections were not counterstained, and were covered with PBS/glycerol (9:1).

Preparation of subcellular protein fractions

Sample preparation was performed as described previously (20) with some modifications. To inhibit proteolytic enzyme activities, all solutions were kept on ice and 0.055 TIU/ml aprotonin, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 1 µg/ml pepstatin were added just before use. To inhibit tyrosine phosphatase activity, 100 µM Na_3VO_4 was added. About 800 mg pancreatic tissue was homogenized in 4 ml extraction buffer (20 mM HEPES, pH 7.2; 1 mM $MgCl_2$; 1 mM EDTA-Na_2; 1 mM dithiothreitol; 10% glycerol) and centrifuged at 800 g, 30 min at 4°C to get rid of cell debris. The supernatant was centrifuged at 48,000 g, for at least 30 min at 4°C. The supernatant (cytosol fraction) was removed and the pellet was resuspended in solubilisation buffer (20 mM HEPES, pH 7.2; 20 mM Mg-acetate; 5 mM NaF; 0.2 mM EDTA-Na_2; 0.8 mM EGTA; 1 mM dithiothreitol; 0.5% Nonidet P-40) and centrifuged at 48,000 g, 30 min, 4°C. The supernatant (soluble membrane fraction) was removed and the pellet was resuspended in radio immunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 8.0; 150 mM NaCl; 10 mM NaH_2PO_4; 5 mM EDTA; 1 mM dithiothreitol; 10% glycerol; 1% Nonidet P-40; 1% Na-desoxycholate; 0.1% SDS) and centrifuged at 48,000 g, 30 min, 4°C. The supernatant (cytoskeleton fraction) was collected.

Immunoprecipitation of c-src.

Immunoprecipitations were performed as described previously (10). Protein concentrations were standardized between the corresponding fractions of the various tissue samples, according to the method described by Bradford (33). The various subcellular fractions were incubated with the mouse monoclonal antibody Ab-#327 (Oncogene Science Inc., Uniondale, NY, USA; 1 µg/400 µl protein extract, 1 hr at 4°C), an antibody that recognizes c-src and does not inhibit the tyrosine kinase activity (34). As a check for the specificity of precipitation, control precipitations were performed with a non-relevant mouse immunoglobulin (SIGMA). Subsequently, the antibody-protein complex was precipitated by incubation with 3 mg protein-A Sepharose, 2 hrs at 4°C and collected by centrifugation. The pellet was washed three times with wash-buffer (20 mM HEPES, pH 7.5; 150 mM NaCl; 0.1% Triton X100; 10% Glycerol; 1 mM Na_3VO_4) and one time with a second wash-buffer (20 mM HEPES, pH 7.5; 5 mM $MgCl_2$; 1mM Na_3VO_4). Supernatants collected after precipitation with both the c-src and the control antibodies were tested in the tyrosine kinase ELISA. The pellets collected were tested in the immune complex tyrosine kinase assay.

Immune Complex Tyrosine Kinase Assay

c-Src tyrosine kinase activity assay was performed as described before (20) with some modifications. c-Src immunoprecipitates from the various subcellular fractions were collected as described in the previous paragraph. c-Src autophosphorylation and phosphorylation of acid-denatured rabbit muscle enolase (Boehringer-Mannheim, Germany) was carried out in 40 µl phosphorylation buffer supplemented with 3 mM $MnCl_2$ and 4 µg enolase. The reaction was started by the addition of 0.5 µl [^{33}P]ATP (Dupont NEN, Stevenage, England; 10 µCi/µl). After 7 min at room temperature the reaction was stopped, 40 µl 2x concentrated sample buffer was added and the samples were heated at 95°C. Proteins were size separated by electrophoresis in 8% SDS-PAGE and electroblotted to a polyvinylidene difluoride (PVDF) filter. Marker proteins were visualized by staining with Coomassie blue. Phosphate incorporation was analyzed in a PhosphorImager coupled to ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Tyrosine Kinase ELISA

When determining the amount of precipitation by the c-src antibody, the kinase assay was carried out based on an ELISA as described in detail by Schraag *et al.* (35). Fifty µl of supernatant with a protein concentration ranging from 50-400 ng were added

in triplicate to the wells of a poly(glutamic acid : tyrosine, 4:1) coated 96-well plastic plate. The phosphorylation reaction was started by adding 50 µl 1 mM ATP containing 1 mM Na_3VO_4 in PBS, and allowed to continue at 37°C for 15 min. The reaction was stopped by washing three times with PBS-Tween (0.1% Tween-20 in PBS; pH 7.4). Subsequently, the plates were incubated with 100 µl/well mouse-anti-P-Tyr monoclonal antibody (Clone IG1, Amersham, UK; diluted 1:1000 in 0.1% BSA in PBS) for one hour at room temperature. The wells were washed four times with 250 µl PBS-Tween/well and subsequently, incubated with 100 µl/well peroxidase conjugated rabbit-anti-mouse antibody (Dako a/s; dilution 1:300 in 1% fish gelatin in PBS) for 60 min at room temperature. Following four washing steps, 100 µl 3,3'-5,5'-tetramethylbenzidine was added at a 1:20 dilution in water, and after 10 min the reaction was stopped with 100 µl/well 1 M H_2SO_4. The extinction in the wells was measured at 450 nm using an automated plate reader.

7.4 RESULTS

Immunohistochemistry

Immunohistochemical localization of the c-src protein revealed a very weak cytoplasmic signal in pancreatic tissue of rats not treated with the carcinogen azaserine (**Figure 7.1**). Ductular and centro-acinar cells stained stronger than acinar cells. However, pancreatic tissue of rats treated with the carcinogen azaserine showed pronounced cytoplasmic immunoreactivity of the c-src antibody in 'normal' acinar cells (**Figure 7.2**). Moreover, putative preneoplastic atypical acinar cell nodules (AACN) stained even stronger, than the surrounding normal acinar cells (**Figure 7.2**). In azaserine-treated rats, the localization of the protein in normal acinar cells was different from that in putative preneoplastic AACN: in normal cells the signal was found mainly in the cytoplasm, whereas in atypical acinar cells the signal was found both in the cytoplasm and the nuclei. This basolateral nuclear staining of c-src was most prominent in AACN of caerulein-treated rats and also clearly visible after incubations with fluorescent antibodies and subsequent visualization using confocal laser scanning microscopy (**Figure 7.3**). Moreover, confocal laser scanning microscopy demonstrated clearly the increased immunoreactivity in the centro-acinar/small ductular cells in the 'normal' acinar cells (**Figure 7.3**).

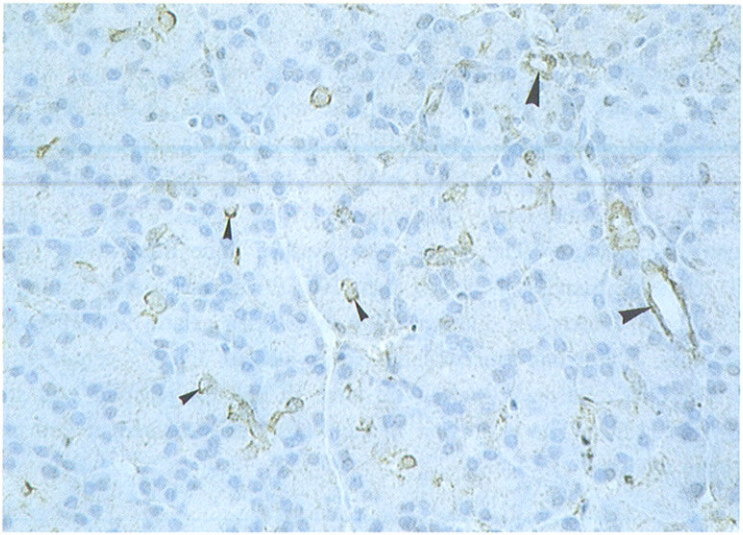

Figure 7.1 c-Src immunoreactivity in normal rat pancreas (rat not treated with azaserine). Weak staining in acinar cells. Arrowheads point to positive staining in small duct (large arrowhead) and in centroacinar cells (small arrowhead).

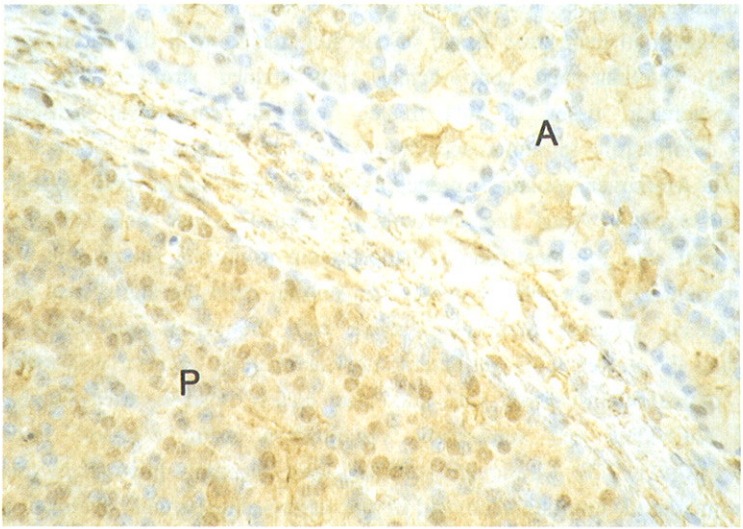

Figure 7.2 c-Src immunoreactivity in a pancreatic section of an azaserine-treated rat. Enhanced nuclear and cytoplasmic staining in the putative preneoplastic atypical acinar cell nodule (P). A='normal' acinar cells (with higher staining intensity than the normal cells in figure 7.1).

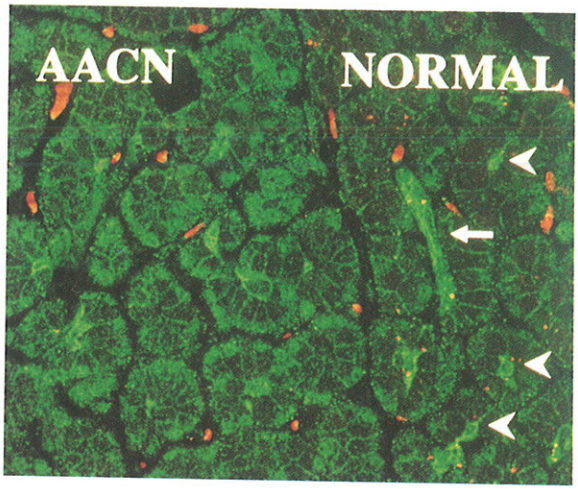

Figure 7.3 Enhanced nuclear c-src immunoreactivity detected by confocal laser scan microscopy in a putative preneoplastic pancreatic nodule of an azaserine-treated rat. Normal='normal' acinar cells, AACN=atypical acinar cell nodule. Arrow points to c-src-positive small duct; arrowheads point to c-src-positive centroacinar cells.

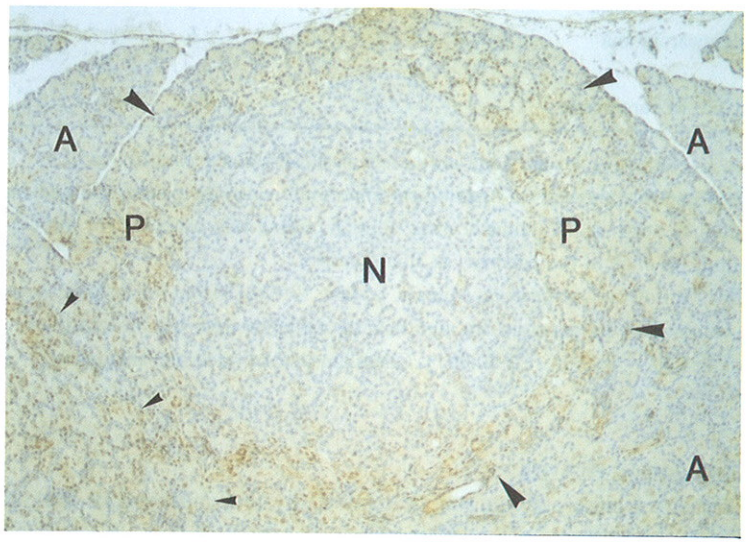

Figure 7.4 c-Src immunoreactivity in the pancreas of an azaserine-treated rat. A='normal' acinar cells, P=putative preneoplastic atypical acinar cell nodule (AACN), N=nodule-in-nodule. Small arrowheads point to AACN in lower left corner, large arrowheads point to AACN around nodule-in-nodule.

In some pancreata from caerulein-treated rats some 'nodules-in-nodules' developed, which have been described as secondary transformations in atypical acinar cell nodules (31). Surprisingly, these nodules-in-nodules did not show immunoreactivity to c-src anymore (**Figure 7.4**). Endocrine cells of the islets of Langerhans also stained for c-src, but no differences in staining intensities were found between azaserine-treated and untreated rats (not shown).

Table 7.1 Number of pancreatic lesions in azaserine-treated rats after 15 months caerulein treatment[a]

	Post-initiation treatment	
	No caerulein	caerulein
No. of rats	19	19
No. of tumour-bearing animals (%)[b]	6 (32)	13 (68)[c]
(Pre)neoplastic lesions observed:		
AACN 1-3 mm^2	15	274***
Adenoma	3	50**
Carcinoma *in-situ*	5	25*
Adenocarcinoma	6	12
Total no. of carcinomas	11	37*

[a]Values are totals per group. Data are based on H&E-stained paraffin sections. The untreated controls showed no histological abnormalities in the pancreas based on H&E-stained sections. [b]Tumour-bearing animal, animal that bears one or more carcinoma (*in-situ*) in the pancreas at final autopsy.
Statistics: no. of tumour-bearing animals, χ^2-test, [c]P=0.0184; no. of lesions, 2-sample *t* test, values with superscripts are significantly different from the 'no caerulein' group (*P<0.05, **P<0.01, ***P<0.001). Caerulein, cholecystokinin analogue.

c-Src Tyrosine Kinase Activity

c-Src tyrosine kinase activity was determined by four independent immune complex tyrosine kinase assays in pancreatic tissues obtained from i) non-carcinogen-treated rats (control rats) with normal acinar tissue, ii) azaserine-treated rats with only a few atypical acinar cell lesions, and iii) azaserine-treated rats, which are injected with caerulein resulting in a significantly increased number of atypical acinar cell nodules

(AACN) and neoplastic lesions, as determined in H&E-stained parallel sections (**Table 7.1**).

Table 7.2 c-Src protein tyrosine kinase activities in rat pancreas homogenates[a]

			Protein tyrosine kinase activity			
Substrate	Subcellular fraction	Treatment group	Exp.1	Exp.2	Exp.3	Exp.4
Enolase	Cytosol	Untreated	nd	nd	nd	1.6
		Azaserine	1.3	7.9	1.1	11.1
		Azaserine + caerulein	1.8	5.8	1.5	37.3
	Soluble membrane	Untreated	nd	nd	nd	4.6
		Azaserine	2.2	18.7	1.8	22.1
		Azaserine + caerulein	4.3	20.9	3.7	97.3
	Cytoskeleton	Untreated	nd	nd	nd	2.3
		Azaserine	4.6	95.0	3.7	18.5
		Azaserine + caerulein	31.5	398	29.0	95.3
c-Src	Cytosol	Untreated	nd	nd	nd	0
		Azaserine	0	1.7	0	0
		Azaserine + caerulein	0	1.3	0	0
	Soluble membrane	Untreated	nd	nd	nd	0
		Azaserine	0	1.7	0	5.6
		Azaserine + caerulein	0	2.0	0	6.3
	Cytoskeleton	Untreated	nd	nd	nd	0
		Azaserine	1.2	13.0	1.0	1.6
		Azaserine + caerulein	5.6	67.5	5.1	12.6

[a]Values represent the relative amounts of radioactive phosphorus incorporated in the substrate, determined in four independent immune-precipitation tyrosine kinase assay experiments, calculated per microgram subcellular fraction protein introduced at precipitation. nd = not done; 0 = value below detection level, not taken into calculations.

Figure 7.5 shows c-src tyrosine kinase phosphorylation activity on the ± 50 kD substrate enolase and on the 60 kD c-src itself (autophosphorylation) in pancreatic

homogenates of azaserine-treated rats with a few (A: not-treated with caerulein) or many (C: treated with caerulein) AACN in the various subcellular fractions. In this figure it is demonstrated, that the tyrosine kinase activity parallelled the increased number of lesions (caused by caerulein) only in the cytoskeleton subcellular fraction.

The amount of c-src protein appeared too low to be detectable by Western blotting. Therefore the relative protein tyrosine kinase activities were calculated in the total amount of protein in the various fractions collected from 0.8 g rat pancreas. Although this report is not a quantitative study, all independently performed experiments pointed clearly in the same directions. Therefore, figures are shown just to give an indication of the direction of the effects (**Table 7.2**).

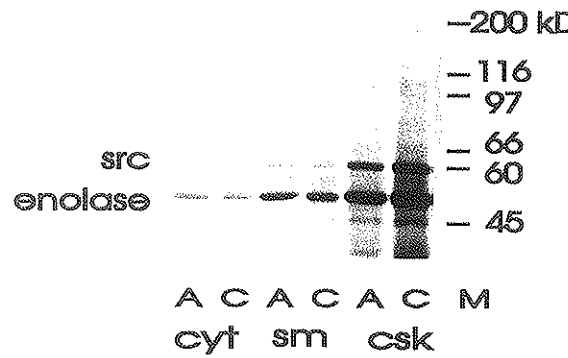

Figure 7.5 c-Src protein tyrosine kinase activities. Phosphorylation on itself (src) and on enolase, in pancreatic subcellular fractions from azaserine-treated rats, either injected (C) or not injected (A) with caerulein. cyt = cytosol, sm = soluble membrane, csk = cytoskeleton, M = molecular weight marker. The amounts of protein in the respective subcellular fractions relate as; cytosol : soluble membrane : cytoskeleton = 64:12:15.

In azaserine-treated rat pancreas, the c-src protein tyrosine kinase activity towards he substrate enolase in the cytoskeleton fraction was on average 3-fold (compared to the cytoplasmic fraction) and 5-fold (compared to the soluble membrane fraction) higher (in **Figure 7.5** the amounts of protein were only standardized *within* the various subcellular fractions, not *between* the subcellular fractions). Moreover, c-src autophosphorylation was detected in the cytoskeletal fraction and occasionally, weakly in the soluble membrane fraction, but neither in the cytosolic subcellular fractions of the pancreas of azaserine-treated rats, nor in the pancreas of control rats. c-Src autophosphorylation activity was on average 7-fold (compared to the cytoplasmic fraction), and 13-fold (compared to the soluble membrane fraction) higher in the cytoskeleton fraction.

In total pancreatic lysates from azaserine-treated rats a 7-fold increase of c-src protein tyrosine kinase activity towards the substrate enolase was found, when compared to that observed in untreated control rats. Furthermore, c-src protein tyrosine kinase activity in the cytoskeletal subcellular fraction appeared to be positively correlated with the number of atypical acinar cell lesions present in the pancreas (**Figure 7.6**). Pancreata from azaserine plus caerulein-treated rats with a large number of AACN (**Table 7.1**) showed approximately 6-fold higher tyrosine kinase activities towards both enolase and c-src, than pancreata from rats treated with azaserine alone, with a low number of AACN. However, in the cytosolic and in the soluble membranes subcellular fractions, only some moderate c-src protein tyrosine kinase activities were detectable on enolase (increment approximately 2-fold), but not on c-src itself.

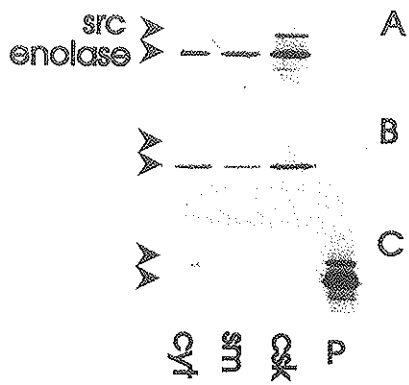

Figure 7.6 c-Src protein tyrosine kinase activities. Phosphorylation on itself (src) and on enolase, in pancreatic subcellular fractions from rats injected with azaserine and caerulein (A), from rats injected with azaserine (B), and from untreated control rats (C). Cyt=cytosol, sm=soluble membrane, csk=cytoskeleton, P=positive control (c-src tyrosine kinase activity in blood platelets). The amount of protein on the different blots is normalized on 200 µg total protein.

Contribution of c-src to total tyrosine kinase activity.

The overall protein tyrosine kinase activity in lysates from rat pancreas was determined by ELISA. Pilot studies revealed linearity of the assay at 200 ng protein. Stimulation of pancreatic carcinogenesis by caerulein resulted in a significant increase in tyrosine kinase activity in both the cytosol and soluble membrane fractions (**Figure 7.7**). Moreover, the increase was most pronounced in the cytoskeleton subcellular fraction (**Figure 7.7**), which was in agreement with the immunoprecipitation data. The contribution of c-src tyrosine kinase activity to the overall tyrosine kinase activity in pancreatic homogenates was determined by measuring the tyrosine kinase activity before and after precipitation of c-src. After correction for aspecific precipitation with a non-

relevant mouse immunoglobulin (control IgG), c-src tyrosine kinase contributed for 41% ± 20 (mean ± SD, n=4, over all groups and over all subcellular fractions) to the total tyrosine kinase activity. In the cytosol fractions the values were too low to determine c-src protein tyrosine kinase contributions. The tyrosine kinase activities due to c-src ranged from 22% ± 12 (in azaserine- + caerulein-treated rats), and 47% ± 1.5 (in azaserine-treated rats), to 83% ± 24 (in non-treated control rats), when averaged over soluble membrane and cytoskeleton fractions (**Figure 7.8**).

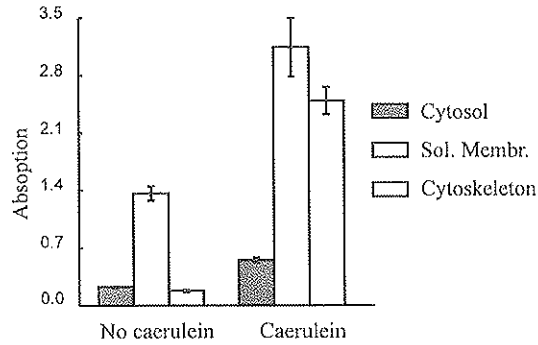

Figure 7.7 Relative protein tyrosine kinase activities determined by ELISA in 200 ng subcellular pancreatic protein lysates from azaserine-treated rats. Values are means (± standard deviation) of three observations. CCK, cholecystokinin analogue caerulein.

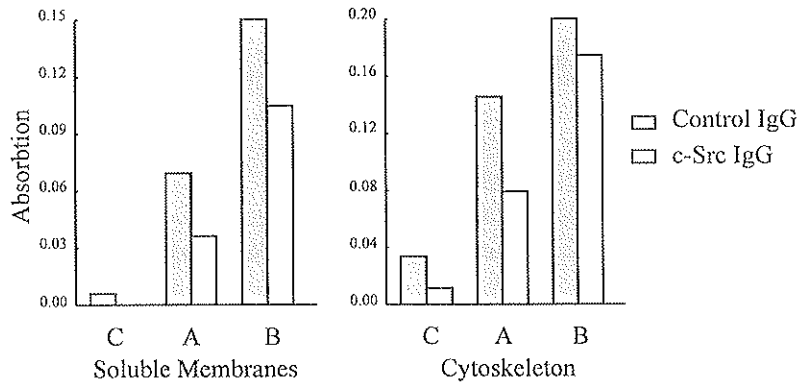

Figure 7.8 Relative protein tyrosine kinase activities determined by ELISA in 200 ng protein supernatants of soluble membrane- and cytoskeleton-pancreatic fractions, after precipitation with a control IgG, or a specific anti-c-src IgG (reduction of absorption indicates c-src involvement). C, control rats, no carcinogen; A, azaserine-treated rats; B, azaserine + caerulein-treated rats.

7.5 DISCUSSION

In the present paper we reported c-src expression and c-src tyrosine kinase activity in the pancreas of azaserine-treated rats. In pancreatic tissues obtained from untreated control rats, no or slight c-src expression was detected by immunohistochemistry, located predominantly in the cytoplasm of ductular and centro-acinar cells, and also in cells of the islets of Langerhans. Moreover, using two different tyrosine kinase assays, c-src protein tyrosine kinase activity in pancreatic homogenates of untreated control rats was very weak or absent. These findings point to a strictly negatively regulated role for c-src in normal adult rat pancreas physiology.

Rats treated with the carcinogen azaserine, demonstrated increased c-src protein expression and protein tyrosine kinase activities. In these animals, pancreatic tissue showed increased cytoplasmic immunoreactivity to anti c-src antibodies in the acinar cells, whereas the staining in ductular-, centro-acinar-, and endocrine cells was similar to that observed in untreated rat pancreata. Moreover, 7-fold increased c-src tyrosine kinase activities were detected in pancreatic homogenates of rats treated with azaserine compared to untreated rats. Previous studies demonstrated significantly increased BrDU labeling indices, indicative for enhanced mitogenic activity in acinar pancreatic cells after injecting rats with azaserine (36). These findings, in combination with the findings shown in the present study, support the hypothesis that c-src is involved in mitogenesis (22,23,37-39). Unfortunately, the c-src protein levels appeared to be too low for detection by Western blotting. Therefore, the present results do not allow to draw conclusions on the extent of activation of the c-src protein. However, since the increased c-src protein tyrosine kinase activity is accompanied by microscopically observed increased immunoreactivity of the c-src antibody, the results suggest an increased c-src tyrosine kinase activity just as a consequence of more c-src protein present (rather than an increased specific c-src protein tyrosine kinase activity).

The enhanced c-src tyrosine kinase activity in the cytoskeletal subcellular fraction reflects a translocation of c-src from the plasma membrane to the cytoskeleton and might suggest increased transforming capacity (26,27). The reason for this cytoskeletal localization is not clear yet, but it is suggested that c-src plays an important role during the remodelling of the cell architecture during mitosis (40).

Rats injected with the cholecystokinin (CCK)-analogue caerulein showed a significant increase in the number of atypical acinar cell lesions compared to non-caerulein treated rats. CCK is known to regulate phosphorylation of a number of proteins, most of which have not been characterized (41). In the present study, overall

protein tyrosine kinase activity increased 6-fold in pancreas homogenates of azaserine-treated rats injected with caerulein, when compared to pancreas homogenates from azaserine-treated rats not injected with caerulein. Moreover, c-src protein expression was increased in putative preneoplastic pancreatic lesions in caerulein injected rats when compared to the surrounding morphologically normal acinar cells. However, in these rats, no increase of c-src protein expression was observed in the morphologically normal acinar cells when compared to acinar pancreatic cells from azaserine-treated rats that were not injected with caerulein. This observation indicates that caerulein treatment does not induce c-src expression in normal pancreatic acinar cells. Therefore, the reported increased c-src activity in pancreatic homogenates of caerulein-treated rats is the result of the increased number of putative preneoplastic lesions caused by caerulein treatment, rather than a direct stimulation of c-src expression by caerulein.

c-Src immunoreactivity is increased in the pre-neoplastic lesions when compared to the surrounding normal acinar cells. Moreover, the localization in the putative preneoplastic AACN in rat pancreas appeared to be mainly nuclear, whereas in normal acinar cells only a cytosolic staining was observed. The reason for this translocation is not clear. Translocation from cytosol to the nucleus has also been observed with MAP-kinase after stimulation of cells by mitogens (42,43). The nuclear localization has been associated with a role for MAP-kinase in gene expression by phosphorylation of transcriptional factors such as c-myc, c-fos and $p62^{tcf}$. CCK also stimulates expression of transcription factors such as c-myc, c-fos and c-jun (44). Therefore, the nuclear localization of c-src (most prominent after stimulation of pancreatic carcinogenesis by caerulein), may reflect a role for c-src in the regulation of gene expression.

In some sections, secondary AACN developing from primary AACN (so-called nodules-in-nodules) were observed in the pancreas of azaserine-treated rats after stimulation with caerulein. In these secondary lesions, c-src immunoreactivity was absent, whereas strong c-src immunoreactivity was found in the primary AACN. Bax *et al.* (45) demonstrated a similar phenomenon for the ATPase activity. They showed increased ATPase activity in primary AACN and a reduced ATPase activity in the secondary transformed nodules-in-nodules and acinar adenocarcinomas, when compared to normal acinar cells. In a previous study we have demonstrated absence of EGFR immunoreactivity in (ATPase negative) nodules-in-nodules as well as in acinar pancreatic adenocarcinomas, whereas EGFR was present in the surrounding normal acinar cells (Chapter 4 of this thesis).

From the above described results it is not possible to conclude whether the increase in c-src activity leads to enhanced acinar cell proliferation, or whether the increase in c-

src activity is caused by the azaserine-induced increase in cellular proliferation. However, recent studies on colon cancer demonstrated that the increased c-src activity in colon carcinomas is responsible for the increased cell proliferation; inhibition of c-src transcription by specific anti-sense probes resulted in decreased cell proliferation of colon cancer cell lines (46). These combined observations suggest that c-src might play a role in the preneoplastic transformation of pancreatic acinar cells -possibly in concert with the activation of growth factor receptors-, although its importance is doubtful in the neoplastic stage of acinar pancreatic carcinogenesis. Secondary transformed pancreatic acinar cells, such as present in nodules-in-nodules or acinar adenocarcinomas, seem to be able to proliferate independently from extracellular signals via EGFR (Chapter 4 of this thesis), and intracellular signals via c-src.

7.6 ACKNOWLEDGEMENTS

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7.7 LITERATURE

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CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

8.1 ROLE OF HORMONES IN EXPERIMENTAL EXOCRINE PANCREATIC CARCINOGENESIS

Chapters two and three of the present thesis deal with the effects of hormones on experimental exocrine pancreatic carcinogenesis. In humans, oestrogen is thought to inhibit, whereas testosterone is thought to promote the carcinogenic process of the pancreas (1,2). Results obtained with two animal models for pancreatic cancer, i.e. the azaserine-treated rat and the *N*-nitrosobis(2-oxopropyl)amine (BOP)-treated hamster, demonstrated that this concept is much more complicated. Based on the observation that in intact male rats, pancreatic tumours developed faster than in female or castrated male rats (3-7), it is concluded that in rats (alike it is suggested in humans), testosterone has a promoting and oestrogen an inhibitory effect on pancreatic carcinogenesis (8). However, in castrated male hamsters or in female hamsters, pancreatic tumours (either transplanted or chemically induced), will develop faster than in intact males. From these experiments it was concluded, that in hamsters oestrogen has a promoting effect on pancreatic carcinogenesis, whereas testosterone inhibits it (9-11) (**Figure 8.1**).

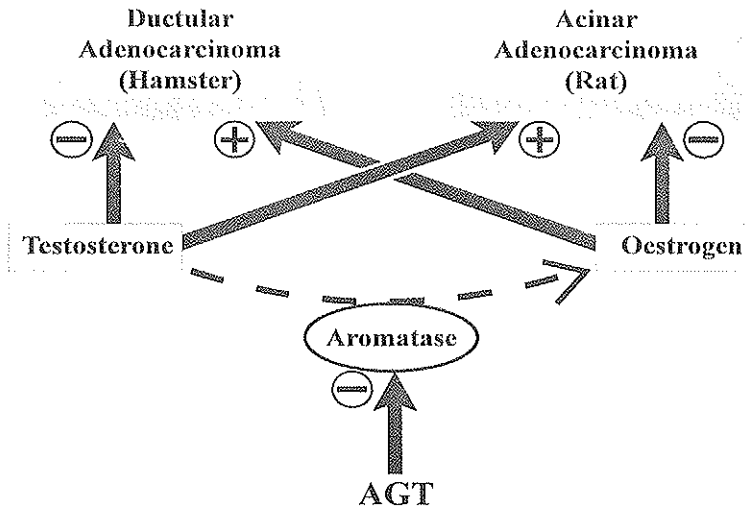

Figure 8.1 Schematic representation of inhibition (-) or promotion (+) of ductular and acinar pancreatic carcinogenesis by testosterone or oestrogen. Testosterone can be converted into oestrogen by the enzyme aromatase. Aromatase activity is inhibited by aminoglutethimide (AGT).

However, not all experiments pointed in the same direction for the effects of testosterone. A possible explanation for these conflicting results may be the conversion of testosterone into oestrogen by the enzyme aromatase. Testosterone might exert inhibitory effects on pancreatic carcinogenesis in the rat after being metabolized into oestrogen. On the other hand, in hamsters, testosterone might exert promoting effects on pancreatic carcinogenesis via this pathway. Treatment with the aromatase inhibitor aminoglutethimide (AGT) has been described to induce regression of hormone-dependent breast tumours both in rats and in humans (12-17). To investigate the relevance of the conversion of testosterone into oestrogen, a study was performed using AGT as therapeuticum in the rat and in the hamster model for exocrine pancreatic carcinogenesis.

In the study presented in chapter two of this thesis, AGT appeared to have inhibitory effects on the development of acinar adenocarcinomas induced in rat pancreas by azaserine. In contrast, AGT did not have an inhibitory effect on the development of ductular tumours induced in hamsters. In some cases even a promoting effect was observed! In humans, most pancreatic tumours (>95%) have a ductular histology and, therefore, the ductular adenocarcinomas induced in hamsters by BOP appear to be more comparable than the acinar pancreatic lesions induced in rats by azaserine. Consequently, it seems justifiable to conclude, that AGT will be an irrelevant and sometimes even dangerous drug to be used for the treatment of human pancreatic *ductular* adenocarcinomas. However, in (the sporadic) case of an *acinar* adenocarcinoma, AGT might be an effective therapeuticum. Combination therapy including a treatment aiming at a reduction of serum testosterone level, appears to be the best option, since in azaserine-treated rats, the inhibitory effects were most pronounced when AGT-treatment was combined with orchiectomy. Chemical castration (treatment with analogues of LH-RH) might be a good alternative, since chemical castration sometimes has been found an adequate alternative for surgery in experimental pancreatic carcinogenesis (18-20).

On the other hand it can be argued, that humans may be more alike rats in their response to AGT, because it has been suggested that testosterone stimulates the growth of pancreatic ductular adenocarcinomas in humans, like it does with the acinar tumours in rats. A possible explanation may be that the activity of aromatase in human pancreatic adenocarcinomas is higher than in the BOP-treated hamster model. Since we cannot supply any proof from the experiments we performed to support this suggestion, it is not justifiable to draw any conclusions on this extent.

Although (as discussed above) the therapeutic value of AGT might be marginal,

from a scientific viewpoint it may be worthwhile to further elucidate the mechanisms of action of AGT. AGT blocks the conversion of testosterone into oestrogen, hence leading to increased serum testosterone levels and decreased oestrogen levels. Since testosterone is found to enhance and oestrogen to inhibit pancreatic carcinogenesis in rats, AGT-treatment should theoretically result in enhanced pancreatic carcinogenesis in rats. AGT, however, caused opposite effects in our experiments. Therefore, it seems to exert its action via another mechanism. Apart from inhibition of aromatase-activity, AGT has also been found to inhibit the conversion of cholesterol into pregnenolone, resulting in increased adrenal cholesterol and plasma adrenocorticotrophin (ACTH) levels, ultimately leading to adrenal enlargement (21) and sometimes even Cushing's syndrome (22). Although in our experiments the doses of AGT were too low to cause adrenal enlargement, this inhibition of cholesterol conversion might, at least partly, have modulating effects on acinar pancreatic carcinogenesis.

It is possible that AGT exerts its action via an inhibition of pregnenolone formation from cholesterol. In the pancreas, pregnenolone can be converted into progesterone by the enzyme δ-5-3 beta-hydroxysteroid dehydrogenase when coupled with steroid-δ 5-4-isomerase (23). Progesterone receptors have been localized in certain pancreatic neoplasms such as cystic tumours (24,25) and endocrine tumours (26). Consequently, in the latter tumours, AGT treatment may cause beneficial effects. However, immunoreactivity to progesterone receptors could not be determined on human ductular pancreatic adenocarcinomas or normal exocrine pancreas (26). Therefore, if the major effect of AGT is exerted via the progesterone receptors, the absence of modulating effects on pancreatic ductular carcinogenesis in hamsters could be explained by the lack of progesterone receptors.

In contrast to what we expected, the blood testosterone levels were *not* increased in AGT-treated rats or hamsters when compared to untreated animals, at final autopsy. It could be, that the doses (which were based on previously performed experiments) were too low to exert an effect. However, it is more likely, that AGT initially did cause an increase in testosterone levels. In our experiments AGT was given during eight months. Therefore, apart from the mechanisms via ACTH or pregnenolone, it is also possible that the longer lasting increase of testosterone triggered a feed-back mechanism through the pituitary gland, which down-regulated the synthesis of testosterone to normal levels, as such abolishing the AGT effect on testosterone metabolism.

In chapters two and three, it has been described that orchiectomy significantly inhibited the development of (pre)neoplastic acinar lesions in azaserine-treated rats. Body weight gain was also significantly inhibited compared to intact controls, probably

caused by the concomitantly reduced food intake by the orchiectomized rats. In hamsters these growth inhibitory effects were also present, but to a much lesser extent. In hamsters, orchiectomy did not significantly inhibit pancreatic carcinogenesis. Reduction of food intake or increase of energy expenditure has been reported to significantly inhibit the carcinogenic process in various organs (27-30). These data support the hypothesis postulated by Meijers (31), that the inhibitory effects of orchiectomy on pancreatic carcinogenesis might be caused by a reduction in food intake, rather than by a direct effect of the reduction in blood testosterone levels.

In chapter three, the effects of the long-acting somatostatin-analogue Sandostatin on experimental pancreatic carcinogenesis have been described. Although Sandostatin treatment alone inhibited the development of AACN and acinar tumours (which is consistent with the finding of somatostatin receptors in acinar tumours), this analogue did not enhance the inhibition caused by surgical castration in azaserine-treated rats. In BOP-treated hamsters, Sandostatin inhibited the development of pancreatic ductular lesions. Although somatostatin receptors could not be determined on pancreatic ductular tumours in humans (32), the present effects with Sandostatin on ductular tumours induced in hamsters point to a possible beneficial role for Sandostatin in patients with pancreatic cancer. Indeed, Klijn (33) reported that most patients with pancreatic cancer experienced a subjective improvement in the absence of serious side effects after Sandostatin therapy. Therefore, Sandostatin apparently does not act directly on the exocrine pancreatic cells, but rather has an indirect effect, e.g. via the suppression of the secretion and/or action of gastrointestinal hormones such as cholecystokinin, secretin or gastrin (34). Another possible mechanism by which Sandostatin acts, is an inhibitory effect on the activity of the epidermal growth factor receptor (EGFR; 35). Inhibition is caused by a stimulatory activity of somatostatin on the dephosphorylation of EGFR. The EGFR is often reported to be functionally involved in carcinogenesis, not only of the pancreas (36-40), but also in several other epithelial tumours, such as in breast (41-43), colon (44) and prostate (45,46). Therefore, a reduction of the activity of EGFR may result in inhibition of cell proliferation.

It has been demonstrated that the effects of Sandostatin could be enhanced by chemical castration with the luteinising hormone-releasing hormone (LH-RH) analogues Zoladex or Buserelin (47). We demonstrated a similar interaction between Sandostatin treatment and orchiectomy on pancreatic carcinogenesis (chapter three). In azaserine-treated rats, surgical castration significantly inhibited the development of acinar (pre)neoplastic lesions, but Sandostatin treatment did not enhance this effect. In contrast,

in BOP-treated hamsters, inhibitory effects of surgical castration on the development of putative preneoplastic borderline lesions were only found in combination with Sandostatin treatment. Surgical castration alone did not have significant inhibitory effects on the development of ductular lesions in BOP-treated hamsters. From these data it can be concluded that testosterone does not seem to play an important role in the development of ductular tumours induced in hamsters by BOP, whereas an enhancing effect cannot be excluded in the development of acinar pancreatic lesions induced in rats by azaserine. Although the mechanism of action remains unclear, the indirect inhibitory effects on pancreatic carcinogenesis in BOP-treated hamsters exerted by Sandostatin, appeared to be enhanced by surgical castration.

8.2 ROLE OF GROWTH FACTORS IN EXOCRINE PANCREATIC CARCINOGENESIS

In chapter four, decreased epidermal growth factor receptor (EGFR) peptide expression is reported in putative preneoplastic atypical acinar cell nodules (AACN) induced in rats by azaserine. Moreover, in more advanced lesions, such as acinar adenocarcinomas, EGFR expression was absent both on the mRNA and peptide levels. In chapter five it is described, that TGF-α mRNA and peptide levels are increased due to azaserine-treatment, only in the histologically normal acinar cells of the pancreas of azaserine-treated rats. In putative preneoplastic acinar cell lesions and in acinar adenocarcinomas reduced TGF-α mRNA and peptide levels were found. Differences in EGF peptide levels could not be observed, although azaserine-treatment resulted in reduced EGF-mRNA levels in pancreatic homogenates. TGF-α and EGF are both exerting their effects on cell differentiation and proliferation via binding to EGFR. In chapter four it is described that strong EGFR peptide expression was only observed in histologically normal acinar pancreas. Consequently, it can be concluded that, in case TGF-α or EGF have effects on the development of acinar pancreatic cell lesions, this seems to take place very early in the carcinogenic process: during the transformation of histologically normal acinar cells into putative preneoplastic atypical acinar cell foci at the initiation phase.

Because the results discussed above have a descriptive nature -only *correlations* can be observed between carcinogenic stages and growth factor expression- a definitive conclusion cannot be drawn, whether changes in either EGF or TGF-α expressions result in, or are the result of the formation of pancreatic acinar cell lesions. In other words: it

can not be established whether changes in EGF or TGF-α expression are the cause or the result of pancreatic carcinogenesis. Cell line and transgenic animal studies have to be performed to get more insight into the role of EGF, TGF-α and EGFR in acinar pancreatic carcinogenesis. Transgenic mice overexpressing TGF-α, exhibit acinar cell proliferation and develop (pseudo)ductular metaplasia in the exocrine pancreas (48,49). Mice, with a disfunctional TGF-α gene did not demonstrate any abnormalities in their pancreata (50,51). To our knowledge, no studies have been performed trying to initiate pancreatic carcinogenesis in these knock-out mice. It will be interesting to investigate whether it is possible to induce pancreatic tumours in these mice. If pancreatic tumour development is inhibited in these mice compared to control mice, a definitive role for TGF-α as a causal factor in pancreatic carcinogenesis can be proven, if injections of TGF-α enhance the development of experimentally-induced pancreatic lesions in these knock-out mice.

In chapter six, studies on the expression of EGF, TGF-α and the EGFR in normal, preneoplastic and neoplastic hamster pancreas are described. In contrast to acinar cells in rat pancreas, EGFR expression was not detected in acinar cells of hamster pancreas. In hamsters, EGFR expression was located in the larger ducts, characterized by columnar ductular epithelium. High EGFR expression was also found in ductular pancreatic adenocarcinoma cells. As mentioned above, overexpression of EGFR has also been reported in human ductular pancreatic adenocarcinomas. However, immunohistochemical studies did not clearly demonstrate increased EGFR expression on ductular cells of ductular adenocarcinomas, when compared with the ductular cells (of main ducts) in normal pancreatic tissues. Therefore, the conclusion, that EGFR is overexpressed *in* tumourous ductular cells, could not be drawn. The significantly increased EGFR mRNA expression in ductular adenocarcinomas may also be a reflection of the increased number of ductular cells in ductular adenocarcinomas.

TGF-α mRNA expression was also significantly increased in ductular adenocarcinomas induced by BOP in the hamster. Immunohistochemical studies demonstrated increased expression of TGF-α peptides in ductular tumour cells, compared to normal ductular cells. Consequently, there is no doubt of overexpression of TGF-α in ductular pancreatic adenocarcinoma cells. In contrast, *decreased* EGF mRNA levels, which were not accompanied by decreased peptide levels, were demonstrated in neoplastic hamster pancreas. These observations point to the conclusion that TGF-α, but not EGF, might be an important factor in the stimulation of pancreatic ductular proliferation via concomitantly expressed EGFR. Other investigators have demonstrated differential effects of TGF-α binding to EGFR when compared to EGF binding to EGFR (52). In

contrast to EGF, TGF-α does not cause down regulation of EGFR after binding. EGF has a low signal frequency with a long signalling time, whereas TGF-α has a high signal frequency (due to extensive recycling) and TGF-α signalling time is short (53). Therefore, it may be hypothesized, that TGF-α replaces EGF in stimulating pancreatic carcinogenesis via binding to EGFR. Further research will be needed to verify this hypothesis. An observation supporting this hypothesis, is the finding described in chapter five of this thesis, that a replacement of EGF by TGF-α also occurs in acinar pancreatic carcinogenesis initiated by azaserine in rats.

We have found that stimulation of EGFR-overexpressing human pancreatic cancer cell lines such as PANC-1 and COLO-357 by EGF increases the proliferation rate of these cells (unpublished personal observations). From the overexpression of TGF-α with concomitant expression of EGFR in hamster ductular adenocarcinomas a paracrine or autocrine stimulatory role in pancreatic carcinogenesis can be concluded (although it still has to be proven whether this mechanism really is a necessary (rate-limiting) step in the multistep process of exocrine pancreatic carcinogenesis). It is harder to understand how a decrease in TGF-α, EGF and EGFR expression, such as observed in our experiments with rats, could contribute to the development of exocrine pancreatic tumours. Several suggestions have been proposed, pointing to a bivalent role of EGFR in rat acinar cells. As pointed out above, EGFR (over)expression is often found to be involved in increased cell proliferation or cellular transformation, also in rat pancreatic acinar cells (54-57). However, Morisset *et al.* (58) demonstrated decreased thymidine incorporation into pancreatic DNA in male Sprague-Dawley rats due to EGF-treatment, and Brockenbrough *et al.* (59) demonstrated reduced EGF binding in regenerating rat pancreas after partial pancreatectomy. Therefore, it may be concluded, that in the situation of pancreatic carcinogenesis in azaserine-treated rats, the abrogation of negative constraints, caused by EGFR, leads to increased acinar cell proliferation.

The concomitantly increased cell proliferation and EGFR reduction, can also be explained by the presence of EGFR-like peptides; which take over the function of EGFR in such a way, that EGFR expression could be down-regulated. The most likely candidate is the EGFR-like peptide c-erbB2 (in the rat: neu). This peptide has an almost identical intracellular domain as the EGFR, but it lacks the extracellular (ligand binding) region. Therefore, it has been suggested that c-erbB2 is the oncogenic counterpart of EGFR, because it is constitutively in the activated state, independent of an extracellular signal. c-ErbB3 and c-erbB4 also have structural similarities with EGFR, but they have extracellular domains and are known to bind other peptides. In order to investigate the involvement of these peptides in rat and hamster pancreatic carcinogenesis, we

performed an immunohistochemical study, using polyclonal antibodies directed against neu, c-erbB3 and c-erbB4 (Santa Cruz Biochemicals, CA, USA) using the method described for EGFR in chapter four.

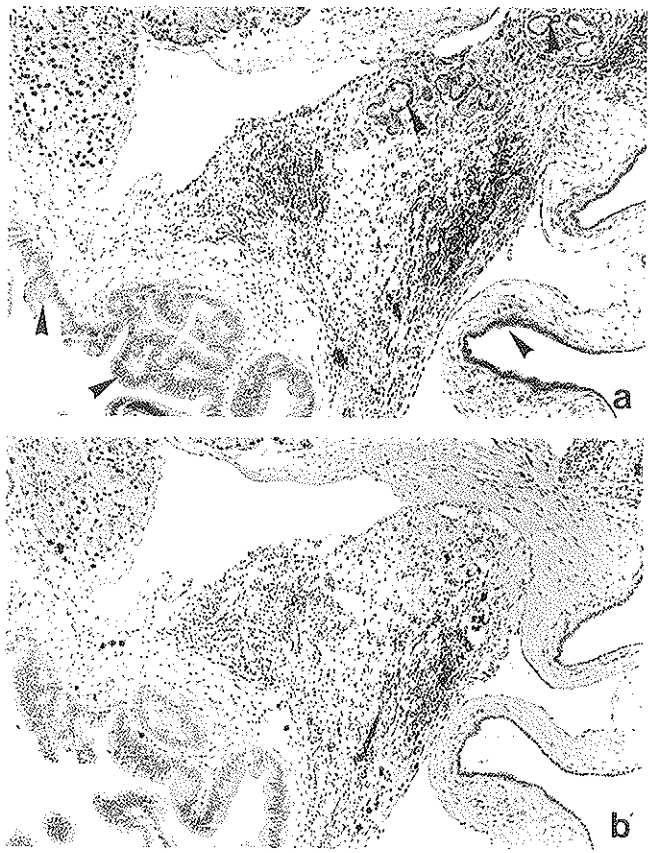

Figure 8.2 DAB-immunohistochemistry in the pancreas of a BOP-treated hamster, counterstained with haematoxylin. a. Localization of c-erbB3 mainly in ductular cells (arrowheads); b. negative control after absorption of the c-erbB3 polyclonal antibody with a specific epitope.

From these studies it appeared that none of the normal or pathological *rat* pancreatic tissues showed immunoreactivity for these antigens. In contrast, in pancreatic tissues of BOP-treated *hamsters*, advanced ductular tubular complexes, putative preneoplastic borderline lesions and ductular adenocarcinomas demonstrated (often strong) immunoreactivity for all these antibodies. The immunoreactivities were localized both in the cytoplasm and on the membranes, and could be abolished by absorbing the

primary antibodies with control-peptides; indicating epitope specificity of the observed staining patterns (**Figure 8.2**). It was concluded, that EGFR expression in rat acinar pancreatic cells is not 'substituted' by an EGFR-like peptide. In contrast, in hamsters, all EGFR-like peptides are present in advanced pancreatic lesions, pointing to a possible role for these peptides in pancreatic carcinogenesis in BOP-treated hamsters. The findings in hamsters are in agreement with the findings observed in human pancreatic carcinogenesis, where overexpression of c-erbB2 (60) has also been reported in ductular adenocarcinomas. To our knowledge, no studies have been published yet, on the expression of c-erbB3 and -B4 in exocrine pancreatic cancer.

Comparison of the present results obtained in rats and hamsters with the results described in human pancreatic cancer, clearly demonstrates that the hamster model is more similar to humans than the rat model. Actually, most results in the rat are different (sometimes even the opposite) from the results obtained in humans or hamsters. This is most probably related to the difference in histology. Apparently, the acinar pancreatic tumours induced in rats by azaserine develop via different (molecular) biological mechanisms, than the ductular pancreatic tumours induced in hamsters by BOP. However, these differences do not exclude acinar involvement in the development of ductular tumours. On the contrary, the increased acinar cell proliferation in pancreata of BOP-treated hamsters, which we reported recently (61), indicates the possibility of involvement of acinar cells in ductular carcinogenesis. Therefore, the azaserine-rat, still has to be considered a valuable model to study exocrine pancreatic carcinogenesis.

In almost all studies described in this thesis, the development of pancreatic tumours could be modulated either by hormones or by dietary factors. For example, caerulein has repeatedly been demonstrated to be a strong enhancer of acinar pancreatic carcinogenesis, whereas orchiectomy had inhibitory effects. In numerous publications, high fat diets have been reported to stimulate pancreatic carcinogenesis, both in hamsters and rats (62-71) and also in humans (72-74). In chapter six, hamster pancreatic carcinogenesis was stimulated by maintaining one group of animals on a high fat diet. In a similar experiment performed in azaserine-treated rats (chapter five), one group of animals was also given a high fat diet (**Table 8.1**). However, in these rats, we did not observe any enhancing effect of fat on pancreatic carcinogenesis. In contrast, the number of lesions was even slightly lower than in the low fat control group (**Table 8.1**). An explanation for this unusual finding can be found in the composition of the high fat diet. In order to make the high fat and the low fat diets isocaloric, in the high fat diet the

high-energy starch component was substituted with the low-energy fibre component cellulose (**Table 8.2**).

Table 8.1 Number of pancreatic lesions in azaserine-treated rats after 6 or 15 months post-initiation treatments[a]

(Pre)neoplastic lesions observed	No. of lesions			
	Low fat		High fat	
	6 months	15 months	6 months	15 months
No. of rats				
No. of tumour-bearing animals (%)[b]	15	19	15	16
AACN (∅1-3 mm)[c]	-	6 (32)	-	4 (25)
Adenoma	1	15	4	21
Carcinoma *in-situ*	0	3	0	1
Adenocarcinoma	0	5	1	1
	0	6	0	3
Total no. of carcinomas				
	0	11	1	4

[a]Values are totals per group. Data are based on H&E-stained paraffin sections. The untreated controls showed no histological abnormalities in the pancreas based on H&E-stained sections. [b]Tumour-bearing animal, animal that bears one or more carcinoma (*in-situ*) in the pancreas at final autopsy. Statistics: no. of tumour-bearing animals, χ^2-test; no. of lesions, 2-sample *t* test. [c]AACN, Atypical Acinar Cell Nodule.

Several studies demonstrated inhibitory effects of fibres not only on pancreatic cancer (75-77), but also on colon cancer (78-81) and breast cancer (82). Moreover, in several studies fibres have been demonstrated to be degraded in the colon by bacteria into short-chain fatty acids, such as butyrate (83). Butyrate has been found to inhibit cell proliferation and to promote cell differentiation *in vitro* (84,85), and also *in vivo* (86-89). Apart from a mechanism via butyrate (fermentation of the fibre component cellulose probably does not result in a high butyrate production in comparison with e.g. fermentation of wheat bran), high fibre diets also cause increased intestinal motility, resulting in an increased faecal excretion rate. Increased faecal excretion rates will result in increased excretion of potentially harmful compounds (fat) to be absorbed into the body. Therefore, it seems conceivable to conclude, that the high concentration of

cellulose in the high fat diet caused the inhibition of the carcinogenic process. This inhibition appeared to be that strong, that the promoting effect of high fat on pancreatic carcinogenesis was completely abolished. These results point to an inhibitory effect of high fibre diets on pancreatic carcinogenesis. This conclusion is supported by a previous study, in which a fibre-rich crude diet demonstrated inhibitory effects on azaserine-induced pancreatic carcinogenesis in rats, when compared to rats fed a semi-synthetic AIN-diet (90).

Table 8.2 Weight percentage composition of the AIN76-based diets[a]

	Low fat	High fat
Casein	25.0	25.0
DL-Methionine	0.38	0.38
Wheat starch	54.47	20.72
Cellulose	7.5	26.25
Choline bitartrate	0.25	0.25
AIN-76-AM minerals	4.38	4.38
AIN-76-AM vitamins	1.25	1.25
CaH_2PO_4	1.77	1.77
Corn oil	5.0	20.0
Total	100.0	100.0
Calories (MJ/kg)	15.5	15.5

[a]The diets were prepared freshly every 2 months. All diets were stored at -20°C until use.

One of the main aims of the studies presented in this thesis was to investigate whether growth factors play a role in the modulation of pancreatic carcinogenesis by diet or hormones. From our studies it appears that EGF, TGF-α and EGFR only play a minor role (if any) in the modulation of pancreatic carcinogenesis in rats and hamsters by caerulein, caloric restriction or fat. Since we have studied only EGF and TGF-α, other growth factors still may be involved, or other dietary factors or hormones may modulate pancreatic carcinogenesis via growth factors. However, immunohistochemical studies performed in our laboratories to investigate the expression of IGF-II, PDGF-A and PDGF-B also did not demonstrate any involvement of these growth factors in the modulating effects of caerulein, caloric restriction, or fat (unpublished results), indicating

that our findings with EGF, TGF-α and EGFR may be representative for the role of growth factors in pancreatic carcinogenesis.

8.3 ROLE OF C-SRC IN EXPERIMENTAL EXOCRINE PANCREATIC CARCINOGENESIS

In chapter seven a study investigating the expression and activity of the cellular proto-oncogene c-src in the pancreas of azaserine-treated and untreated rats has been described. Both EGFR (chapter five) and c-src expression (chapter seven) appeared to be decreased in acinar pancreatic adenocarcinomas induced by azaserine in the rat. Recent studies in various human cancer cell lines point to a role of c-src in EGFR-mediated signalling (91,92). The observed correlation between c-src and EGFR expression in our experiments, might also suggest a functional linkage between those proteins in the exocrine pancreas of azaserine-treated rats.

In chapter seven, we reported translocation of c-src from the cytosol to the cytoskeleton due to carcinogen treatment, from which we suggested a functionally role for c-src in organizing the cell architecture. Cell-surface integrin receptors are thought to be involved in (pancreatic) tumour invasion through the basement membrane (93). Recent studies point to the importance of the association of integrins with cytoskeletal proteins such as α-actinin (αA), vinculin (Vin), talin and paxillin (Pax), for the construction of signalling complexes (94) (**Figure 8.3**). The peptides mentioned above are also closely related with α-actinin and actin cytoskeleton filaments. One model for integrin signalling suggests that integrin clustering activates focal adhesion kinase (FAK), inducing its phosphorylation on a tyrosine and subsequent binding to c-src. c-Src may then phosphorylate another tyrosine on FAK, ultimately resulting in Ras activation. EGFR-signalling (e.g. after binding EGF or TGF-α) has also been found to increase c-src expression and activation, especially in cytoskeleton subcellular fractions (92). Moreover, it has been suggested that EGFR may influence key-steps in the processes of tumour invasion and dissemination (95,96). Since integrins are involved in extracellular matrix binding and, therefore, are thought to be involved in invasive and metastatic capacity of tumour cells, it is tempting to hypothesize that the influence of EGFR on cellular invasiveness and metastatic capacity is mediated through integrins, possibly via c-src in a way as suggested in **Figure 8.3**.

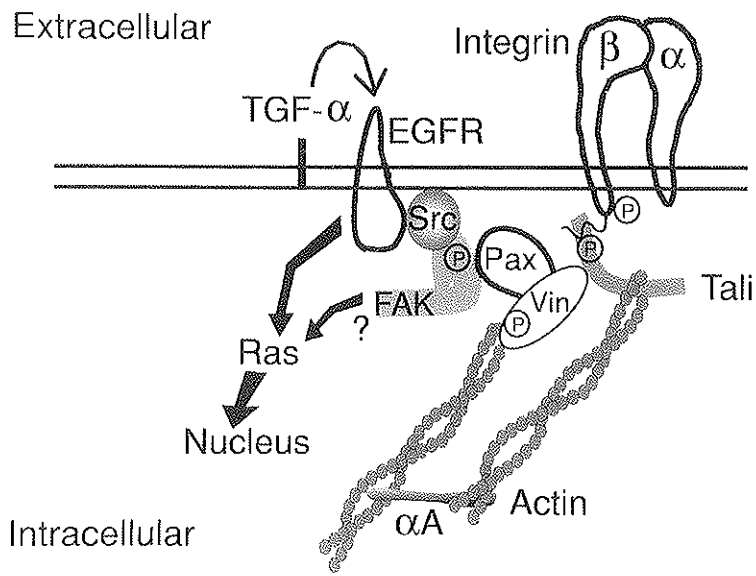

Figure 8.3 Schematic representation of a hypothetic functional correlation between EGFR, c-src, cytoskeleton elements and cell adhesion elements. P indicates possible phosphorylation sites for c-src and/or EGFR. See text for explanation.

The results of the present experiments in rats point to a role for EGFR and c-src early in the carcinogenic process; immediately after initiation by azaserine, causing the development of putative preneoplastic atypical acinar cell nodules (AACN) from histologically normal acinar cells. Both EGFR mRNA expression and c-src activity and expression were significantly increased in AACN, whereas EGFR protein localization was disturbed, in comparison with histologically normal pancreas or pancreas homogenates from untreated control rats. Because we studied c-src in the acinar model for pancreatic carcinogenesis only, the relevance of c-src in the ductular carcinogenic process in human pancreas still has to be elucidated. In recent experiments with the human pancreatic cancer cell lines PANC-1 and COLO-357 (a generous gift from prof. dr. M. Korc, University of California in Irvine, USA) we demonstrated mRNA coding for c-src (unpublished results), indicating that there might be a role for c-src in human pancreatic cancer. Further studies with these cell lines are in progress, in order to examine whether c-src plays an essential role in (EGF-induced) cell proliferation. Moreover, studies are in progress, to examine the expression and activity of c-src in BOP-treated hamsters, the model for ductular pancreatic carcinogenesis. In this model c-src expression was also demonstrated in ductular lesions (unpublished results).

8.4 SUMMARIZING CONCLUSIONS

From the data presented in this thesis, the following conclusions were drawn:

1. Sex hormones as well as growth factors are involved in the development of pancreatic acinar and ductular tumours, induced in rats and hamsters by azaserine and BOP, respectively.
2. Aminoglutethimide does not inhibit ductular pancreatic carcinogenesis in hamsters and consequently, it does not seem to be of any therapeutic value in the treatment of human pancreatic ductular adenocarcinomas.
3. Aminoglutethimide, especially in combination with surgical castration, inhibits the development of acinar adenocarcinomas induced in rats by azaserine. Therefore, aminoglutethimide might have a therapeutic potency for treatment of pancreatic acinar adenocarcinomas, which occasionally occur in humans.
4. The somatostatin analogue Sandostatin inhibits, particularly in combination with surgical castration, the development of ductular adenocarcinomas induced in hamsters, and to a lesser extent the development of acinar adenocarcinomas induced in rats. Therefore, Sandostatin might be of therapeutic value for the treatment of human pancreatic tumours.
5. In azaserine-treated rats, the expression of transforming growth factor-α (TGF-α), epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR) is reduced in putative preneoplastic and absent in neoplastic acinar cell lesions. However, azaserine-treatment caused an increase in TGF-α expression, accompanied by a decrease in EGF expression in histologically normal pancreatic acinar cells. It was concluded that these growth factors may play a role in pancreatic acinar carcinogenesis in rats at an early stage, during the transformation of histologically normal acinar cells into putative preneoplastic atypical acinar cell foci.
6. In BOP-treated hamsters, TGF-α and EGFR, but not EGF, are overexpressed in pancreatic ductular adenocarcinomas. Therefore, TGF-α might have a paracrine or autocrine stimulatory effect via binding to EGFR on the development of pancreatic ductular adenocarcinomas.
7. Both in azaserine-treated rats and in BOP-treated hamsters, EGF appears to be replaced by TGF-α in the carcinogenic process.
8. Both in azaserine-treated rats and in BOP-treated hamsters, TGF-α, EGF or EGFR are most probably not involved in the modulation of pancreatic carcinogenesis by

dietary fat, caloric restriction and caerulein injections.

9. With respect to the expression of EGF, TGF-α and EGFR, pancreatic tumours induced in hamsters by BOP correspond better to human pancreatic tumours, than the pancreatic tumours induced in rats by azaserine.
10. Increased c-src immunoreactivity and c-src protein tyrosine kinase (PTK) activity was observed in putative preneoplastic atypical acinar cell nodules induced in rats by azaserine. However, c-src peptides were not detectable in more advanced lesions. From these results it was concluded, that c-src (alike EGF, TGF-α and EGFR) might be involved in the carcinogenic process of azaserine-treated rats at an early stage.
11. The increase of c-src PTK-activity in the cytoskeleton subcellular fraction in pancreatic carcinogenesis initiated in rats by azaserine, suggests a functional role for c-src in regulating the cell-architecture during cell proliferation.

Overall it was concluded that growth factors most probably do not play a prominent role in the modulation of exocrine pancreatic carcinogenesis by dietary factors or hormones. The investigation of intra-cellular factors, such as c-src, might offer more insight into the mechanisms by which hormones and diet modulate exocrine pancreatic carcinogenesis.

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SUMMARY

The present thesis deals with the role of hormones and growth factors in exocrine pancreatic carcinogenesis. Patients suffering from exocrine pancreatic cancer have an expected five-year survival of 2-3%. This very poor prognosis is caused by the propensity of exocrine pancreatic cancer to have metastasized at the time of diagnosis. Therefore, the lack of early diagnosis and subsequent adequate treatment make exocrine pancreatic cancer a leading cause of death due to cancer. Since early diagnosis and treatment still have to be improved considerably, prevention seems to be the most promising way to deal with pancreatic cancer. For this reason, research on the modulating effects of diet and hormones on pancreatic cancer, are highly relevant. In chapters two and three of this thesis, two studies are described on the role of hormones, viz. testosterone and somatostatin, in pancreatic carcinogenesis. In chapters four to seven studies are described, in which the role of growth factors in the modulating effects of hormones and diet on pancreatic carcinogenesis are investigated.

Two animal models were used for these studies. Wistar rats injected i.p. with azaserine develop putative preneoplastic atypical *acinar* cell foci (AACF) within about four months and *acinar* adenocarcinomas after about nine months. Syrian golden hamsters injected s.c. with *N*-nitrosobis(2-oxopropyl)amine (BOP) develop putative preneoplastic tubular *ductular* complexes within about four months and *ductular* adenocarcinomas after about six months. More than 95% of the human exocrine pancreatic tumours have a ductular histology, whereas the rest of these tumours has an acinar or a mixed acinar/ductular structure. The progenitor cell-type from which the pancreatic ductular tumours arise is still a matter of debate in literature. Some investigators are convinced that ductular cells are the progenitor cells, whereas other investigators demonstrated acinar cell involvement in the development of the ductular tumours. Therefore, it is worthwhile to investigate the mechanisms of pancreatic carcinogenesis in azaserine-treated rats as well as in BOP-treated hamsters.

Male sex-hormones are thought to be involved in stimulating the development of pancreatic cancer, since pancreatic tumour incidences are higher in men than in women, and since testosterone levels in patients with pancreatic cancer were found to be significantly lower than in healthy controls. Testosterone is one of the most important sex-hormones in men. In chapters two and three of the present thesis, research is described on the role of testosterone in the development of exocrine pancreatic tumours. In rats, testosterone appeared to enhance pancreatic carcinogenesis, whereas its hormonal counterpart, oestrogen, was found to have an inhibitory effect on this process. In the

hamster model it appeared to be just the other way around: testosterone inhibited, whereas oestrogen enhanced pancreatic carcinogenesis. It was hypothesized, that the conversion of testosterone into oestrogen by the enzyme aromatase, might be responsible for these seemingly conflicting results.

In the study described in chapter two, rats and hamsters were treated as follows: A) surgical castration, B) oral administration of the aromatase inhibitor aminoglutethimide (AGT), or C) both castration and aadministration of AGT. Group D were untreated controls. To mimic therapeutic treatment of human pancreatic cancer, treatments were started four months after carcinogen treatment. At that time pancreatic lesions already had developed, the number of which was established using an extra control group (E), sacrificed four months after initiation of pancreatic carcinogenesis. Our results demonstrated inhibition of exocrine pancreatic carcinogenesis due to castration in rats, but not in hamsters. AGT treatment also inhibited pancreatic carcinogenesis in rats, but slightly increased the pancreatic tumour-load in hamsters. From these results it was concluded, that AGT does not seem useful as a therapeutic modality for human pancreatic ductular tumours. However, it might be useful (especially in combination with castration) for the treatment of pancreatic acinar tumours, which occasionally do develop in humans. Furthermore, the conversion of testosterone into oestrogen does not appear to play an important role in ductular pancreatic cancer induced in hamsters.

In the study described in chapter three, the role of testosterone alone, and in combination with the hormone somatostatin, was investigated. Previous research indicated inhibitory capacities of somatostatin in pancreatic cell proliferation. Therefore, somatostatin may be useful as a drug against pancreatic cancer. The experimental set-up was similar to that described in chapter two. Rats and hamsters were divided into the following groups: A) surgical castration, B) Sandostatin (a long-acting analogue of somatostatin, administrated by subcutaneously implanted osmotic pumps), C) castration and Sandostatin, or D) control group (physiological saline-solution instead of Sandostatin). The results from this study confirmed the enhancing potency of testosterone in the development of acinar tumours in azaserine-treated rats, and the marginal role of testosterone in the development of ductular pancreatic tumours in BOP-treated hamsters. In the rat-model, Sandostatin did not enhance the inhibitory effects of surgical castration. In contrast, in the hamster-model Sandostatin inhibited the development of pancreatic ductular tumours, particularly when combined with surgical castration. From these results it was concluded, that Sandostatin may be useful as a therapeutic modality against human pancreatic ductular adenocarcinomas.

Growth factors can be considered locally acting hormones: whereas hormones

mostly act in an endocrine way, growth factors mostly act in a paracrine or autocrine way. In chapters four, five and six of the present thesis, research is described on the involvement of growth factors in the modulating effects of dietary factors or hormones on exocrine pancreatic carcinogenesis. For reasons beyond the scope of this summary, we have focused on the epidermal growth factor (EGF), the transforming growth factor-α (TGF-α) and their mutual receptor, the epidermal growth factor receptor (EGFR).

In chapter four, the expression of EGFR on normal pancreatic cells and on putative preneoplastic AACF and acinar adenocarcinomas induced in rats, is described. Surprisingly, whereas EGFR expression was detected in normal pancreatic cells of the rat, acinar adenocarcinomas did not express EGFR. In putative preneoplastic foci, EGFR mRNA levels were increased. However, immunohistochemical investigations pointed to a change in localization of EGFR: in normal acinar cells EGFR was found both on cell-membranes and in the cytoplasm, whereas in putative preneoplastic lesions, EGFR was localized only in the cytoplasm. From these results, it was concluded that if EGFR plays a role in the development of acinar pancreatic tumours, it ought to be at a very early stage of the carcinogenic process, e.g. during the development of putative preneoplastic acinar lesions (AACF) from histologically normal acinar cells.

In chapter five, EGF and TGF-α expression in pancreas of azaserine-treated rats is described. To investigate the expression of these growth factors during modulation of exocrine pancreatic cancer, the rats were treated as follows: A) low-fat diet (control group), B) promotion by caerulein injections (a cholecystokinin-analogue, known to enhance acinar pancreatic carcinogenesis), C) inhibition by a caloric restricted/high fibre diet. An extra control group (D) consisting of rats not treated with the carcinogen azaserine and maintained on a normal low-fat diet was included. From these experiments it appeared that both EGF and TGF-α expression decreased in putative preneoplastic AACF and acinar adenocarcinomas. However, after injection of the carcinogen azaserine, an increase in TGF-α mRNA and a decrease in EGF mRNA were detected in pancreatic homogenates. Immunohistochemical studies revealed that these effects occurred in histologically normal acinar cells of the pancreas of azaserine-treated rats. Although caerulein enhanced and caloric restriction inhibited the development of acinar pancreatic lesions in the rat, these treatments did not affect the expression of EGF or TGF-α. From these results it was concluded that TGF-α (but probably not EGF) may exert an enhancing effect on acinar cell proliferation early in pancreatic carcinogenesis. This conclusion was supported by the finding described in chapter four, that EGFR (the receptor for TGF-α) is expressed in the pancreas of azaserine-treated rats, also only during the development of early AACF from normal acinar cells.

The experiments described in chapter six were similar to those described in chapters four and five, but performed in BOP-treated hamsters instead of azaserine-treated rats. In BOP-treated hamsters, pancreatic cancer was also inhibited by caloric restriction, or enhanced by caerulein. Previous studies demonstrated conflicting results when hamsters were injected with caerulein. Some investigators reported inhibition, while others reported enhancement of ductular pancreatic carcinogenesis by caerulein. Therefore, apart from the caerulein-treated group, an extra group maintained on a high-fat diet was included to stimulate ductular pancreatic carcinogenesis in BOP-treated hamsters. The results from these experiments demonstrated overexpression of TGF-α and EGFR in pancreatic tumours in BOP-treated hamsters. The expression of EGF, however, appeared to change hardly at the protein level, whereas a decrease was observed at the mRNA level. In this animal model the modulation of pancreatic carcinogenesis also did not cause any change in EGF, TGF-α or EGFR expression. The overexpression of TGF-α with a concomitant expression of EGFR in BOP-induced pancreatic tumours suggest a (paracrine or autocrine) stimulatory role for TGF-α in the development of ductular pancreatic tumours in hamsters.

c-Src is a cytoplasmic protein with protein tyrosine kinase (PTK)-activity, recently found to be involved in EGFR-mediated signal transduction. Therefore, research was performed, described in chapter seven of the present thesis, to investigate the expression and the activity of the proto-oncogene *c-src* in normal and (pre)neoplastic pancreas of azaserine-treated rats. In putative preneoplastic AACF both c-src protein levels and c-src PTK-activity increased significantly, mainly in the cytoskeleton subcellular fraction. However, in more advanced lesions such as secondary transformed acinar "nodules-in-nodules", c-src protein could not be detected. From these results it was concluded, that c-src might be involved at an early stage of pancreatic carcinogenesis induced in rats by azaserine. The cytoskeletal localization of c-src in preneoplastic pancreas suggests involvement of c-src in the organization of cell architecture in the early phase of pancreatic carcinogenesis.

The findings described in the present thesis have been summarized and discussed in chapter eight, which resulted in the hypothesis that TGF-α, through binding EGFR, via c-src, might enhance the capacity of cancer cells to become invasive and ultimately, to metastasize.

From the data presented in this thesis, the following conclusions were drawn:

1. Sex-hormones and growth factors are involved in the development of pancreatic

acinar and ductular tumours, induced in rats and hamsters, respectively.

2. Aminoglutethimide is not useful as a therapeutic modality for the treatment of ductular pancreatic tumours.
3. Aminoglutethimide, especially in combination with surgical castration, might have therapeutic potential for the treatment of acinar pancreatic tumours.
4. Sandostatin may be useful for the treatment of pancreatic cancer.
5. TGF-α, EGF and EGFR most probably play a role in acinar pancreatic carcinogenesis induced in rats by azaserine, but only at an early stage: during the transformation of histologically normal acinar cells into putative preneoplastic AACF.
6. TGF-α might have a paracrine or autocrine stimulatory effect on the development of pancreatic ductular adenocarcinomas via binding to EGFR.
7. In azaserine-treated rats and in BOP-treated hamsters, EGF appears to be replaced by TGF-α in the carcinogenic process.
8. In azaserine-treated rats and in BOP-treated hamsters, EGF, TGF-α and EGFR are most probably not involved in the modulation of pancreatic carcinogenesis by dietary fat, caloric restriction or caerulein injections.
9. With respect to the expression of EGF, TGF-α and EGFR, ductular pancreatic tumours induced in hamsters by BOP resembles human pancreatic tumours more, than acinar pancreatic tumours induced in rats by azaserine.
10. c-Src might be involved in pancreatic carcinogenesis in azaserine-treated rats at a rather early stage.
11. A functional role is suggested for c-src in regulating cell-architecture during pancreatic acinar cell proliferation in azaserine-treated rats.

Overall it was concluded that growth factors most probably do not play a prominent role in the modulation of exocrine pancreatic carcinogenesis by dietary factors or hormones. The investigation of intra-cellular factors, such as c-src, might offer more insight into the mechanisms by which hormones and diet modulate exocrine pancreatic carcinogenesis.

SAMENVATTING

In dit proefschrift zijn experimenten beschreven, waarin de rol van hormonen en groeifaktoren bij het ontstaan van pancreaskanker onderzocht werd. De *pancreas* (= de alvleesklier) is een in de buikholte gelegen orgaan, dat grofweg twee funkties vervult. 1) Het zogenaamde *endocriene deel van de pancreas* (de eilandjes van Langerhans) zorgt voor het op peil houden van de suikerspiegels in het bloed. Insuline is één van de belangrijkste hormonen die daarvoor door de pancreas worden gemaakt en aan het bloed worden afgegeven. Soms treedt er een defect op in de insuline-produktie door de pancreas, hetgeen kan leiden tot suikerziekte. 2) Het zogenaamde *exocriene deel van de pancreas* zorgt voor de produktie van verterings-enzymen. Voorbeelden van deze verteringsenzymen zijn chymotrypsine en trypsine, die afgegeven worden aan de dunne darm, waar ze helpen bij de vertering van eiwitten. Zowel in het endocriene, als het exocriene deel van de pancreas komt kanker voor. In dit proefschrift wordt echter alleen onderzoek beschreven, uitgevoerd aan tumoren die zijn ontstaan in het exocriene deel van de pancreas (Zie ook Figuur 1.1, in de inleiding).

Kanker van de exocriene pancreas is over het algemeen ernstiger dan kanker van de endocriene pancreas. De reden is, dat kanker van de exocriene pancreas vrijwel altijd te laat ondekt wordt. In tegenstelling tot kanker van de endocriene pancreas, waar de patienten vaak al in een redelijk vroeg stadium met ernstige verschijnselen van suikerziekte een dokter raadplegen, hebben patienten met exocriene pancreas kanker heel lang geen klachten. Tegen de tijd dat ze wel klachten krijgen (vaak pijn, uitstralend naar de rug), is het daardoor meestal al te laat voor adequate behandeling; de tumor heeft zich al uitgezaaid naar andere organen (lever, milt, longen). Pancreastumoren zijn ongevoelig voor de meeste vormen van chemotherapie. Soms wordt er nog wel eens een ingrijpende operatie uitgevoerd ("een Whipple operatie"), dit leidt echter nauwelijks tot een verbetering van de prognose. Mede door het ontbreken van een afdoende therapie, is de prognose van exocriene pancreas kanker erg slecht; na vijf jaar zijn nog slechts 2 of 3 van de 100 mensen waarbij pancreaskanker werd vastgesteld, in leven. Omdat de therapeutische mogelijkheden beperkt zijn, is het -náást onderzoek naar betere therapieën- van groot belang, meer over de oorzaak en het verloop van deze ziekte te weten te komen, zodat pancreaskanker in een vroeger (nog behandelbaar) stadium vastgesteld kan worden, of -beter nog- geheel voorkómen kan worden.

De exocriene pancreas bestaat globaal uit twee typen cellen: kliercellen (= *acinaire cellen*), die de verteringsenzymen produceren en uitscheiden en cellen, die de afvoerbuisjes (= ductuli) vormen waardoor de verteringsenzymen worden afgevoerd naar

de dunne darm, deze cellen worden de *ductulaire cellen* genoemd. Doordat er in de exocriene pancreas dus grofweg twee typen cellen te onderscheiden zijn, zijn er ook twee typen kanker te onderscheiden; acinaire tumoren (= kliercel tumoren) en ductulaire tumoren (= tumoren aan de afvoerbuisjes).

Het in dit proefschrift beschreven onderzoek is uitgevoerd bij ratten en hamsters (alleen mannetjes). De ratten worden gebruikt als model voor de acinaire pancreastumoren, de hamsters worden gebruikt als model voor de ductulaire tumoren. Ratten van ongeveer drie weken oud worden in de buikholte ingespoten met *azaserine*, een stof die er voor zorgt, dat de dieren acinaire pancreastumoren gaan ontwikkelen. Al na vier tot zes maanden zijn er bij deze ratten microscopisch kleine afwijkingen in de pancreas te zien, die als voorstadia van de uiteindelijke tumoren beschouwd kunnen worden; de zogenaamde '*preneoplastische* acinaire *cel-lesies*'. Na ongeveer 9-12 maanden zijn de eerste acinaire tumoren in de pancreas aantoonbaar. In het algemeen wordt een ratte-proef na 12 tot 15 maanden beëindigd. Om ductulaire pancreastumoren op te wekken bij hamsters, worden deze als ze zo'n zes weken oud zijn, onderhuids ingespoten met een stof die *N*-nitrosobis(2-oxopropyl)amine (afgekort: *BOP*) heet. Hierna ontwikkelen de eerste preneoplastische ductulaire cel-lesies zich al na ongeveer twee tot vier maanden en de eerste ductulaire tumoren kunnen na ongeveer acht maanden aangetoond worden. Omdat hamsters in het algemeen wat eerder dood gaan dan ratten, wordt een hamster-proef meestal al na 12 maanden beëindigd.

Bij de mens is meer dan 95% van de exocriene pancreastumoren van het ductulaire type. De rest van de exocriene pancreastumoren is van het acinaire of van een gemengd acinair/ductulair type. Daardoor lijkt het hamster-model beter vergelijkbaar met de mens, dan het ratte-model. Het is echter nog steeds van belang om het ratte-model ook te bestuderen, omdat het cel-type van waaruit de ductulaire tumoren in de mens zijn ontstaan, nog niet met zekerheid is vastgesteld. Er zijn onderzoekers die denken, dat alleen de ductulaire cellen ontsporen, ongeremd gaan delen en daardoor de ductulaire tumoren vormen, terwijl een andere groep onderzoekers denkt, dat het juist de acinaire cellen zijn, die ontsporen, ductulair worden, en daarna pas uitgroeien tot tumoren. Om meer inzicht te krijgen in de mogelijke rol van acinaire cellen bij het ontstaan van humane exocriene pancreastumoren, is het daarom van belang ook het acinaire ratte-model te bestuderen.

Omdat pancreaskanker meer voorkomt bij mannen, dan bij vrouwen, is het aannemelijk te veronderstellen, dat hormonen die betrokken zijn bij de geslachtsdifferentiatie bij de man, het ontstaan van pancreaskanker bevorderen. Eén van de belangrijkste geslachtshormonen bij de man is testosteron. In de hoofdstukken twee

en drie van dit proefschrift, wordt de rol van testosteron bij het ontstaan van exocriene pancreas kanker beschreven. Eerder onderzoek in ratten heeft aangetoond, dat testosteron een versterkend (= promoverend) effect op het onstaan van pancreaskanker (= pancreas *carcinogenese*) had, terwijl de hormonale tegenhanger van testosteron, oestrogeen, een remmend effect op dit proces zou hebben. In het hamster-model blijkt dit juist omgekeerd te zijn; oestrogeen promoveert, testosteron remt. De omzetting van testosteron in oestrogen, veroorzaakt door het enzym aromatase, zou de mogelijke oorzaak van deze schijnbare tegenstelling kunnen zijn. Effecten die van testosteron verwacht werden, zouden dan tegengewerkt kunnen worden, doordat testosteron omgezet wordt in oestrogeen.

In hoofdstuk twee is een onderzoek beschreven met ratten en hamsters die als volgt behandeld werden: A) gecastreerd (testosteron wordt in de testikels gemaakt, dus castratie leidt tot testosteron depletie), of B) ze kregen aminoglutethimide (AGT) via een maagsonde toegediend (AGT is een aromatase remmer waardoor de omzetting van testosteron naar oestrogeen wordt geblokkeerd), of C) ze werden gecastreerd en kregen ook AGT toegediend. Groep D) was een onbehandelde controle groep. Om een behandeling van pancreaskanker bij de mens na te bootsen, werd de behandeling pas vier maanden na de carcinogeen injectie gestart; op het moment dat er al pancreas lesies waren ontstaan. Dit werd gecontroleerd door na vier maanden een extra groep (E) op te offeren en de pancreas te bestuderen. Castratie bleek de ontwikkeling van pancreastumoren in de rat wel, maar in de hamster niet te remmen. AGT behandeling bleek in de rat ook te remmen, maar in de hamster leek er zelfs een verhoging van het aantal pancreastumoren op te treden. Uit deze resultaten werd geconcludeerd, dat AGT niet bruikbaar is als therapeuticum voor humane ductulaire tumoren. Misschien kan het wel gebruikt worden (vooral in combinatie met castratie) voor de behandeling van de -zeldzame- acinaire pancreastumoren bij de mens. Verder bleek uit het onderzoek dat het niet erg waarschijnlijk is, dat de omzetting van testosteron naar oestrogeen een belangrijke rol speelt bij de ductulaire pancreas carcinogenese.

In hoofstuk drie werd ook de rol van testosteron bij de pancreas carcinogenese onderzocht, maar nu in combinatie met een ander hormoon; het somatostatine. Uit eerder onderzoek bleek, dat somatostatine een remmend effect heeft op de celdeling in pancreastumoren. Daaruit kan worden afgeleid, dat somatostatine misschien wel eens gebruikt kan worden als therapeuticum tegen pancreaskanker. In hoofdstuk drie wordt een onderzoek beschreven, naar het effect van castratie op de effecten van somatostatine. Als lang-werkend analoog voor somatostatine gebruikten we het geneesmiddel Sandostatin (somatostatine zélf wordt erg snel afgebroken na toediening). De proefopzet

was weer hetzelfde als die in het vorige hoofstuk; start van behandeling na vier maanden, vier groepen: A) castratie, B) Sandostatin (onderhuidse pompjes gaven het geneesmiddel in constante doses af), C) castratie + Sandostatin, D) controle groep (zij kregen een fysiologische zout-oplossing, i.p.v. Sandostatin). De resultaten van deze studie bevestigden de geringe rol van testosteron bij het ontstaan van ductulaire pancreastumoren in het hamster-model en een promoverende rol voor testosteron bij het ontstaan van acinaire tumoren in het ratte-model. In dit laatste model bleek Sandostatin geen versterkend effect op de gevolgen van castratie te hebben. In het hamster model daarentegen, werd de ontwikkeling van pancreastumoren geremd, vooral wanneer Sandostatin gecombineerd werd met castratie. Hieruit werd geconcludeerd, dat Sandostatin misschien wel als therapeuticum voor de behandeling van ductulaire pancreaskanker bij de mens gebruikt kan worden.

De rest van het onderzoek beschreven in dit proefschrift, is meer fundamenteel van aard; er wordt nagegaan waarom normale pancreascellen zich soms tot tumoren ontwikkelen. Wij hebben daarbij de rol die groeifaktoren in dit proces zouden kunnen spelen onderzocht. Groeifaktoren zijn in principe vergelijkbaar met hormonen, met dit verschil, dat hormonen (b.v. testosteron) in een bepaald orgaan (b.v. de testikels) geproduceerd worden, dan aan de bloedbaan afgegeven worden en vervolgens in een ander doel-orgaan (b.v. de pancreas) hun funktie vervullen (= een *endocriene werking*), terwijl groeifaktoren meer lokaal werken; zij beïnvloeden cellen in de directe omgeving van de cellen waar ze in geproduceerd worden (= *paracriene werking*), of zij vervullen hun funktie in dezelfde cellen die de groeifaktoren produceren (= *autocriene werking*). Zie ook Figuur 1.4, in de inleiding. Groeifaktoren komen vaak ook in normaal weefsel voor, maar het ontsporen van groeifaktoren (b.v. een overproduktie) zou wel eens de oorzaak voor het ontstaan van een tumor kunnen zijn. Er zijn vele groeifaktoren bekend, maar elk celtype is maar gevoelig voor een 'beperkt' aantal van deze groeifaktoren. In andere woorden; een groeifaktor is vaak specifiek voor bepaalde celtypen. Deze specificiteit wordt bepaald door de aanwezigheid van het juiste type 'groeifaktor ontvangers' (= groeifaktor *receptoren*) op deze cellen. Om redenen waarop in deze samenvatting niet zal worden ingegaan, hebben we gekozen voor het bestuderen van de mogelijke betrokkenheid van de zogenaamde "epidermale groeifaktor" (*EGF*), de "transformerende groeifaktor-α" (*TGF-α*) en hun receptor; de "epidermale groeifaktor receptor" (*EGFR*) bij de exocriene pancreas carcinogenese.

In hoofdstuk vier is onderzoek beschreven naar de expressie van EGFR op normale pancreas cellen en ook op door azaserine opgewekte preneoplastische acinare cel-lesies en op acinaire tumoren in de pancreas van de rat. Tot onze verbazing, bleken acinaire

tumoren geen EGFR meer te bezitten. In de voorstadia blijkt er op synthese-niveau een toename van EGFR te zijn. Echter, indien de aanwezigheid in de cellen onderzocht wordt, blijkt er een re-localisatie plaats te hebben gevonden; in normale acinaire cellen zit EGFR op de cel membraan en *cytoplasmatisch* (= intra-cellulair), terwijl in de preneoplastische lesies EGFR alleen nog maar in het cytoplasma voorkomt. Uit deze resultaten werd geconcludeerd, dat indien EGFR al een rol speelt in de pancreas-carcinogenese in het azaserine-ratte model, dit alleen in een vroeg stadium (bij de overgang van 'normale' acinaire cellen naar preneoplastische acinaire cellen) zal zijn.

In hoofdstuk vijf zijn studies beschreven met de twee belangrijkste groeifaktoren die EGFR binden; EGF en TGF-α. Hiervoor werd ook weer het azaserine-rat model gebruikt. De met azaserine ingespoten ratten werden in verschillende behandelings-groepen ingedeeld waarbij de pancreas-carcinogenese óf gestimuleerd, óf geremd werd door de volgende behandelingen: A) een normaal laag-vet dieet (controle groep), B) een laag-vet dieet en stimulatie van de groei van pancreastumoren met behulp van caeruleine (een analoog van het darm-hormoon cholecystokinine), C) remming van de pancreas carcinogenese, door de ratten een dieet met een verlaagde calorische waarde te geven. Een extra controle groep, bestaande uit ratten die niet met het carcinogeen azaserine geïnjecteerd werden, werd toegevoegd. Uit deze experimenten bleek een afname van zowel EGF als TGF-α in de preneoplastische acinaire cel-lesies en in de acinaire tumoren. Kort na het toedienen van het carcinogeen azaserine, was er wel een toename van TGF-α en een afname van EGF te detecteren. Dit bleek plaats te vinden in de histologisch nog normale acinaire cellen. Ondanks dat caeruleine een significante toename en calorische beperking een duidelijke afname van het aantal acinaire cel-lesies veroorzaakten, hadden deze behandelingen geen effect op de expressie van EGF of TGF-α in de ratte-pancreas. Uit deze resultaten werd geconcludeerd, dat vooral TGF-α, vroeg in de acinaire pancreas carcinogenese een cel-deling stimulerende rol zou kunnen vervullen. Vooral omdat we uit resultaten van de experimenten beschreven in hoofdstuk vier weten, dat de receptor voor TGF-α ook alleen maar op een vroeg tijdstip in de acinaire pancreas-carcinogenese tot expressie komt.

In hoofdstuk zes, zijn weer vergelijkbare experimenten als in hoofdstuk vier en vijf beschreven, maar nu in de BOP-behandelde hamsters, model voor de ductulaire pancreas carcinogenese. Ook nu werd de pancreas carcinogenese gestimuleerd, of geremd. Omdat het niet helemaal zeker was of caeruleine in BOP-behandelde hamsters de ductulaire pancreas-carcinogenese wel zou stimuleren, werd er een groep toegevoegd die een vet-rijk dieet (i.p.v. een normaal laag-vet dieet) te eten kreeg. De resultaten uit deze proeven toonden een overexpressie van TGF-α en EGFR aan in de ductulaire pancreastumoren in

BOP-behandelde hamsters. De expressie van EGF daarentegen, bleek nauwelijks te veranderen, of zelfs wat af te nemen. Ook in dit proefdier-model hadden de verschillende pancreas carcinogenese stimulerende of remmende behandelingen geen effect op de expressie van EGF, TGF-α of EGFR. De overexpressie van TGF-α met een gelijktijdige aanwezigheid van EGFR in BOP-geïnduceerde pancreastumoren suggereert een stimulerende funktie van deze groeifaktor op de cel-vermenigvuldiging in het carcinogenese proces.

Als een groeifaktor (b.v. TGF-α) aan zijn receptor (b.v. EGFR) bindt, wordt hierdoor een signaal aan de cel afgegeven. Dit signaal wordt afgegeven door het intracellulaire deel van de receptor, via -vaak vele- cytoplasmatische eiwitten, naar de kern van de cel. Het afgeven van het signaal door de groeifaktor-receptor naar de kern, wordt *signaal transductie* genoemd. In de kern zit al het erfelijke materiaal (het *DNA*) van de cel in de *chromosomen* opgeslagen. Het signaal van de groeifaktor receptor zorgt er voor, dat het juiste stukje (= het juiste *gen*) van een chromosoom op het juiste moment gebruikt wordt, zodat de juiste funktie in (of door) de cel uitgevoerd kan worden (b.v. de cel gaat delen). De genen coderen namelijk voor bepaalde eiwitten, die op hun beurt hun funktie in de cel weer vervullen. Bij het funktioneren van een normale cel zijn vele genen betrokken. Nu gebeurt het wel, dat er een verandering (= een *mutatie*) in (de regulatie van) een gen optreedt, waardoor deze zich abnormaal gaat gedragen. Soms leidt zo'n verandering tot het ontstaan van tumoren. Een gen, dat na 'activatie' tot gevolg heeft dat er een tumor ontstaat, noemt men een *oncogen*. Als de verandering nog niet heeft plaatsgevonden, noemt men zo'n gen een *proto-oncogen*. *c-Src* is zo'n proto-oncogen, dat betrokken lijkt te zijn bij de signaal transductie van EGFR.

In hoofdstuk zeven wordt de betrokkenheid van c-src bij de acinaire pancreas carcinogenese in de azaserine-rat beschreven. c-Src is een eiwit, dat in het cytoplasma van de cel voorkomt en daar een bepaalde enzym-funktie vervult. Deze enzym-funktie wordt de *tyrosine kinase aktiviteit* genoemd. Het voert te ver om dit in detail uit te leggen, maar van belang is, dat deze aktiviteit een belangrijke parameter is voor de betrokkenheid van c-src in de pancreas carcinogenese. In de preneoplastische acinaire cel-lesies werd een toename van het c-src eiwit en ook van c-src (tyrosine kinase) activiteit gedetecteerd. De meeste c-src aktiviteit werd in het cel-skelet gevonden (net als de mens heeft een cel ook een soort 'skelet'). In tumoren was c-src echter niet meer aanwezig. Uit deze resultaten blijkt dus betrokkenheid van c-src, op een vroeg tijdstip in het acinaire pancreas carcinogenese proces in de azaserine-rat. De lokalisatie van de toegenomen c-src aktiviteit, suggereert betrokkenheid van c-src bij de regulatie van de

cel-architectuur (-vorm) gedurende het carcinogenese proces.

In de algemene discussie (hoofdstuk acht) is uit bovenstaande resultaten een hypothese gedestilleerd, waarin geopperd wordt, dat bij de pancreas-carcinogenese, TGF-α door binding aan EGFR, via c-src, wel eens invloed zou kunnen hebben op de potentie van een kankercel om in ander weefsel door te dringen en zich uit te gaan zaaien (te *metastaseren*).

Uit de in dit proefschrift beschreven experimenten zijn de volgende conclusies getrokken:

1. Zowel geslachtshormonen als groeifaktoren zijn betrokken bij het ontstaan van acinaire of ductulaire tumoren in de pancreas van, respectievelijk, met azaserine-behandelde ratten, of met BOP-behandelde hamsters.
2. Aminoglutethimide is niet bruikbaar voor de behandeling van ductulaire pancreaskanker.
3. Aminoglutethimide zou bruikbaar kunnen zijn als therapie tegen acinaire pancreaskanker.
4. Sandostatine lijkt bruikbaar te zijn voor de behandeling van pancreaskanker.
5. Indien TGF-α, EGF en EGFR een rol spelen bij de ontwikkeling van pancreas-tumoren bij de rat, dan zal dit op een vroeg tijdstip in het carcinogenese proces zijn: met name bij de overgang van normale acinaire cellen naar de pre-neoplastische cel-lesies.
6. TGF-α zal via de EGFR een paracrien of autocrien stimulerend effect op de pancreas-carcinogenese in BOP-behandelde hamsters kunnen hebben.
7. TGF-α lijkt EGF te vervangen in het carcinogenese proces, zowel bij met azaserine-behandelde ratten als bij met BOP-behandelde hamsters.
8. Zowel bij met azaserine-behandelde ratten als bij met BOP-behandelde hamsters, zijn TGF-α, EGF en EGFR hoogstwaarschijnlijk niet betrokken bij de modulering van de pancreas-carcinogenese, door een vet-rijk dieet, een calorisch beperkt dieet, of door caeruleine-injecties.
9. Met betrekking tot de expressie van groeifaktoren is het hamster pancreas-carcinogenese model beter vergelijkbaar met pancreaskanker bij de mens, dan het ratte-model.
10. Het proto-oncogen c-src zou op een vroeg tijdstip betrokken kunnen zijn bij de pancreas-carcinogenese in azaserine-behandelde ratten.

11. De organisatie van het cel-skelet gedurende de cel-vermeerdering, zou een funktie van c-src in de pancreas-carcinogenese bij azaserine-behandelde ratten kunnen zijn.

De algemene conclusie kan worden getrokken, dat bij de toename van pancreaskanker door b.v. een vet-rijk dieet, of bij de afname van pancreaskanker door b.v. een calorisch beperkt dieet, groeifaktoren hoogst waarschijnlijk geen prominente rol spelen. Het bestuderen van intra-cellulaire eiwitten zoals het proto-oncogen c-src, lijkt voor het begrijpen van de mechanismen waarmee b.v. hormonen en diëten de exocriene pancreas carcinogenese moduleren, meer perspectief te bieden.

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VERANTWOORDING

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CURRICULUM VITAE

The author of this thesis (Corjan Visser) was born July 1st, 1964 in Spijkenisse, The Netherlands. He graduated Atheneum-B in August 1983 at highschool in Den Briel, whereafter he started studying Biological Science at the State University Leiden. In an advanced phase of this study, he was put on track of basic research by one of his preceptors, dr. Menno Kruk (Dept. Pharmacology, State University Leiden). He received his "doctorandus" (MSc) degree in Biological Science (medical differentiation) on August 26th, 1989. Then, he did an alternative period for the military army services at the TNO Nutrition and Food Research Institute in Zeist, The Netherlands. In this 16 months' period, he (amongst other things) revised a previously rejected Dutch Cancer Society (KWF) grant application for a PhD-student project. This project entitled "Role of Growth Factors in Experimental Pancreatic Carcinogenesis in Rats and Hamsters: Modulation by Dietary Fat and Cholecystokinin" was funded by the KWF in 1991. Hence, he was appointed as a PhD-student (Assistent-in-Opleiding) on this collaborative project between the departments of Pathology in the TNO-Institute in Zeist and in the University Hospital in Utrecht, under direct supervision of dr. Ruud Woutersen and dr. Roel de Weger, respectively (1991-1995). During this PhD-study he also lived in Irvine, California, USA, where he worked and studied in the laboratory of Prof. dr. Murray Korc for seven months. From September 1995 he is associated with a collaborative project between the Molecular Biological Laboratory of the Department of Pathology in the University Hospital Utrecht (dr. Marcel Tilanus) and the Laboratory of Immunology and Histocompatibility, Saint Louis Hospital in Paris, France (Prof. dr. Dominique Charron). On this project concerning "new genes" in the HLA genomic region, he will start to work in Paris, January 1st, 1996.

"Let's just say I was
testing the bounds of
reality. I was curious to
see what would happen.
That's all it was:
just curiosity."

Jim Morrison (The Doors)
Los Angeles, 1969.

Stellingen

Bij het proefschrift "Hormones and growth factors in experimental exocrine pancreatic carcinogenesis"

1) Zowel hormonen als groeifaktoren zijn betrokken bij de ontwikkeling van kanker aan de exocriene pancreas.

2) Hoewel zowel het hormoon cholecystokinine als bij voorbeeld een hoog-vet dieet kan bijdragen aan het ontstaan van exocriene pancreastumoren, lijkt dit niet te worden gereguleerd via groeifaktoren (in ieder geval voor zover het EGF, TGF-α en de EGFR betreft).

3) Voor het bestuderen van moleculair biologische mechanismen die ten grondslag liggen aan het ontstaan van het ductulaire pancreascarcinoom in de mens, lijkt het hamster-model beter aan te sluiten dan het ratte-model voor pancreas-carcinogenese.

4) Naast een al vaker gedemonstreerd inhiberend effect op de ontwikkeling van colon- en borstkanker, zou de inname van vezelrijk voedsel ook wel eens preventief kunnen werken op het ontstaan van pancreastumoren.

5) Al vroeg in het pancreascarcinogenese-proces lijkt TGF-α de rol van EGF over te nemen.

6) Het is de vraag of de toegenomen economische welvaart in Japan door de 'ver-westerlijking' opweegt tegen de daardoor eveneens toegenomen incidentie van kanker o.a. aan de pancreas.

7) De te hoge bevolkingsdichtheid ligt ten grondslag aan alle sociale, c.q. maatschappelijke complicaties in de hedendaagse samenleving.

8) Stelling 7 leidt er toe, dat het aantal kinderen dat door een (echt)paar van mijn generatie op de wereld wordt gezet, omgekeerd evenredig is met de maatschappelijke betrokkenheid van dit stel.

9) Verdraagzaamheid tegenover de onverdraagzamen is misschien de moeilijkste taak die deze tijd van ons verlangt. (The world according to Garp. John Irving)

10) Naarmate er meer 'boven-natuurlijke' zaken wetenschappelijk verklaard kunnen worden, zal het aantal religieus-gelovigen meer en meer afnemen.

11) In Nederland is de windhonden-rensport 'humaner' dan de paarden-rensport.

12) Motor-rijders zijn betere weggebruikers dan automobilisten.

Corjan Visser, 14 november 1995.