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Hormonal modulation of carcinogenesis chemically induced in exocrine pancreas of rat and hamster

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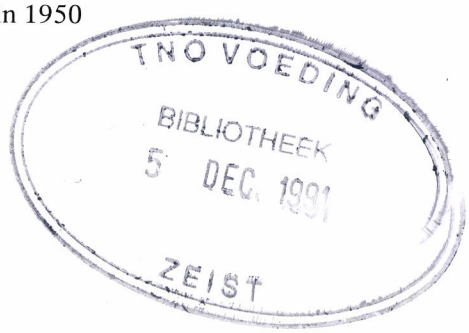

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Het leven is vol ellende en ook nog te snel voorbij

Woody Allen

Abbreviations

AACF	atypical acinar cell focus/foci
AACN	atypical acinar cell nodule(s)
AGT	aminoglutethimide
AR	androgen receptor
BBS	bombesin
BOP	N-nitrosobis(2-oxopropyl)amine
CA	cyproterone acetate
CCK	cholecystokinin
CR-1409	lorglumide
EGF	epidermal growth factor
ER	oestrogen receptor
IGF	insulin-like growth factor
LHRH	luteinizing hormone-releasing hormone
LHRH-A	LHRH agonist
PR	progesterone receptor
RSF	raw soya flour
TI	trypsin inhibitor(s)

Contents

CHAPTER 1

General introduction

Pancreatic cancer is a relatively common disease in most Western countries (1, 2, 3). Like in most other Western countries, the incidence of pancreatic cancer has increased rapidly in the Netherlands in the past decades, both in men and women (1, 2, 3). Early diagnosis of pancreatic cancer is difficult due to the lack of symptoms. Usually no curative therapy is possible at the time of diagnosis. The mortality rate is almost identical to the incidence because of the bad prognosis of pancreatic cancer (2, 4, 5).

In a study in the Dr. Daniel den Hoed Cancer Center concerning 49 patients with unresectable cancer of the pancreas, it appeared that 25% of the patients died within 3 months, 48% within 6 months, 77% within 1 year, while only 6% survived more than 2 years. Therefore, a better insight into the factors involved in the pathogenesis of pancreatic cancer is needed.

Epidemiological and toxicological studies have not identified unequivocally factors increasing the risk for pancreatic cancer (2, 4, 6). Since no factor exclusively responsible for pancreatic cancer has been identified, it is believed that factors promoting the effects of environmental carcinogens play an important role in its pathogenesis (2, 4, 7, 8).

As epidemiological and nutritional studies have shown that the incidence of pancreatic cancer parallels the intake of fat and protein, nutrition seems to play an important role in the pathogenesis of pancreatic cancer (1, 2, 4, 6). For example, in Japan, westernization of the diet was accompanied with a rapid increase in the incidence of pancreatic cancer (2, 4, 6). Because fat and protein are potent stimulants of the secretion of cholecystokinin (CCK), CCK may be an important factor (9).

With respect to nutrition, it has been suggested that CCK, a hormone released from the upper small intestine with trophic effects on the pancreas, is involved in pancreatic carcinogenesis (2, 4, 7, 10–13). Recent studies have shown that CCK is trophic to acinar cells of the pancreas (14–17). Administration of CCK to animals resulted in both hypertrophy and hyperplasia of the pancreas (14–17). CCK probably acts by sensitizing the pancreas to chemical carcinogens (18, 19), but a direct carcinogenic effect of chronically elevated plasma CCK concentrations cannot be excluded. Howatson and Carter suggested that CCK also acts as a co-carcinogen or promotor of pancreatic carcinogenesis in the hamster nitrosamine model (20).

It is believed that the effect of raw soya flour on the pancreas is mediated by increased plasma levels of CCK. This assumption is based on the following findings:

1. Studies in rats have shown that raw soya flour, a diet stimulating the secretion of CCK, induces hypertrophy, nodular and adenomatous hyperplasia, dysplasia and cancer of the pancreas (11).
2. CCK and raw soya flour induce similar changes in the pattern ot pancreatic enzymes (9, 11, 14–17, 21).
3. Proximal intestinal resection, resulting in removal of the CCK-producing cells, prevents the trophic action of raw soya flour on the pancreas (13).
4. Feeding a raw soya flour diet to rats induces increases in plasma CCK as determined by a gall-bladder bioassay (22).

It has further been demonstrated that a raw soya flour diet renders the pancreas more vulnerable to the carcinogenic effect of azaserine and di(2-hydroxypropyl)-nitrosamine (DHPN) in experimental animals (10). In fact, in one study azaserine did not produce pancreatic cancer unless the rats were fed raw soya flour (23). The mechanism underlying the increased plasma CCK concentrations during raw soya flour nourishment is probably related to the presence of heat-labile trypsin inhibitors in raw soya flour. It has been suggested that these trypsin inhibitors interfere with a postulated feedback mechanism between intraduodenal trypsin and CCK secretion (13, 23, 24).

CCK is not only believed to be a mediator in the action of trypsin inhibitors, but also in other gastrointestinal hormones. For example, bombesin appeared to have a strong pancreotrophic effect, which could be mediated by CCK (25–29).

Until now the importance of endogenous CCK in pancreatic carcinogenesis has not been elucidated due to the lack of a reliable immunoassay for CCK. The recent development of a specific and sensitive radioimmunoassay for CCK (30) enabled us to determine the role of endogenous CCK in the pathogenesis of pancreatic cancer. Also animal studies could be conducted to determine whether CCK has promoting or co-carcinogenenic properties with respect to the development of pancreatic cancer induced in hamsters and rats and whether the action can be inhibited by CCK-receptor antagonists.

Besides the possible role of diet, and hence gastrointestinal hormones, in the aetiology of human pancreatic cancer, there is increasing evidence that the pancreas is sensitive to hormones that play a role in sex steroid hormone metabolism. Recently, some hypothalamic hormones have been identified in pancreatic tissue. In addition, high activities of sex steroid biosynthetic enzymes have been measured in pancreatic tumours (31, 32). These enzymes are often found in tissues containing receptors for their respective products (31, 33). These findings suggest that sex steroids are involved in the physiology of foetal, adult and malignant pancreatic tissue. It has been demonstrated in human pancreatic carcinoma xenografts growing in nude mice that testosterone enhanced tumour growth, whilst other hormone therapies such as anti-androgens inhibited tumour growth compared to control tissue (32, 34). It is well known that testosterone may act via the oestradiol receptor after being metabolized to oestrogen by the enzyme aromatase (35, 36). Consequently, on the basis of studies in animal models new approaches to the therapy for hormone-dependent tumours are being developed (37, 38). Such a new approach has been the

use of recently produced analogues of hypothalamic hormones, especially of luteinizing hormone-releasing hormone (LHRH) (37–42). Hypothalamic hormones are not only detected in the hypothalamus, but also in the pancreas and the gastrointestinal tract. These hypothalamic hormones probably have a local regulatory function and cannot only influence cell function, but also DNA synthesis and cell growth (37, 38). Receptors for LHRH are found in different organs and cell types (37, 38, 43). Therefore, analogues of hypothalamic hormones are very interesting with regard to their potential usefulness as therapeutic agents in the treatment of cancer. In experimental studies they appeared effective in the treatment of various tumours (37–42).

Experimental studies are required to further substantiate the role of hormones in carcinogenesis and the value of endocrine treatment modalities in the treatment of pancreatic cancer.

Animal models of pancreatic carcinogenesis

Carcinomas of the pancreas usually refer to neoplasms arising in the exocrine pancreas, whereas neoplasms of the endocrine pancreas are collectively named islet cell tumours. Cubilla and Fitzgerald (44) and Moroshi et al. (45) have classified the various histological types of 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 show tubular structures, which is interpreted as evidence that pancreatic cancer arises from ducts or ductules. However, no conclusive evidence is available yet for this suggestion. 'Spontaneous' pancreatic neoplasms apparently originating from ductal or ductular epithelium are known to be less common in mammals, except in man and hamster (46). On the contrary, tumours apparently arising from acinar cells occur in several species (47–49). Interestingly, in rats prone to spontaneous acinar cell tumours, only this tumour type could be induced experimentally. On the other hand, in Syrian golden hamsters only ductular cell neoplasms could be induced; this type of neoplasm is a common spontaneous disease in this species (50). Acinar cell tumours in hamsters neither occur spontaneously nor under experimental conditions (46).

Pancreatic cancer model in hamsters (46, 50-54)

Pancreatic ductal (ductular) tumours can be induced in Syrian golden hamsters by several specific N-nitroso compounds such as N-nitrosobis(2-hydropropyl)amine (BHP), N-nitroso(2-acetoxypropyl)amine (BAP), N-nitrosomethyl(2-oxopropyl)-amine (MOP) and N-nitrosobis(2-oxopropyl)amine (BOP). BOP has a rather specific pancreotrophic effect with a narrow tumour spectrum in other organs (lungs, liver, gall-bladder and kidneys).

Pancreatic neoplasms can be induced upon repeated weekly s.c. injection of BOP in doses of 10 mg/kg body weight (b.w.), as early as after 8 weeks. After a single dosis

of 2.5 mg/kg b.w. or 40 mg/kg b.w., Pour (46) found neoplastic lesions after 54 and 21 weeks, respectively.

Pancreatic cancer model in rats (55-59)
Induction of pancreatic hyperplastic nodules, adenomas and carcinomas in rats by intraperitoneal injection of azaserine was first reported by Longnecker et al. (55, 56). Adenocarcinomas or poorly differentiated carcinomas developed in the pancreas of rats repeatedly treated with azaserine as early as 11 months after initial azaserine treatment. Two months after azaserine treatment, the earliest manifestations have been found (57). These focal lesions have been described as hyperplastic nodules or atypical acinar cell nodules (AACN). The number of these nodules increased with time upon azaserine exposure and was related to animal age and rate of pancreatic DNA synthesis during growth (58). Wistar and W/LEW rats are highly responsive to nodule induction (59). Virtually all azaserine-treated rats developed multiple nodules after an interval of four months. Female and male rats developed approximately similar numbers of lesions, but the size of the lesions was larger in males than in females.

Histogenesis of pancreatic cancer in experimental animals and its relevance to man

There is no general agreement as to the origin of pancreatic cancer, either in man or in the animal models mentioned above. Pour et al. (53) considered the hamster model to provide a unique opportunity to study pancreatic cancer, especially because of the remarkable similarity of the induced lesions to those occurring in man in terms of morphological, biological and biochemical characteristics. Pour's group stated that the precursor cells are those of putative 'intra-insular' ductules associated with newly formed endocrine islet cells. Pour et al. did not find direct transformation of acinar to islet cells, but stated that many tumours seem to develop within islets (46).

Electron microscopic studies of others (60, 61) suggest that the BOP-induced hamster tumours arise from existing ductules, without acinar characteristics. Electron microscopic investigations by Flaks et al. (62–64), however, provided evidence that hamster pancreatic adenocarcinomas also may develop from acinar cells that have dedifferentiated, frequently resulting in the formation of pseuductular structures and multifocal cystic lesions and ultimately adenomas and adenocarcinomas (65).

In man only a small proportion of tumours, namely those with a clearly recognizable acinar cell differentiation, are classified as being of acinar origin. Most other tumours contain duct-like structures and have been classified as ductal. There is ultrastructural evidence, however, of acinar cell characteristics in all human pancreatic tumours studied by electron microscopy (66, 67). Furthermore, acinar dysplasia appears to be common in patients with pancreatic cancer (68).

Longnecker (69) proposed that early biochemical and perhaps even immunological markers might differ between neoplasms of acinar and those of ductal

cell origin. Furthermore, it seems likely that factors that promote or inhibit progression of early stages of carcinogenesis are different in lesions originating from either cell types. The above considerations indicate that not only the BOP-treated hamster, but also the azaserine-treated rat might be an animal model suitable to provide evidence relevant to man.

Moreover, no conclusive data are available on the relevance of the preneoplastic lesions as precursor of pancreatic carcinomas. Therefore, it was considered worthwhile to study the effects of hormonal manipulation of pancreatic carcinogenesis in both species and to compare the results of chronic studies with the findings of short-term studies.

The main purposes of the investigations described in this thesis were to study:
(1) the role of CCK and CCK-related factors in the development of putative preneoplastic ductular lesions in the pancreas of BOP-treated hamsters;
(2) the role of CCK and CCK-related factors in the development of AACN and acinar adenocarcinomas in azaserine-treated rats;
(3) short and long-term effects of hormonal manipulation of pancreatic carcinogenesis in rats and hamsters in order to gain more insight into the mechanism underlying hormonal therapy of pancreatic cancer in man.

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CHAPTER 2

Histogenesis of early preneoplastic lesions induced by N-nitrosobis-(2-oxopropyl)amine in exocrine pancreas of hamsters

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Summary

The histogenesis of early putative preneoplastic lesions, arising in exocrine pancreas of Syrian hamsters after treatment with N-nitrosobis(2-oxopropyl)amine (BOP), was evaluated by electron microscopy and immunohistochemistry. Electron microscopical examination of pseudoductular lesions, present in hamster pancreas 2–4 months after treatment with BOP, demonstrated that acinar cells forming part of these lesions frequently lose their zymogen granules. However, convincing evidence of dedifferentiation of acinar cells to proliferating ductal/ductular cells has not been found. Most ductal/ductular cells of the BOP-induced pseudoductular lesions stained positively with cytokeratins specific to ductal/ductular cells. Acinar cells were all negative and, moreover, those lining the pseudoductular lesions were frequently surrounded by cytoplasmic processes of adjacent cells that stained strongly positive with the cytokeratin antibody.

The present findings indicate that the early pseudoductular lesions induced in exocrine pancreas of hamsters by BOP originate from proliferating ductal/ductular rather than from proliferating dedifferentiated acinar cells.

Introduction

By far the greatest number of human adenocarcinomas of the exocrine pancreas are of the ductal type and only a small percentage show an acinar cell differentiation (1). Rats and hamsters treated with azaserine or propylated nitrosamines, respectively, are the most frequently used experimental models to study pancreatic carcinogenesis (2, 3). These two animal models differ markedly with respect to the histomorphology of the induced pancreatic tumours, being of ductal/ductular cell type in the hamster and of the acinar cell type in the rat (2, 4, 5). Because of the similarity of the induced tumours to those occurring in man, the hamster model has been considered to

provide a unique opportunity to study pancreatic carcinogenesis (6). The histogenesis of the ductal/ductular adenocarcinomas induced in hamsters, however, is a topic of debate in the literature. In BOP-treated hamsters, many tumours have been 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. postulated that ductular and islet precursor cells are the foundation of the 'pseudoductular lesions' and, hence, of the adenocarcinomas (5, 6). Recently, the centroacinar cell origin has been emphasized (7, 8). Electron microscopical examination of pancreatic tumours, induced in hamsters by nitrosamines, has led to the conclusion that the tumours arise from existing ducts without any involvement of acinar cells (8–10). Other workers, however, claim that pancreatic adenocarcinomas in hamsters develop from acinar cells by dedifferentiation (11–15).

The differences in view on the histogenesis of pancreatic ductal/ductular adenocarcinomas, induced in hamsters by propylated nitrosamines, concentrate on two key questions:

1. Are early pseudoductular lesions formed by proliferating dedifferentiated acinar cells or by proliferating ductal/ductular cells?
2. Which category of the early, putative precancerous lesions is linked to a high progression probability and, hence, most relevant to the formation of pancreatic cancer?

The purpose of the present study was to find additional information to answer the first question by means of electron microscopic as well as immunohistochemical techniques.

Materials and methods

Seventy-eight male weanling Syrian golden hamsters were obtained from Charles River, Montreal, Canada. They were kept on softwood bedding in macrolon cages, five animals per cage, under standard laboratory conditions. The animals were fed a semi-purified diet, the composition of which has been given in a previous paper (16). Seventy-two hamsters were injected subcutaneously (SC), once weekly with 20 mg BOP/kg body wt at 5, 6 and 7 wk of age. Six animals served as untreated controls. BOP (Ash Stevens, 5861 John C. Lodge Freeway, Detroit, MI) was dissolved in 0.9% NaCl solution immediately before use. Twenty-six animals, including two untreated controls, were sacrificed 2, 3 or 4 mo after the last treatment with the carcinogen.

The animals were anaesthesized by ether, exsanguinated by cannulating the abdominal aorta, and then examined for gross pathological changes. The entire pancreas and liver from each animal were excised and weighed. At each interval, part of the pancreata from 12 BOP-treated animals and one untreated control were processed for electron microscopy. For this purpose, the tissue was fixed for 20 h in 2% paraformaldehyde with 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.35,

900 mOsm) and stored in 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.35, 820 mOsm). Graded aceton-water mixtures were used for dehydration. An Epon-Araldite mixture was used for embedding. Semi-thin sections, stained with 1% toluidine blue in 1% borax solution, and ultra-thin sections, contrasted with magnesium uranyl acetate and lead citrate, were cut on a Reighert Ultracut E and examined with a Zeiss photomicroscope and a Philips EM410LS electron microscope at 60 kV. The remaining parts of the pancreata were fixed in 10% buffered formalin and processed for microscopy by conventional methods, step-sectioned at 4 μm, and stained with haematoxylin and eosin (H&E). The pancreata of the 12 remaining BOP-treated animals and one untreated control killed at each time interval were fixed for 18 h in formaldehyde sublimate and subsequently processed in paraffin for immunohistochemical, as well as for routine (H&E), light microscopy. Sections 4 μm thick were mounted on glass slides and dried.

The polyclonal antiserum to cytokeratin filaments contained subunits against 48, 51, 52, 56, 58 and 60 kDa, as previously described (17, 18), and differentiated ductal, ductular, and centroacinar cells from acinar cells in the hamster pancreas. Specific antiserum to hamster acinar cells was a monoclonal antibody raised in mice, as reported by Parsa et al. (19). All aforementioned antibodies were obtained from Dakopatts a/s, Glostrup, Denmark, except for the antibody against hamster acinar cells, which was kindly provided by I. Parsa.

After deparaffination, mercury deposits were removed by lugol, and the slides were bleached with sodium thiosulphate. Subsequently, endogenous peroxidase was blocked by 0.3% hydrogen peroxide in methanol. Thereafter, the slides were washed in phosphate-buffered saline (PBS, 0.01 M, pH 7.4). The cytokeratin antigen was demonstrated by the peroxidase-anti-peroxidase method and the acinar cell antigen was demonstrated by an indirect method (20). The specificity of the cytokeratin reaction was verified by absorption with purified keratin and by replacing the specific antiserum with PBS.

Results

At each sacrifice, the pancreata of the hamsters showed pseudoductular lesions lined by flattened, cuboidal, or columnar epithelium. The incidence and size of the pseudoductular lesions demonstrated an increase in time resulting in the presence of more lesions with a greater diameter at 4 mo than at 2 mo after treatment. However, at each sacrifice, the size of the various pseudoductular lesions observed varied considerably. Qualitatively, the pseudoductular lesions did not reveal striking differences at the three intervals except that after 2 mo relatively more acinar cells appeared to form part of the pseudoductular lesions than after 4 mo.

In normal hamster pancreas, strong specific positivity was seen in ductal, ductular, and centroacinar cells. Much fainter staining was seen in the pancreatic

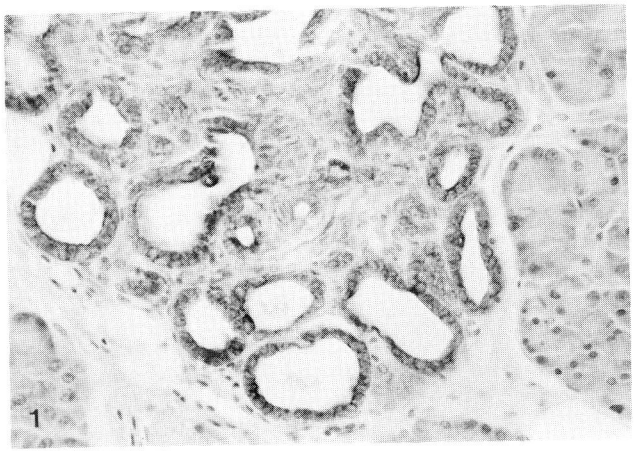

Fig. 1. Pseudoductular lesion lined by cuboidal epithelium in hamster pancreas 4 mo after treatment with BOP. Immunoperoxidase stain with antibody to cytokeratin. Note the strong positive staining of the cuboidal pseudoductular cells (× 280).

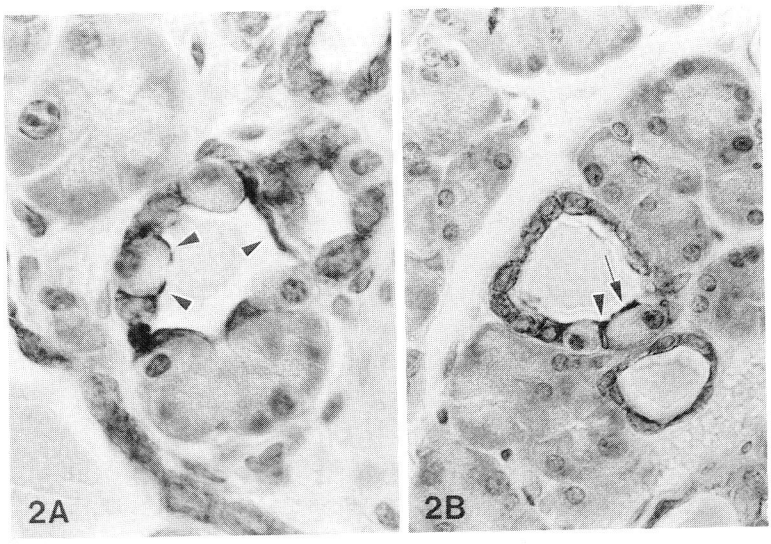

Fig. 2. A. Acinar cells forming part of a pseudoductular lesion in hamster pancreas 4 mo after treatment with BOP. Immunoperoxidase stain with antibody to cytokeratin. Pseudoductular cells are clearly positive, whereas the acinar cells are negative. Note the cytokeratin-positive cytoplasmic processes of the adjacent pseudoductular cells (arrowheads) on the luminal surface of the acinar cell (× 700). B. Pancreas of a hamster 3 mo after treatment with BOP. Immunoperoxidase staining with antibody to cytokeratin. Cytokeratin-positive pseudoductular cell between two negative acinar cells (arrowhead). Note the pseudoductular cell growing over the luminal surface of the neighbouring acinar cell (arrow) (× 700).

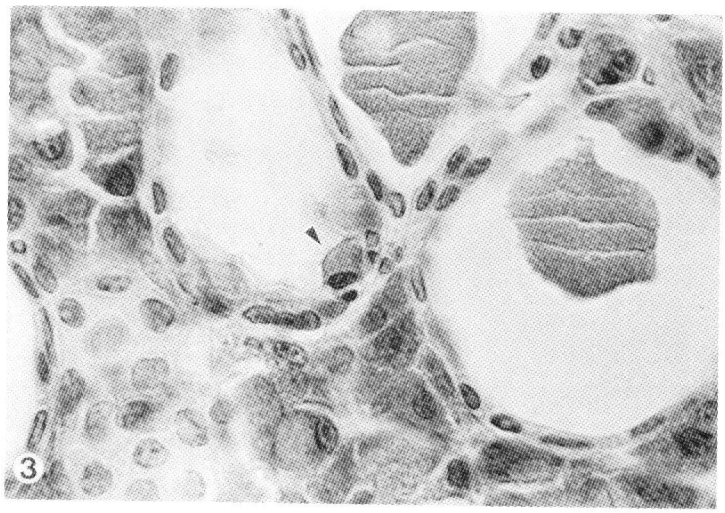

Fig. 3. Acinar cells within pseudoductular lesions in hamster pancreas 4 mo after BOP treatment. Immunoperoxidase stain with antibody to acinar cells. Note the unstained pseudoductular cells situated around the beneath the acinar cell giving the impression of the acinar cell being pushed into the lumen (arrowhead) (× 700).

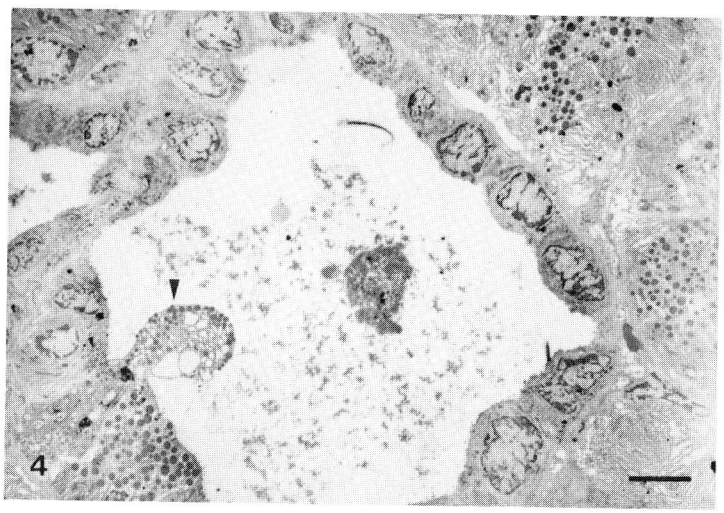

Fig. 4. Pancreas of a hamster 2 mo after treatment with BOP. The acinar cell has been expelled into the lumen of a pseudoductular lesion (arrowhead). Note the dilated rough endoplasmic reticulum. Bar = 2 μm.

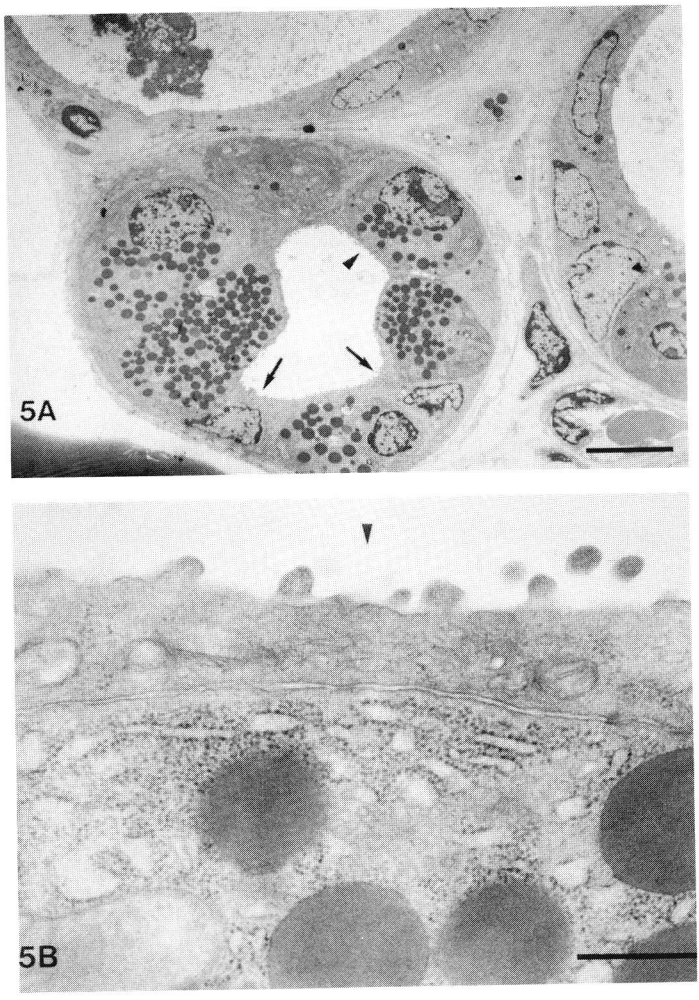

Fig. 5. A. Pseudoductular lesion containing acinar cells in hamster pancreas, 4 mo after treatment with BOP. One acinar cell showing a strong reduction of zymogen granules is completely covered on the luminal surface by cytoplasmic processes of adjacent centroacinar cell(s) (arrowhead). Note the (enlarged) centroacinar cells adjacent to the acinar cells (arrows). Bar = 5 μm. B. Detail of Fig. 1. The cytoplasmic processes covering the acinar cell is part of the cell that lacks much endoplasmic reticulum and zymogen granules, indicating a ductal/ductular rather than an acinar origin. Bar = 0.5 μm.

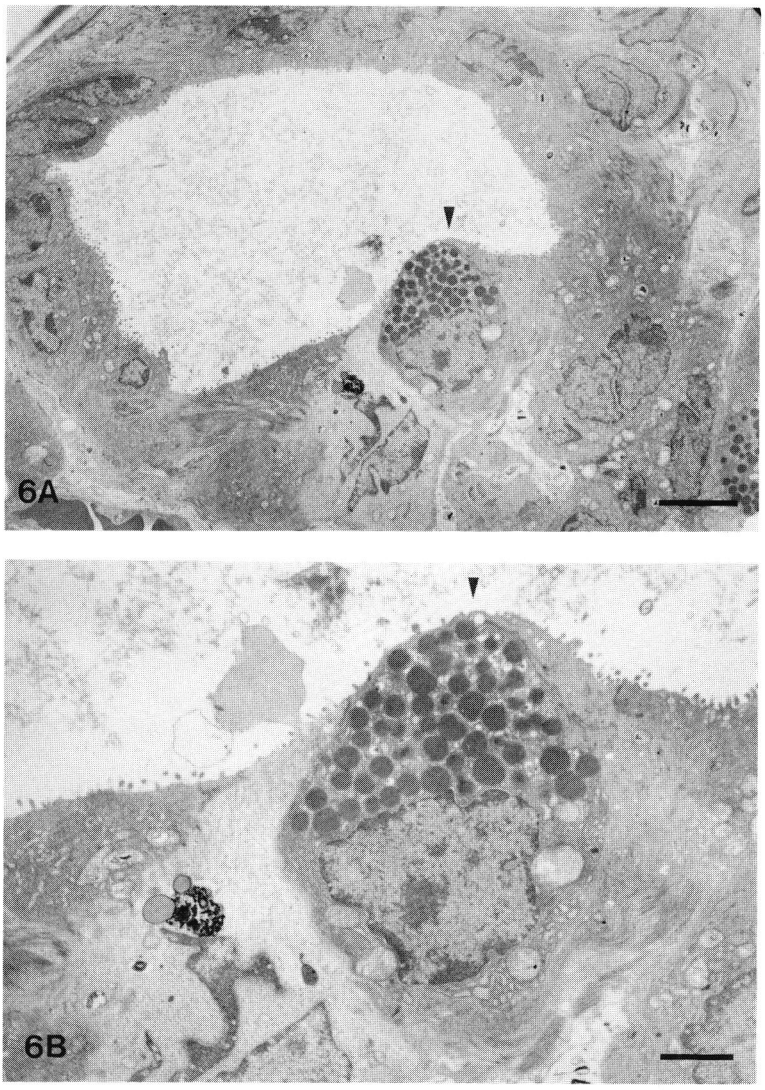

Fig. 6. A. Hamster pancreas 3 mo after treatment with BOP showing a pseudoductular lesion containing one acinar cell. Note the cytoplasmic process of the adjacent centroacinar cell on the luminal surface (arrowhead). Bar = 5 μm. B. Detail of Fig. 2A. Note the swollen mitochondria. Bar = 2 μm.

islets. No staining at all was observed in normal acinar cells. The ductal/ductular cells lining the pseudoductular lesions stained positively with the cytokeratin antibody (Fig. 1). The acinar cells forming part of the pseudoductular lesions could be easily recognized by their zymogen granules; they did not stain at all with the cytokeratin antibody (Figs. 2A, B). When applying an antibody specific to hamster acinar cells, the pseudoductular lesions frequently appeared to be lined by one or more acinar cells that occasionally were seen to be expelled into the lumen of the pseudoductule (Fig. 3). This 'pushing away' of acinar cells was also observed with electron microscopy (Fig. 4).

Two months after the last injection of BOP, electron microscopical examination of the exocrine pancreas revealed several acinar cells with dilatation of the rough endoplasmic reticulum and marked mitochondrial swelling. At subsequent sacrifices, distension of the lumina of some acini and loss of zymogens in several acinar cells became more prominent. Occasionally, autophagic vacuoles packed with zymogen granules were seen. The luminal surface of the pseudoductular lesions was frequently covered with cytoplasmic processes of adjacent centroacinar cells (Figs. 5A, B and 6A, B), which were stained with the polyclonal cytokeratin antiserum (Figs. 2A, B).

Discussion

Several workers have postulated that not only ductal/ductular cells, but also acinar cells, may play an important role in the development of early pseudoductular lesions and ultimate ductular adenocarcinomas in BOP-treated hamsters (14, 15, 21). According to Flaks, the development of ductular adenocarcinomas comprises two steps: dedifferentiation of the acinar cells to duct-like cells, followed by proliferation of these dedifferentiated acinar cells.

It is difficult to understand, however, how cells that can hardly be distinguished from ductular or centroacinar cells can be classified with certainty as former acinar cells. The main characteristics of the acinar cells are the abundance of rough endoplasmic reticulum and zymogen granules. Cells with loss of zymogens could be considered either as transitional cells between acinar and ductal/ductular cells, or degenerating cells. However, since these cells did not show a typical ductal cytokeratin pattern or signs of proliferating activity, their participation in the development of pseudoductules is rather speculative. Loss of zymogen granules, whether or not by selective autophagy, and dilatation of rough endoplasmic reticulum have been interpreted as signs of a dedifferentiation process to which acinar cells of BOP-treated hamster pancreas are thought to be subject (14, 15). These features, however, are in no way indicative of a proliferative process and can also be found in cells subjected to various toxic substances (22).

The changes seen in the acinar cells are, in our view, indicative of a toxic effect exerted by the carcinogen. It is not illogical to assume that the loss of acinar cells results in proliferation of centroacinar cells. Using a multi-injection protocol, the number of acinar cells, as well as centroacinar cells, affected by the cytotoxic or

carcinogenic action of BOP will be increased. We observed that acinar cells, being part of pseudoductular lesions, are either pushed away into the lumen of the pseudoductule or completely surrounded by processes of adjacent centroacinar cells.

Our electron microscopical observations are in accordance with those of Pour et al. (5, 7, 8, 24), who also described formation of cytoplasmic processes of centroacinar cells surrounding the acinar cells, leading to the isolation of acinar cells from the lumen and subsequent degeneration. We did not find any evidence for dedifferentiation of acinar cells to proliferating duct-like cells. Essentially, our findings are comparable with those of Willemer et al. (23) who induced pancreatitis in rats by cerulein and oleic acid and found that acinar cells, forming part of pseudoductules or tubular complexes, represent degenerating acinar cells that have no regenerative potency and lost their secretory and membrane characteristics (23). Therefore, it may be concluded from the present findings that the early pseudoductular lesions found in hamster pancreas 2, 3 and 4 months after treatment with BOP originate from proliferating ductal/ductular cells rather than from dedifferentiated acinar cells. On the other hand, it is possible that degeneration of the acinar cells caused by the toxic effect of BOP plays an important role, if not a *conditio sine qua non*, in the carcinogenesis process. Bell and Ray (25) found that BOP-induced ductular adenocarcinomas in exocrine pancreas of hamsters stained positively with the cytokeratin antibody. It is tempting to combine their findings with those presented in this paper and those reported by Pour et al. (8, 24) and to conclude that proliferating centroacinar cells are mainly involved in the formation of pseudoductular lesions and, hence, in the development of ductular adenocarcinomas in the exocrine pancreas of hamsters treated with BOP or its analogues. However, the relationship between the various types of early putative preneoplastic pseudoductular lesions and the ultimate ductular adenocarcinomas is not fully elucidated and needs to be further evaluated.

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CHAPTER 3

Role of cholecystokinin in the development of BOP-induced pancreatic lesions in hamsters

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Summary

Cholecystokinin (CCK) has been shown to promote pancreatic growth and azaserine-induced pancreatic carcinogenesis in rats. The present study was carried out to determine effects of CCK on pancreatic growth and carcinogenesis in the BOP-hamster model.

One hundred male Syrian golden hamsters were injected s.c., once weekly with 20 mg BOP/kg body wt at 6, 7 and 8 weeks of age, and divided into 4 groups of 25 animals each, which received one of the following treatments (once daily, 3 days per week for 16 weeks): (a) gelatin; (b) CR-1409, a potent CCK-receptor antagonist; (c) CCK-8, 2.5 μg/kg body wt, (d) CCK-8 in combination with CR-1409 (30 s before CCK treatment).

The animals were killed after 19 weeks. The growth of the pancreas, but not the incidence of pancreatic (pre)neoplastic lesions, was enhanced by CCK-8. CR-1409 did not influence the effect of CCK on pancreatic growth.

Introduction

Raw soya bean flour and trypsin inhibitors cause an increase in plasma cholecystokinin (CCK) levels in rats and have been found to promote carcinogenesis in the azaserine-rat model (1, 2). Furthermore, exogenously administered caerulein (a synthetic CCK analogue) promotes pancreatic carcinogenesis in rats (3). Moreover, it has recently been demonstrated that CR-1409, a highly specific CCK-receptor antagonist, inhibits the promoting effect of CCK on both pancreatic growth and the development of putative preneoplastic acinar lesions induced in rat pancreas by azaserine (4, 5, 6), pointing to a possible role of CCK in pancreatic carcinogenesis in rats. However, by far the greatest number of human adenocarcinomas of the

exocrine pancreas are of the ductal type and only a small percentage show an acinar cell differentiation. Because of the similarity of the induced tumours to those occurring in man, the N-nitrosobis(2-oxopropyl)amine (BOP) hamster model is considered to provide a unique model to study pancreatic carcinogenesis (7). In the BOP-hamster model, however, the effects of CCK on pancreatic carcinogenesis are rather inconsistent. Enhancing as well as inhibitory effects and also absence of any effect of CCK on the development of ductular pancreatic tumours induced in hamsters by nitrosamine-derivatives have been reported (8, 9, 10, 11, 12). These inconsistent findings most probably can be ascribed to variations in study design. In most studies CCK has been injected concurrently with the nitrosamine derivative, thus not discriminating between the initiation and the promotion process. In the present study CCK administration started one week after the last injection with carcinogen. The injection protocol used was comparable with a previously performed rat study where a slightly supraphysiological dose (2.5 μg/kg body wt) was injected once daily, 3 days/week, for 18 weeks resulting in a promoting effect on pancreatic carcinogenesis (6).

The present study was conducted to find out whether CCK also has promoting effects on the development of ductular lesions induced in hamster pancreas by BOP, using incidences of putative preneoplastic and neoplastic lesions as parameters.

Materials and methods

One hundred male Syrian golden hamsters (obtained at 5 weeks of age from Charles River Wiga, Sulzfeld, FRG) were injected s.c., once weekly, with 20 mg N-nitrosobis(2-oxopropyl)amine (BOP)/kg body weight at 6, 7 and 8 weeks of age according to an injection protocol as previously described (13). BOP (Ash Stevens, Inc., 5861 John C. Lodge Freeway, Detroit, MI 48202) was dissolved freshly in 0.9% NaCl solution. The hamsters were housed in macrolon cages under standard laboratory conditions, 5 hamsters per cage, and fed the Institute's stock diet which is low in fat (5%) and compounded from natural feed ingredients. The composition of the diet was as reported previously (14). The animals were allocated to 4 different groups by a computerized randomization procedure. Each group consisted of 25 animals and received one of the following treatments (once daily, 3 d/wk for 16 wk): (a) gelatin (controls); (b) CR-1409 (12 mg/kg body wt, s.c.); (c) CCK-8 (2.5 μg/kg body wt, s.c.); (d) CCK (2.5 μg/kg body wt, s.c.) in combination with CR-1409 (12 mg/kg body wt, s.c.). CR-1409 was dissolved in distilled water to a concentration of 0.4% and adjusted to pH 9 with NaOH (0.1 M) and administered 30 min before injection of CCK.

The dose of cholecystokinin octapeptide (CCK-8) used in the present study was based on plasma concentration time curves for CCK-8 obtained from a previously described 2-week study in rats and hamsters (4). In this 2-week study it had been

shown that subcutaneous administration of 2.5 μg/kg body wt of CCK-8 (Cambridge Research Biochemical, Cambridge, UK) stimulated pancreatic growth and DNA synthesis both in rats and in hamsters. In rats CCK-8 caused a maximum increase of plasma CCK levels 30 min after administration. The increase in pancreatic growth was significantly inhibited by the highly specific CCK-receptor antagonist CR-1409 (kindly provided by Rotta Research Laboratories, Milan, Italy). Subcutaneous injection of 2.5 μg/kg body wt of CCK-8, dissolved in 16% hydrolysed gelatin (Hospital Pharmacy, University Hospital, Leiden, Netherlands) resulted in plasma CCK levels which were only slightly supraphysiological and comparable with those seen after dietary administration of maize oil. Plasma CCK levels were determined using a highly specific and sensitive radioimmunoassay as previously described in detail (15, 16). Treatment of the hamsters started one week after the last injection with BOP. Body weights were recorded weekly and food intake monthly. The general condition and behaviour of the animals were checked daily.

Terminal autopsy was conducted 19 weeks after the first injection with BOP. The animals were anaesthetized by ether and exsanguinated by cannulating the abdominal aorta 30 min after the s.c. administration of 2.5 μg/kg body wt of CCK-8; they were examined for gross pathological changes and pancreas and liver were weighed. These organs were fixed in 10% buffered formalin. The pancreata were completely processed for microscopy by conventional methods, step-sectioned at 5 μm (about 5 per hamster pancreas), stained with haematoxylin and eosin (H&E) and examined by light microscopy.

The different types of BOP-induced putative (pre)neoplastic ductular lesions were evaluated as described previously (17, 13). Major attention was directed to tubular ductal complexes showing dysplastic or anaplastic (cellular) changes, desmoplasia and/or inflammation, suggestive of progression to malignancy. A tubular ductal complex exhibiting one or more of the characteristics suggestive of a neoplastic change was classified as 'borderline' (Figure 1). A ductular adenocarcinoma is a lesion mainly composed of atypical dysplastic ductules. Such lesions are most characteristically surrounded by an inflammatory reaction (Figure 2). When such a lesion did not show local invasion in the surrounding acinar and fibrous tissue it was classified as carcinoma *in situ* if it exhibited local invasion as stage I ductular carcinomas or microcarcinomas. Advanced stages were classified as plain carcinomas. 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 generalized linear model with a normal error distribution. The relevant sources of variation were: treatment with gelatin; treatment with CCK-8; combination of treatment with CCK-8 and CR-1409, and their interaction.

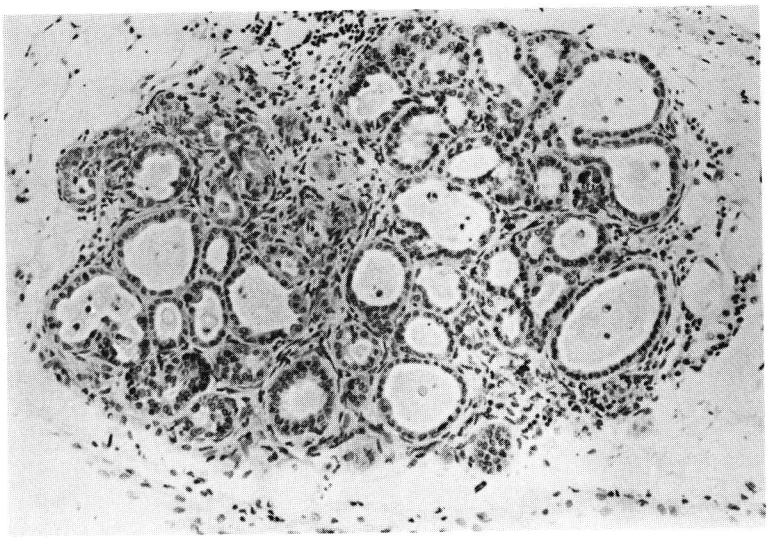

Fig. 1. Borderline lesion: tubular ductal complex showing dysplastic epithelium, desmoplasia and inflammatory cells. (H&E, × 320.)

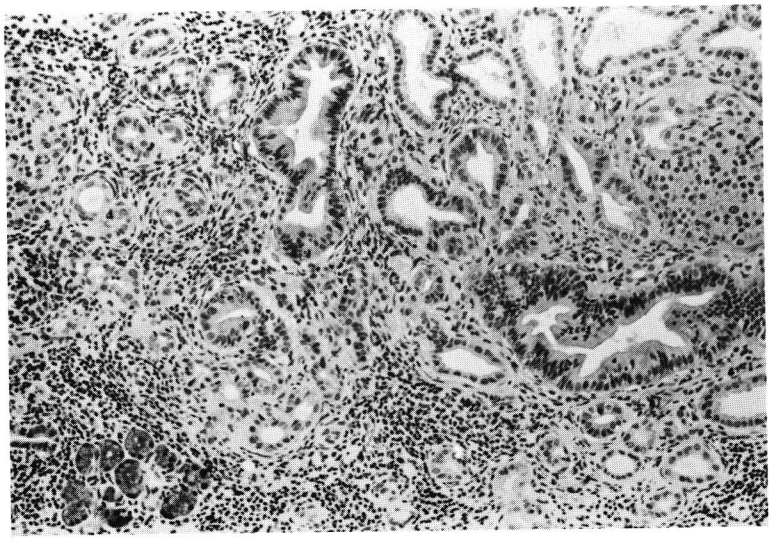

Fig. 2. Ductular adenocarcinoma, mainly composed of atypical, dysplastic ductules. Note the inflammatory reaction. (H&E, × 320.)

Results

The CCK-8 content of the stock solution was constant throughout the experiment. The results show that the plasma CCK concentration increased significantly ($P < 0.01$) as compared to the controls 30 min after a single injection with CCK-8, either alone or in combination with CR-1409 (Table 1).

The effects of CCK-8, either alone or in combination with CR-1409, on body, pancreatic and liver weights are shown in Table 2, and the quantitative analyses of pancreatic H&E-stained paraffin sections are shown in Table 3.

The influence of CR-1409 and CCK-8, as well as their interaction, on the (square root of) the number of borderline lesions and the natural logarithms of body and pancreatic weight was assessed with analysis of variance techniques.

No significant interaction of CCK and CR-1409 appeared with respect to the above parameters. CCK increased significantly ($P < 0.05$) absolute and relative pancreatic weight, but not body weight. Body weight and absolute pancreatic weight ($P < 0.01$), but not relative pancreatic weight, were significantly decreased by CR-1409.

Table 1. Effects of CCK, either alone or in combination with CR-1409 (a specific CCK-receptor antagonist) on plasma CCK of hamsters (mean ± SEM).

Treatment	Plasma CCK level 0.5 h after injection (pM)
Control (gelatin)	3.3 ± 0.30
CR-1409 + gelatin	3.6 ± 0.13
CCK	18.8 ± 2.19 **
CR-1409 + CCK	18.1 ± 2.03 **

Statistics: Student's t-test; ** $P < 0.01$ compared to control.

Table 2. Effects of CCK, either alone or in combination with CR-1409 on mean body/organ weights (± SEM) of BOP-treated hamsters.

Treatment	Body wt (g)	Pancreatic weight		Liver weight	
		absolute (g)	relative (g/kg)	absolute (g)	relative (g/kg)
Control (gelatin)	121.8 ± 5.1	0.491 ± 0.026	4.11 ± 0.23	4.728 ± 0.219	38.80 ± 0.70
CR-1409 + gelatin	112.0 ± 2.5	0.452 ± 0.025	4.03 ± 0.18	4.252 ± 0.107	37.92 ± 0.75
CCK	119.1 ± 2.6	0.528 ± 0.021[1]	4.47 ± 0.19[1]	4.500 ± 0.129	37.76 ± 0.66
CR-1409 + CCK	114.7 ± 2.4	0.544 ± 0.034[1]	4.73 ± 0.26[1]	4.369 ± 0.080	38.27 ± 0.68

Statistics: one-way analysis of variance followed by Student's t-test (two-tailed)
[1] Increased in animals treated with CCK compared to animals not treated with CCK; in combined treatment the effect could be ascribed to treatment with CCK: $P < 0.05$.

Table 3. Effects of CCK, either alone or in combination with CR-1409 on development of (pre)neoplastic lesions in exocrine pancreas of BOP-treated hamsters.

Treatment	Effective number of animals	Average number of advanced ductular lesions			
		borderline lesion	carcinoma *in situ*	micro-carcinoma	carcinoma
Control (gelatin)	24	5.5	0.1	0.1	0.1
CR-1409 + gelatin	23	7.0	0.1	0.2	0.0
CCK	25	6.7	0.2	0.1	0.0
CR-1409 + CCK	24	5.3	0.0	0.2	0.0

Statistics: linear model with a normal error distribution: * $P < 0.05$.

Statistical analyses of the data did not reveal a significant effect of CCK-8 and CR-1409 on the (square root of) borderline ductular lesions. The number of pancreatic carcinomas was too small to justify statistical analyses.

Discussion

The main findings of the present study in which Syrian golden hamsters were injected subcutaneously (once daily for 3 days per week during 16 weeks) with a slightly supraphysiological dose of 2.5 μg CCK-8/kg body wt are (a) a significantly stimulated pancreatic growth and (b) no effect on the development of early putative preneoplastic ('borderline') or neoplastic ductular lesions.

The results of the present study are in full agreement with those obtained by Pour et al. (12), who also found that CCK given after BOP injection did not have any effect on pancreatic carcinogenesis in hamsters. The design of the present study, however, differed noticeably from the study performed by Pour and co-workers, who injected the hamsters only once with BOP (20 mg/kg body wt) in combination with CCK (20 IDU/kg body wt), either 3 h before, simultaneously or 3 h after BOP treatment. Injection of CCK after BOP did not influence development of pancreatic tumours, whereas CCK injected 3 h before or simultaneously with BOP inhibited the development of pancreatic tumours. In the same paper, Pour et al. describe that weekly injections of hamsters with 2.5 mg BOP/kg body wt accompanied with CCK (3 h before, simultaneously or 3 h after BOP treatment) for 20 weeks did not have any effect on pancreatic carcinogenesis. Interestingly, in hamsters treated with di-isopropanol nitrosamine (DIPN, 250 mg/kg body wt) Johnson also found an inhibitory effect of CCK (20 IDU/kg body wt) on development of pancreatic and liver tumours when injected simultaneously for 40 weeks (8).

The results of the present study with BOP-treated hamsters partly contrast with those of our previous study with azaserine-treated rats, which was performed

according to the same protocol. In rats, treatment with CCK-8 caused a significant increase in pancreatic weight and had an enhancing effect on growth of putative preneoplastic acinar nodules. This enhancing effect of CCK-8 on pancreatic growth and carcinogenesis in rats was significantly inhibited by the highly specific CCK receptor antagonist CR-1409 (5, 6). In hamsters there was no interaction between CCK-8 and CR-1409 and thus in this species the specific action of CR-1409 is doubtful. The aforementioned differences in effects of CCK on pancreatic carcinogenesis in rats as compared to hamsters may indicate that receptors for CCK are present in acinar but not in (pseudo)ductular pancreatic tissue. Up to now CCK receptors have not been demonstrated in ductular pancreatic tissue but they have been found in a transplanted human pancreatic ductular adenocarcinoma (18).

The present results are contradictory to the findings of some other workers. Howatson found a reduction in latency period and an increased incidence of pancreatic tumours in hamsters weekly treated with BOP (5 mg/kg body wt) for life in combination with (day before, simultaneously and day after carcinogen injection) 30 IDU/kg body wt CCK for 6 weeks (10).

Contradictory results have also been observed with caerulein repeatedly injected in hamsters treated with N-nitrosobis(2-hydroxypropyl)amine (BHP). Satake et al. (11) found an enhancing effect of caerulein (20 μg/kg body wt) when injected weekly, simultaneously with BHP (500 mg/kg body wt) for 8–27 wk, whereas Andrén-Sandberg et al. (9) did not find any effect of caerulein (2 μg/kg body wt; twice daily 5 d/wk for 18–22 wk) injected simultaneously weekly with BHP (125–250 mg/kg body wt) for life. An explanation for these inconsistent findings cannot be given easily. Enhancing effects of CCK have been found only in studies using supraphysiological high doses of CCK (20–30 μg/kg body wt). Such high doses repeatedly injected in experimental animals may lead to severe pancreatitis (3). Since pancreatitis is a pathological condition generally accompanied with recurrent tissue damage and repair it is not illogical to assume that such high doses of CCK given simultaneously with a pancreatic carcinogen may induce more tumours as compared to experiments in which almost physiological doses of CCK are used. In the present study the dose of CCK was low and CCK was injected only 3 days per week; this is most probably the cause for the statistically significant increase in pancreatic weight which is consistent with the results of a 2-week study performed with hamsters in our Institute. In that study injection of 2.5 μg CCK injected twice daily, 5 days per week for 2 weeks, also resulted in a significant increase in pancreatic weight (4).

In the previous short-term study (17 wk) in rats performed in our Institute, according to the same protocol as the present study, pancreatic weight also increased in the CCK-treated group as compared to controls.

The observation that the modulating effect of CCK-8 on experimental pancreatic carcinogenesis in rats is different from that in hamsters indicate that, besides long-term studies in rats, also chronic studies with CCK-8 in BOP-treated hamsters have

to be conducted in order to find out whether CCK-8 may have promoting effects on development of ductular adenocarcinomas in hamsters. Such data are needed to evaluate the possible role of CCK in pancreatic carcinogenesis in man.

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CHAPTER 4

Effects of the synthetic trypsin inhibitor camostate on the development of BOP-induced pancreatic lesions in hamsters

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Summary

Trypsin inhibitors have been shown to promote pancreatic growth as well as the development of pancreatic tumours in rats. The present study was carried out to examine the effects of the synthetic trypsin inhibitor camostate on the growth of the pancreas and on the development of pancreatic preneoplastic and neoplastic lesions in hamsters treated with N-nitrosobis(2-oxopropyl)amine. A specific cholecystokinin-receptor antagonist was administered to determine the role of cholecystokinin in camostate action. The animals were killed 19 weeks after the first injection with N-nitrosobis(2-oxopropyl)amine.
Camostate caused an increase in growth of the pancreas and a decrease in the number of (pre)neoplastic ductular pancreatic lesions. Lorglumide (CR-1409) did not influence these effects of camostate. It was concluded that rats and hamsters behave differently with regard to the effect of camostate on pancreatic growth and carcinogenesis.

Introduction

Protease or trypsin inhibitors (TI) are widely distributed in man's food, including not only most of the legumes but also cereal grains, potatoes and eggs (12). However, the safety of soya beans and TI has been increasingly questioned (7, 10, 16, 25) and food processing is legally restricted in many countries to prevent consumption of improperly heated soya flour.

In rats treated with azaserine oral administration of either RSF or synthetic trypsin inhibitors such as camostate leads to enhanced pancreatic hypertrophy and hyperplasia resulting in a significant increase in weight as well as an enhanced development of atypical acinar cell foci/nodules (AACF/AACN), adenomas and acinar adenocarcinomas in the exocrine pancreas (5, 9, 14, 15, 20, 26).

Recently it has been demonstrated that lorglumide (CR-1409), a highly specific CCK-receptor antagonist, inhibits the promoting effect of CCK and TI on both pancreatic growth and development of putative preneoplastic acinar lesions in azaserine-treated rats, pointing to a possible role of CCK in the trophic effects of TI induced pancreatic growth and carcinogenesis in rats (4, 5, 6).

Since there is increasing evidence of a marked species specificity (11), it is of paramount importance to know whether the rat is unusually susceptible to the development of pancreatic AACN and adenomas when the pancreas is stimulated through feedback mechanisms designed to regulate pancreatic function. By far the greatest number of adenocarcinomas of the human exocrine pancreas are of ductal type. Because of the similarity of the induced tumours to those occurring in humans, the N-nitrosobis(2-oxopropyl)amine (BOP) hamster model is considered to be more relevant for the human situation than the azaserine-rat model (17, 22, 24). Therefore, the present study was carried out in order to elucidate the effect of TI, either alone or in combination with CR-1409, on pancreatic carcinogenesis in BOP-treated hamsters using pancreatic weight and numbers of (pre)neoplastic lesions as parameters.

Materials and methods

One hundred male Syrian golden hamsters (obtained at 5 weeks of age from Charles River Wiga, Sulzfeld, FRG) were each injected s.c., once weekly, with 20 mg N-nitrosobis(2-oxopropyl)amine (BOP)/kg body weight at 6, 7 and 8 weeks of age according to an injection protocol as previously described (29). BOP (Ash Stevens, 5861 John C. Lodge Freeway, Detroit, MI 48202) was dissolved freshly in 0.9% NaCl solution. The hamsters were housed in macrolon cages under standard laboratory conditions, 5 hamsters per cage, and fed the Institute's stock diet which is low in fat (5%) and compounded from natural feed ingredients. The percentage composition of the diet has been given previously (28). The animals were allocated to 4 different groups by a computerized randomization procedure. Each group consisted of 25 animals and received one of the following treatments (once daily, 3 d/wk for 16 wk): (a) gelatin (controls, s.c.); (b) CR-1409 (12 mg/kg body wt, s.c.); (c) camostate (200 mg/kg by gavage); (d) camostate (200 mg/kg by gavage) 30 min after CR-1409 injection (12 mg/kg body wt, s.c.).

The control group was given gelatin because the present experiment was part of a larger study in which CCK was administered in gelatin (18).

CR-1409, a highly specific CCK-receptor antagonist (kindly provided by Rotta Research Laboratories, Milan, Italy), was dissolved in distilled water to a concentration of 0.4% and adjusted to pH 9 with 0.1 M NaOH and administered s.c. 30 min before administration of camostate.

Camostate, the protease inhibitor, a generous gift from Sanol Schwarz (Mannheim, FRG), was dissolved in distilled water and administered at a dose of 200 mg/kg by orogastric intubation. For comparative purposes the same dose of

camostate was used as in previous studies, where CR-1409 significantly reduced the increase in pancreatic growth after camostate treatment (2, 5).

Treatment of the hamsters started one week after the last injection with BOP. Body weights were recorded weekly and food intake was measured monthly. The general condition and behaviour of the animals were checked daily.

Terminal autopsy was conducted 19 weeks after the first injection with BOP. The animals were anaesthetized by ether and exsanguinated by cannulating the abdominal aorta 30 min after treatment with camostate; they were examined for gross pathological changes, and pancreas and liver were weighed.

The pancreas and the liver were fixed in 10% buffered formalin. The pancreata were completely processed for microscopy by conventional methods, step-sectioned at 5 μm (about 5 per hamster pancreas) and stained with haematoxylin and eosin (H&E). An area of about 200 mm^2 was screened by light microscopy.

The different types of BOP-induced putative (pre)neoplastic ductular lesions used for analysis were evaluated as described previously (18). Major attention was directed to tubular ductal complexes showing dysplastic or anaplastic (cellular) changes, desmoplasia and/or inflammation, suggestive of progression to malignancy. A tubular ductal complex exhibiting one or more of the characteristics suggestive of a neoplastic change was classified as 'borderline'. A ductular adenocarcinoma is a lesion mainly composed of atypical dysplastic ductules. Such lesions are most characteristically surrounded by an inflammatory reaction. If such a lesion did not show local invasion in the surrounding acinar and fibrous tissue it was classified as carcinoma *in situ*; if it exhibited local invasion it was classified as stage I ductular carcinomas or microcarcinomas. Advanced stages were classified as carcinomas.

Plasma CCK was measured by radioimmunoassay employing antibody T204 as previously described (3).

Statistical analysis of the data was parametric. Influence of CR-1409 and camostate, as well as their interaction, on the square root (because of homogeneity of variance) of the number of lesions as well as on body weight, pancreatic and liver weights were assessed by analysis of variance. The number of lesions was evaluated statistically by a linear model with a normal error distribution. The relevant sources of variation were: treatment with gelatin; treatment with camostate; combination of treatment with camostate and CR-1409, and their interaction.

Results

The results show that the plasma CCK concentration had increased significantly ($P < 0.01$) as compared to the controls 30 min after a single orogastric administration with camostate either alone or in combination with CR-1409 (Table 1).

The effects of camostate, either alone or in combination with CR-1409, on body weight, pancreatic and liver weights are presented in Table 2, and the quantitative analyses of pancreatic H&E-stained paraffin sections are presented in Table 3.

Table 1. Effects of camostate (trypsin inhibitor), either alone or in combination with CR-1409 (a specific CCK-receptor antagonist), on plasma CCK of hamsters (mean ± SEM).[1]

Treatment	Plasma CCK level 0.5 h after injection (pM)
Control (gelatin)	3.3 ± 0.30
CR-1409 + gelatin	3.6 ± 0.13
Camostate	27.8 ± 4.7**
CR-1409 + camostate	35.0 ± 6.1**

[1] Statistics: Student's t-test; ** $P < 0.01$ compared to control.

Table 2. Effects of camostate (trypsin inhibitor), either alone or in combination with CR-1409 on mean body/organ weights (± SEM) of BOP-treated hamsters.

Treatment	Body weight (g)	Pancreatic weight		Liver weight	
		absolute (g)	relative (g/kg)	absolute (g)	relative (g/kg)
Control (gelatin)	121.8 ± 5.1	0.491 ± 0.026	4.11 ± 0.23	4.728 ± 0.21	38.80 ± 0.70
CR-1409 + gelatin	112.0 ± 2.5	0.452 ± 0.025	4.03 ± 0.18	4.252 ± 0.107	37.92 ± 0.75
Camostate	111.2 ± 2.6	0.580 ± 0.022[1]	5.23 ± 0.18[1]	4.144 ± 0.138	37.13 ± 0.47
CR-1409 + camostate	111.1 ± 4.0	0.548 ± 0.027[1]	4.95 ± 0.18[1]	4.416 ± 0.246	39.39 ± 0.90

Statistics: one-way analysis of variance followed by Student's t-test (two-tailed)
[1] Increased in animals treated with camostate compared to animals not treated with camostate; in combined treatment the effect could be ascribed to treatment with camostate: $P < 0.01$.

Table 3. Effects of camostate, either alone or in combination with CR-1409 on development of (pre)neoplastic lesions in exocrine pancreas of BOP-treated hamsters.

Treatment	Effective number of animals	Number of advanced ductular lesions			
		borderline lesion	carcinoma *in situ*	micro-carcinoma	carcinoma
Control (gelatin)	24	133	2	3	1
CR-1409 + gelatin	23	162	2	5	0
Camostate	25	67[1]	7	2	0
CR-1409 + camostate	25	99[1]	3	1	0

[1] Decreased in animals treated with camostate compared to animals not treated with bombesin; in combined treatment the effect could be ascribed to treatment with camostate. Statistics: linear model with a normal error distribution: $P < 0.05$.

Camostate increased significantly ($P < 0.01$) both absolute and relative pancreatic weight, but not liver and body weight. A significant inhibitory effect ($p < 0.05$) of camostate was present on the square root of the number of borderline ductular lesions. This effect persisted also after statistical correction for pancreatic weight.

The number of pancreatic carcinomas was too small to justify statistical analyses. No significant interaction of camostate and CR-1409 was found with respect to the aforementioned parameters.

Discussion

In the present study it was shown that Syrian golden hamsters which were injected subcutaneously with the pancreatic carcinogen BOP at 6, 7 and 8 weeks of age and which were subsequently treated with the synthetic trypsin inhibitor camostate had an increased pancreatic weight accompanied by a significantly decreased number of putative preneoplastic (borderline) ductular lesions. CR-1409, a highly specific and potent CCK-receptor antagonist did not influence these effects of camostate.

The increase of pancreatic weight in hamsters after treatment with TI may be ascribed to the enhanced CCK plasma levels, because in a previously published parallel study with subcutaneously injected CCK-8 the treatment with CCK also enhanced pancreatic weight (18). The stronger increase of pancreatic weight after TI treatment compared to the increase after administration of CCK may be due either to the more enhanced CCK plasma level after TI treatment than after treatment with exogenous CCK (18) or to the endogenous nature of the CCK.

In azaserine-treated rats, administration of TI for a period of 16 weeks leads to an increase in pancreatic weight comparable to that observed in the present study with hamsters (5). The enhanced pancreatic weight was inhibited by pretreatment with CR-1409 in rats but not in hamsters. In a recent 14-day study performed in our Institute (2) administration of camostate, alone and in combination with CR-1409, to both rats and hamsters resulted also in enhanced pancreatic growth in both species. CR-1409 caused a significant decrease in the effect of camostate in rats but not in hamsters. Moreover, in studies on the effects of CCK treatment we have seen the same phenomenon: CR-1409 had no effect on CCK enhanced pancreatic growth in hamsters (18), although the dosis of CR-1409 used effectively inhibited the CCK enhanced pancreatic growth in rats (4). These results allow the conclusion that in hamsters the interaction of CR-1409 with the CCK-receptor may be different to that in rats.

Comparison of the present results with those previously presented indicates that in hamsters and rats the short-term effect of TI on pancreatic growth is comparable to that of RSF and CCK-8 (8, 18). However, long-term (14 months) feeding of RSF caused a significant increase in weight of the pancreas in rats but not in hamsters (11). It may be that prolonged stimulation of hamster pancreas by CCK results in desensitization of acinar cells as has been reported previously (1).

With respect to pancreatic carcinogenesis, the effects of TI in hamsters are even more contrasting with those observed in rats. TI inhibited the development of

preneoplastic ductular lesions in hamsters, but development of the acinar lesions (AACF and AACN) and carcinomas in the azaserine-rat model was stimulated by TI feeding as well as by exogeneously administered CCK (4, 5, 9, 14, 15, 20, 26). Since this effect in rats is inhibited by CR-1409, CCK is considered to be a mediator in TI induced effects on pancreatic carcinogenesis in rats. In hamsters, CR-1409 did not interact with the inhibiting effect of TI on the development of borderline pancreatic lesions, which is consistent with the finding that CR-1409 and CCK did not show interaction in the hamster model either (18). The present findings in hamsters are in agreement with those of Liener and Hasdai, who showed a reduction of pancreatic tumours in hamsters fed unheated RSF for 18 months (11).

The contrasting modifying effects of RSF and TI on pancreatic carcinogenesis in the rat and hamster may be due to a different type of cell of origin of pancreatic cancer in these species. In hamster pancreas an induction of transformation of centroacinar cells into acinar cells by TI (19, 21) might be an explanation for the inhibitory effects of TI on the proliferation of centroacinar cells into ductular lesions. In contrast to the tumours in the hamster model the pancreatic lesions observed in azaserine-treated rats are thought to be of acinar origin (13). Consequently induction of differentiation of centroacinar cells into acinar cells by TI may stimulate the development of pancreatic acinar lesions in the rat.

Up to now studies performed with nitrosamine-treated hamsters resulted in inconsistent findings with respect to the effects of CCK (18). The results of the present study revealed that administration of camostate after the initiation with BOP significantly inhibited the development of putative preneoplastic ('borderline') lesions in hamster pancreas, indicating that CCK may have the potency to inhibit the development of pancreatic tumours not only in the initiation, but also in the promotion phase (18, 23).

An inhibitory effect of protease inhibitors on the carcinogenic process has been described before in skin, breast, colon and liver carcinogenesis (27). The mechanism by which protease inhibitors exert their effect on carcinogenesis is still largely unknown. In case of pancreatic cancer, however, the results of the present study point to a role of CCK in this inhibitory effect.

Since soya bean products are an increasingly important nutritional source of protein in the Western world, effects of the consumption of TI-containing foods on human health deserve further investigation.

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CHAPTER 5

Effects of bombesin on the development of BOP-induced pancreatic lesions in hamsters

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Summary

Bombesin has been shown to promote pancreatic growth as well as the development of pancreatic (pre)neoplasia in rats. The present study was carried out to determine the effects of bombesin on pancreatic growth and on the development of pancreatic (pre)neoplastic lesions in hamsters treated with N-nitrosobis(2-oxopropyl)amine (BOP).

Bombesin caused an increase in growth of the pancreas accompanied with a decrease in the number of (pre)neoplastic ductular pancreatic lesions. CR-1409 did not influence these effects of bombesin. It is concluded that in BOP-treated hamsters the effect of bombesin on the pancreas is not mediated by CCK. These findings support the existence of a specific difference between rats and hamsters with regard to the effect of bombesin on pancreatic carcinogenesis.

Introduction

Bombesin (BBS), the amphibian analogue of gastrin-releasing peptide in mammals, is of great interest with respect to pancreatic carcinogenesis (4). Bombesin-like peptides have been found in neurons in the gastrointestinal tract and the pancreas of several species (11). In man, bombesin-like peptides have also been demonstrated in small-cell lung cancers, where they stimulate tumour growth through an autocrine mechanism (2).

Recently we have conducted a 2-week experiment in which CCK and BBS were administered to both rats and hamsters (7). CCK and BBS were found to stimulate pancreatic growth and DNA synthesis in both species. It was concluded from that study that marked differences exist between rats and hamsters in the pancreatic growth response to CCK and BBS.

Furthermore, previous studies with azaserine-treated rats have demonstrated that administration of BBS, CCK or camostate for 4 months leads to an increase in number and size of atypical acinar cell foci and nodules (AACF and AACN,

respectively) in the exocrine pancreas, indicating a promoting effect on pancreatic carcinogenesis in this species. Using lorglumide (CR-1409), a specific CCK-receptor antagonist which inhibits the peripheral effects of CCK, it was demonstrated in these studies in rats that CCK plays a major role in camostate-enhanced, but not in BBS-enhanced pancreatic carcinogenesis (4, 5).

However, these effects seem to be species-specific. In studies with hamsters treated with N-nitrosobis(2-oxopropyl)amine (BOP), CCK and camostate enhanced pancreatic growth, but the effects of these compounds on (BOP-induced) carcinogenesis were different from those observed in rats. Whereas cholecystokinin did not have any effect, camostate even showed an inhibitory effect in this species. The interaction of CR-1409 with both agents was also different in hamsters (6, 8, 10). Alexander et al. (1) have found that growth of a human ductal adenocarcinoma xenografted into nude mice was significantly inhibited by BBS. Up to now, no studies have been reported concerning the effect of BBS on the development of (pre)neoplastic pancreatic lesions in BOP-treated hamsters.

Because of the similarity of the induced pancreatic ductular tumours to those occurring in man, the BOP-hamster model is considered to be highly relevant to the human situation (9, 12, 13).

The present study was conducted to investigate the effects of bombesin on pancreatic carcinogenesis in BOP-treated hamsters using pancreatic weight and incidence of (pre)neoplastic lesions as parameters.

Materials and methods

One hundred male Syrian golden hamsters (obtained at 5 weeks of age from Charles River Wiga, Sulzfeld, FRG), were injected s.c., once weekly, with 20 mg N-nitrosobis(2-oxopropyl)amine (BOP)/kg body weight at 6, 7 and 8 weeks of age according to an injection protocol as previously described (14). BOP (Ash Stevens, 5861 John C. Lodge Freeway, Detroit, MI 48202) was dissolved freshly in 0.9% NaCl solution.

The hamsters were housed in macrolon cages under standard laboratory conditions, 5 hamsters per cage, and fed the Institute's stock diet which is low in fat (5%) and compounded from natural feed ingredients. The composition of the diet has been reported previously (15). The animals were allocated to 4 different groups by a computerized randomization procedure. Each group consisted of 25 animals and received one of the following treatments (once daily, 3 d/wk for 16 wk): (a) gelatin (controls); (b) CR-1409 (12 mg/kg body wt, s.c.); (c) bombesin (10 µg/kg, s.c.); (d) bombesin (10 µg/kg, s.c.) 30 s after CR-1409 injection (12 mg/kg body wt, s.c.).

CR-1409, a highly specific CCK-receptor antagonist (kindly provided by Rotta Research Laboratories, Milan, Italy), was dissolved in distilled water to a concentration of 0.4%, adjusted to pH 9 with 0.1 M NaOH and administered 30 min before injection of bombesin. Bombesin (Sigma Chemical, St. Louis, MO) was

dissolved in 16% hydrolysed gelatin (Hospital Pharmacy, University Hospital, Leiden, Netherlands). For comparative purposes we selected a dose of bombesin which induced slightly supraphysiological plasma CCK levels in rats (7). The dose of CR-1409 was comparable with that used in previous studies (6, 7). Treatment of the hamsters started one week after the last injection with BOP. Body weights were recorded weekly and food intake monthly. The general condition and behaviour of the animals were checked daily.

Autopsy was conducted 19 weeks after the first injection with BOP. The animals were anaesthetized by ether and exsanguinated by cannulating the abdominal aorta 30 min after treatment with bombesin; they were examined for gross pathological changes and the pancreas and liver were weighed. These organs were fixed in 10% buffered formalin. The pancreata were completely processed for microscopy by conventional methods, step-sectioned at 5 μm (about 5 per hamster pancreas) and stained with haematoxylin and eosin (H&E). An area of about 200 mm^2 was screened by light microscopy.

The different types of BOP-induced putative (pre)neoplastic ductular lesions used for analysis were evaluated as described previously (8). Major attention was directed to tubular ductal complexes showing dysplastic or anaplastic (cellular) changes, desmoplasia and/or inflammation, suggestive of progression to malignancy. A tubular ductal complex exhibiting one or more of the characteristics suggestive of a neoplastic change was classified as 'borderline'. A ductular adenocarcinoma is a lesion mainly composed of atypical dysplastic ductules. Such lesions are most characteristically surrounded by an inflammatory reaction. When such a lesion did not show local invasion in the surrounding acinar and fibrous tissue it was classified as carcinoma *in situ* if it exhibited local invasion as stage I ductular carcinomas or microcarcinomas. Advanced stages were classified as plain carcinomas.

Plasma CCK was measured by radioimmunoassay employing antibody T204 as previously described (3).

Body and organ weight data were evaluated statistically by two-way analysis of variance with initial body weight as covariable. The number of lesions was evaluated statistically by a generalized linear model with a normal error distribution. The relevant sources of variation were: treatment with gelatin; treatment with bombesin; combination of treatment with bombesin and CR-1409, and their interaction.

Results

Thirty minutes after a single injection with bombesin, either alone or in combination with CR-1409, plasma CCK concentrations were not significantly different from controls (Table 1).

The effects of bombesin, either alone or in combination with CR-1409, on body and pancreatic weights are presented in Table 2 and the quantitative analyses of pancreatic H&E-stained paraffin sections are presented in Table 3.

Table 1. Effects of bombesin, either alone or in combination with CR-1409 (a specific CCK-receptor antagonist) on plasma CCK of hamsters (mean ± SEM).[1]

Treatment	Plasma CCK level 0.5 h after injection (pM)
Control (gelatin)	3.3 ± 0.30
CR-1409 + gelatin	3.6 ± 0.13
BBS	3.6 ± 0.54
CR-1409 + BBS	4.3 ± 0.46

[1] Statistics: Student's t-test; * $P < 0.05$ compared to control.

Table 2. Effects of bombesin, either alone or in combination with CR-1409 on mean body/organ weights (± SEM) of BOP-treated hamsters.[1]

Treatment	Body weight (g)	Pancreatic weight		Liver weight	
		absolute (g)	relative (g/kg)	absolute (g)	relative (g/kg)
Control (gelatin)	121.8 ± 5.1	0.491 ± 0.026	4.11 ± 0.23	4.728 ± 0.219	38.80 ± 0.70
CR-1409 + gelatin	112.0 ± 2.5	0.452 ± 0.025	4.03 ± 0.18	4.252 ± 0.107	37.92 ± 0.75
BBS	121.7 ± 3.5	0.605 ± 0.023[1]	5.04 ± 0.21[1]	4.509 ± 0.166	37.05 ± 0.59
CR-1409 + BBS	108.3 ± 2.2	0.624 ± 0.029[1]	5.77 ± 0.26[1]	4.484 ± 0.161	41.25 ± 0.99[2]

Statistics: one-way analysis of variance followed by Student's t-test (two-tailed)
[1] Increased in animals treated with bombesin compared to animals not treated with bombesin; in combined treatment the effect could be ascribed to treatment with bombesin: $P < 0.01$.
[2] Compared to controls: $P < 0.05$.

Table 3. Effects of bombesin, either alone or in combination with CR-1409 on development of (pre)neoplastic lesions in exocrine pancreas of BOP-treated hamsters.

Treatment	Effective number of animals	Number of advanced ductular lesions			
		borderline lesion	carcinoma *in situ*	micro-carcinoma	carcinoma
Control (gelatin)	24	133	2	3	1
CR-1409 + gelatin	23	162	2	5	0
BBS	25	96[1]	3	1	0
CR-1409 + BBS	25	118[1]	4	2	1

[1] Decreased in animals treated with bombesin compared to animals not treated with bombesin; in combined treatment the effect could be ascribed to treatment with bombesin. Statistics: linear model with a normal error distribution: $P < 0.05$.

The influence of bombesin and CR-1409, as well as their interaction, on the square root (because of homogeneity of variance) of the number of borderline lesions and body weight, pancreatic and liver weights were assessed by analysis of variance. Bombesin increased significantly ($P < 0.01$) both absolute and relative pancreatic weight, but not body weight. Relative liver weight was increased significantly ($P < 0.05$) only by treatment with CR-1409 plus bombesin.

Statistical analyses of the data revealed a significant ($P < 0.05$) inhibitory effect of bombesin on the square root of the number of the borderline ductular lesions. This effect was maintained after statistical correction for pancreatic weight.

Data for the CR-1409-treated group have been reported and discussed previously (8). No significant interaction of bombesin and CR-1409 was found with respect to the above parameters.

The number of pancreatic carcinomas was too small to justify statistical analyses.

Discussion

In the present study it was shown that BOP-treated hamsters, injected subcutaneously with bombesin for 4 months, exhibited an increased pancreatic weight. Moreover, BBS-treated hamsters developed significantly less putative preneoplastic (borderline) ductular lesions as compared to controls. These effects of bombesin are not associated with significantly changed plasma CCK levels. These results are in agreement with those observed in a previous 2-week study in which BBS caused hyperplasia and hypertrophy of the pancreas in rats as well as in hamsters (7).

The present inhibitory effect of BBS on pancreatic carcinogenesis in hamsters is in contrast with the effect of BBS on pancreatic carcinogenesis in rats. From the observations in azaserine-treated rats it may be concluded that BBS has a promoting effect on both normal and putative preneoplastic (atypical acinar cell focus) tissue (4, 7).

The present results in hamsters demonstrate trophic as well as inhibitory responses on the pancreas suggesting that the actions of BBS (or possibly the hormones it releases) are site-specific and interact differently in normal and neoplastic pancreatic tissue. Our results demonstrate that BBS has an inhibitory effect on the development of putative preneoplastic ductular tissue. This may be a highly relevant finding because of the similarity of the tumours induced in hamster pancreas by BOP as compared to those occurring in man. This is the more true since Alexander et al. (1) found that growth of human ductal pancreatic adenocarcinoma xenografted into nude mice was significantly inhibited by BBS.

Previously, important interspecies differences were found in the effects of CCK and of TI (8, 10). Similarly, species specificity also seems to exist for the relation between bombesin and CCK. Bombesin stimulated the release of endogenous CCK in rats, but not in hamsters. Therefore, we suggested that the effects of bombesin could not be attributed to stimulation of secretion of endogenous CCK, notwithstanding bombesin stimulated pancreatic growth in rats and hamsters (4, 7).

This conclusion can be maintained in the present study. An explanation for the different carcinogenic effects of BBS in the rat and hamster may be that different cells of origin of pancreatic cancer are affected in these species (8, 9, 12, 13). The growth effects of BBS are probably mainly caused by hypertrophy and hyperplasia of acinar tissue, whereas the ductular lesions found in hamster pancreas would originate from centroacinar cells.

An inhibitory effect of BBS on pancreatic carcinogenesis in hamsters has not been described to date. Since Alexander et al. (1) found comparable effects on growth of transplanted human pancreatic adenocarcinomas, long-term studies into the effects of BBS on the development of ductular carcinomas induced in hamster pancreas by BOP are warranted in order to find out whether BBS may be of value for the treatment of pancreatic carcinomas in man.

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CHAPTER 6

Influence of cholecystokinin and bombesin on azaserine-induced lesions in rat pancreas

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Summary

Cholecystokinin and bombesin have been shown to promote pancreatic growth and development of azaserine-induced acidophilic atypical acinar cell nodules in rat pancreas after treatment for 16 weeks. Lorglumide, a specific cholecystokinin-receptor antagonist, inhibited the stimulating effect of cholecystokinin, but not of bombesin. The present study was carried out to determine effects of cholecystokinin and bombesin, alone and in combination with lorglumide, on pancreatic growth and carcinogenesis after chronic treatment. The animals were killed 8 months after the start of treatment.

Growth of the pancreas and the development of acidophilic atypical acinar cell nodules in exocrine pancreas were enhanced significantly by both cholecystokinin and bombesin, but the number of carcinomas was increased only by bombesin. Lorglumide inhibited the effects of cholecystokinin on both pancreatic growth and on the development of acidophilic nodules. The effects of bombesin on pancreatic growth and development of pancreatic lesions, except for adenomas, were not inhibited by lorglumide.

Introduction

Although the incidence of pancreatic cancer has increased in most Western countries during the past 30 years the aetiology of this disease is still largely unknown. Epidemiological studies show that the incidence of pancreatic cancer is higher among men than among women suggesting that hormonal factors may be involved in the development of this highly fatal type of tumour (1). Dietary factors such as high fat, protein and trypsin inhibitors (TI) have been implicated as relevant causative factors of pancreatic cancer and have also been found to promote carcinogenesis in the azaserine-rat model (2–5).

There is increasing evidence that cholecystokinin (CCK) plays an important role in TI-promoted pancreatic carcinogenesis (4). Plasma CCK levels had increased in rats and hamsters treated with TI (6). Furthermore, exogenously administered CCK enhanced the development of azaserine-induced acidophilic atypical acinar cell nodules (AACN) in the exocrine pancreas of rats (7). Lorglumide (CR-1409), a highly specific CCK receptor antagonist, inhibited the promoting effect of CCK on both pancreatic growth and development of AACN (7).

Although pancreatic growth was enhanced in azaserine-treated rats as well as in BOP-treated hamsters after treatment with CCK for 16 weeks, the effects of CCK on pancreatic carcinogenesis appeared to be different in these models (7, 8). CCK enhanced development of pancreatic acinar lesions in azaserine-treated rats, but not of pancreatic ductular lesions in BOP-treated hamsters. Liener et al. (9) found similar effects with RSF: in rats, but not in hamsters, pancreatic weight still increased after feeding RSF for 15 months. In rats pancreatic carcinogenesis had been enhanced at that time, whereas in hamsters pancreatic carcinogenesis appeared to be inhibited rather than enhanced.

Besides cholecystokinin, bombesin (BBS), the amphibian analogue of the gastrin-releasing peptide in mammals, has been found to stimulate pancreatic growth and carcinogenesis in the azaserine-rat model (10, 11). In the rat, exogenously administered bombesin causes elevation of plasma CCK levels, stimulation of pancreatic growth and enhancement of carcinogenesis (7, 11). Therefore, it has been suggested that bombesin-enhanced pancreatic stimulation is mediated by CCK (11). However, the results of a previously performed 16-week study with azaserine-treated rats, however, did not support this hypothesis (7).

Up to now most animal studies into the role of CCK and bombesin in pancreatic carcinogenesis lasted for 16 weeks at most using number and size of putative preneoplastic lesions as parameters (4, 7, 11). The present study was carried out to establish the long-term effects of CCK and BBS on pancreatic carcinogenesis in azaserine-treated rats.

Materials and methods

Male weanling Wistar Bor rats (Wisw,Cpb; $n = 240$; obtained from F. Winkelmann Versuchstierzucht GmbH, Borchen, FRG) were injected three times i.p. with 30 mg/kg of azaserine (Calbiochem, La Jolla, CA) at 19, 26 and 54 days of age. The animals were allocated to 6 different groups by a computerized randomization procedure. Each group consisted of 40 rats which were kept in stainless steel cages, fitted with wire-mesh floors and fronts. They were housed under standard laboratory conditions, 5 rats per cage, and kept on a high fat/high protein diet (Table 1) for 4 months to enhance the yield of advanced pancreatic lesions.

Treatment started 4 months after the first injection with carcinogen; during treatment the animals were maintained on a low fat/low protein diet (Table 1).

Table 1. Composition of the diets (% w/w)

Ingredients	High fat /high protein	Low fat /low protein
Casein	46.8	9.5
DL-methionine	0.48	0.1
Wheat	–	4.0
Wheat starch	–	60.2
Pregelatinized starch	13.6	5.0
Cellulose	11.8	10.0
Jones-Foster minerals	5.3	4.5
KH_2PO_4	0.71	0.6
Vitamin ADEK	0.53	0.45
Vitamin B mixture	0.36	0.3
Choline chloride	0.47	0.4
Maize oil	20.0	5.0

The animals received one of the following treatments (once daily, 3 d/wk for 16 wk): gelatin (controls); CR-1409 (12 mg/kg body wt, s.c.); CCK-8 (2.5 μg/kg body wt, s.c.); CCK-8 (2.5 μg/kg body wt, s.c.) in combination with CR-1409 (12 mg/kg body wt, s.c.); BBS (10 μg/kg body wt, s.c) and BBS in combination with CR-1409 (12 mg/kg body wt, s.c.). CR-1409 was dissolved in distilled water to a concentration of 0.4% and adjusted to pH 9 with 0.1 M NaOH and subsequently administered 30 min before injection of CCK or BBS.

The dose of cholecystokinin octapeptide (CCK-8) and bombesin used in the study were based on plasma concentration time curves for CCK-8 obtained from a previously described 2-week study in rats and hamsters (11). In this 2-week study it was shown that subcutaneous administration of 2.5 μg/kg body wt of CCK-8 (CCK-8; Cambridge Research Biochemical, Cambridge, UK) or 10 μg/kg body wt of BBS (Sigma Chemical, St. Louis, MO), both dissolved in 16% hydrolysed gelatin (Hospital Pharmacy, University Hospital, Leiden, Netherlands) resulted in plasma CCK levels only slightly exceeding physiological levels and similar to those seen after dietary administration of maize oil. Plasma CCK levels were determined using a highly specific and sensitive radioimmunoassay as previously described in detail (12, 13). The highly specific CCK receptor antagonist lorglumide (CR-1409, kindly provided by Rotta Research Laboratories, Milan, Italy). The general condition and behaviour of the animals were checked daily.

Terminal autopsy was conducted on 136 animals after 8 months of treatment. The rats were anaesthetized by ether and exsanguinated by cannulating the abdominal aorta 30 minutes after the s.c. administration of 2.5 μg/kg body wt of CCK-8 or 10 μg/kg body wt of BBS; they were examined for gross pathological changes and the pancreas and liver were weighed. These organs were 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.

All animals that died beyond 6 months of treatment but before the terminal autopsy were incorporated in the study, provided their pancreata were fit for histopathological evaluation. About 200 mm^2 of pancreatic tissue of each rat was microscopically screened for the number of azaserine-induced AACN, using a grid inside the ocular as described before (14). Only acidophilic AACN with transection areas >0.5 mm^2 were counted and classified on size. AACN, acinar cell adenomas (AACN with a transection area larger than 3 mm^2), and (localized) carcinomas were identified and classified according to criteria of Longnecker et al. (15, 16).

The approval for above procedures was obtained by the animal welfare committee of the Institute.

Mean organ and body weights were evaluated with analysis of variance. The number of pancreatic lesions was evaluated with a generalized linear model (17). With such a model, the effects of type of promoter (none, CCK, bombesin), of presence of receptor-antagonist, and of the interaction between these factors can be assessed. A Poisson error distribution was used for the evaluation of the adenomas and carcinomas in view of the relatively low observed counts. The counted numbers of AACN were considerably larger. Therefore, we may approximate the relevant error distribution with a normal error distribution for the square root of these counts (17). The statistical analyses of AACN reported here are based on this approximation. Analyses based on the Poisson distribution, however, should not substantially alter the conclusions. For evaluation of the prevalence (incidence) of tumours we used again a generalized linear model. The difference with the model used for lesions was the error distribution. For prevalence, the binomial distribution is appropriate.

Results

Plasma CCK levels in subgroups of animals obtained half an hour after administration of gelatin (control), lorglumide and CCK or BBS, alone or in combination with CR-1409, are shown in Table 2. The results demonstrate that CCK concentrations during lorglumide administration were significantly higher ($P<0.01$) than in the control rats that received gelatin. Both CCK and BBS led to a marked increase of plasma CCK concentrations as compared with control animals ($P<0.001$). There was no significant difference between CCK concentrations obtained in rats after CCK or BBS administration. Lorglumide slightly augmented both CCK- and BBS-stimulated plasma CCK values. However

these differences did not reach statistical significance when compared to CCK values obtained after CCK and BBS administration without lorglumide (Table 2).

Table 2. CCK plasma levels (pM) in subgroups of animals obtained half an hour after administration of gelatin, lorglumide and CCK or BBS, alone or in combination with lorglumide.

Animal No.	Controls	CR-1409	CCK-8	CCK-8 + CR-1409	BBS	BBS + CR-1409
1	1.1	3.2	7.6	8.7	7.5	9.3
2	2.2	6.7	8.8	9.7	10.9	9.3
3	3.2	6.8	11.2	12.7	7.9	10.3
4	1.3	1.2	9.3	13.1	9.3	11.5
5	1.2	0.6	8.2	10.2	8.7	9.3
6	2.1	5.3	5.7	9.7	10.0	13.0
7	2.7	4.8	4.8	9.9	6.9	7.7
8	0.5	2.8	7.9	7.1	4.8	7.9
9	0.1		9.2	6.8	11.2	
10	1.3					
Mean	1.57	3.93**	8.08***	9.77***	8.58***	9.79***
± SEM	± 0.31	± 0.83	± 0.64	± 0.72	± 0.68	± 0.63

Statistics: ANOVA; compared to control: ** $P < 0.01$, *** $P < 0.001$

Table 3. Effects of CCK and BBS, either alone or in combination with CR-1409, on mean body and organ weights (± SEM)

Treatment	*n*	Body wt at autopsy (g)	Pancreatic weight		Liver weight	
			absolute (g)	relative (g/kg)	absolute (g)	relative (g/kg)
Control (gelatin)	37	458.4 ± 8.7	1.261 ± 0.045	2.78 ± 0.10	14.27 ± 0.48	31.0 ± 0.8
CR-1409 + gelatin	34	474.1 ± 9.4	1.219 ± 0.043	2.61 ± 0.11	14.98 ± 0.59	31.6 ± 1.1
CCK[1]	31	466.2 ± 8.5	1.918 ± 0.069**	4.13 ± 0.15**	14.59 ± 0.39	31.4 ± 0.8
CR-1409 + CCK[1 2]	34	454.2 ± 8.2	1.492 ± 0.051**	3.29 ± 0.11	13.95 ± 0.41	30.6 ± 0.5
BBS[1]	35	451.2 ± 7.9	2.379 ± 0.086**	5.32 ± 0.21**	14.31 ± 0.37	31.6 ± 0.4
CR-1409 + BBS[1]	32	454.8 ± 8.0	2.306 ± 0.107**	5.11 ± 0.25**	14.40 ± 0.35	31.7 ± 0.5

Statistics: one-way analysis of variance followed by Student's t-test (two-tailed); ** $P < 0.01$.
[1] Compared to control
[2] Compared to CCK alone, the effect was significantly smaller ($P < 0.01$).

The effects of CCK-8 and bombesin, alone or in combination with CR-1409, on body weight and pancreatic and liver weights are shown in Table 3.

Neither body weight nor absolute and relative liver weights were influenced significantly by the respective treatments. CR-1409 alone did not influence pancreatic weight.

Table 4. Effects of CCK-8 and BBS, either alone or in combination with CR-1409 on development of acidophilic AACN and carcinomas in exocrine pancreas of azaserine-treated rats

Treatment	Effective number of animals	Total number of lesions per group			
		AACN trans.area 0.5–1.0 mm^2	AACN trans.area 1.0–3.0 mm^2	adenomas AACN, trans. area >3.0 mm^2	carcinomas
Control (gelatin)	37	62	21	3	16
CR-1409 + gelatin	37	51	35	4	11
CCK	34	357***[1]	231***[1]	19***[1]	16
CR-1409 + CCK	35	163***[2]	86**[2]	10**[2]	16
BBS	37	555***[1]	336***[1]	31***[1]	36**[1]
CR-1409 + BBS	32	356	213	11**[3]	16

Statistics: linear model (square root of AACN) or generalized linear model with Poisson error (adenomas, carcinomas); ** $P < 0.01$, *** $P < 0.001$.
[1] vs controls.
[2] CR-1409 + CCK vs CCK.
[3] CR-1409 + BBS vs BBS.

Table 5. Effects of CCK-8 and BBS, either alone or in combination with CR-1409, on total number and incidence of carcinomas in exocrine pancreas of azaserine-treated rats.[1]

	Total number/incidence of lesions in group					
	A	B	C	D	E	F
a. Total number						
Effective number of animals	37	37	34	35	37	32
Number of pancreatic carcinomas	16	11	16	16	36**[2]	16
b. Incidence						
Effective number of animals	37	37	34	35	37	32
Number of animals bearing a tumour	12	9	12	12	22**[2]	12

[1] Group A, controls; group B, CR-1409; group C, CCK-8; group D, CR-1409 + CCK-8; group E, BBS; group F, CR-1409 + BBS. Statistics: generalized linear model; ** $P < 0.01$.
[2] BBS vs no-BBS.

Absolute as well as relative pancreatic weight was increased ($P < 0.01$) by treatment with CCK or bombesin. The effect of CCK, but not of bombesin, on pancreatic weight was reduced significantly when CR-1409 was administered half an hour before treatment with CCK or bombesin. However, pancreatic weight with the combination of CCK and CR-1409 was still slightly higher than in the control group. The quantitative analyses of pancreatic H&E-stained paraffin sections are presented in Tables 4 and 5.

Treatment with CR-1409 alone did not affect the number of pancreatic lesions. Treatment with CCK or bombesin increased the number of acidophilic AACN of all size categories, including adenomas ($P < 0.001$).
Treatment with CR-1409 inhibited the enhancing effect of CCK on the development of acidophilic AACN, including adenomas ($P < 0.01$). Neither on the total number of carcinomas nor on the incidence of tumours (Table 5) there was an effect of treatment with CCK or CR-1409. In contrast, treatment with bombesin increased both the total number of carcinomas ($P < 0.01$) and the incidence of carcinomas ($P < 0.05$). CR-1409 showed a tendency to inhibit the enhancing effect of bombesin on the number of all types of pancreatic lesions. However, the difference with the group treated with bombesin alone reached statistical significance ($P < 0.01$) for the total number of adenomas only.

Discussion

The main findings of the present 12-month study with azaserine-treated rats can be summarized as follows.

1. CCK as well as bombesin stimulate pancreatic growth even after chronic administration.
2. CR-1409, a specific CCK receptor antagonist, inhibits pancreatic growth stimulated by CCK, but not by bombesin.
3. CCK in a dose slightly higher than the physiological level enhances the development of AACN, but not of carcinomas.
4. CR-1409 inhibits this CCK-stimulated growth of AACN.
5. Bombesin enhances the development of all pancreatic neoplastic lesions including carcinomas.
6. CR-1409 shows a tendency to inhibit the bombesin-promoted effect on these pancreatic lesions, but this inhibitory effect reached the level of statistical significance for the adenomas only.

The observed inhibitory effect of CR-1409 on CCK-stimulated pancreatic growth is in agreement with our previous findings in a 4-month study (7) indicating that the receptors for CCK on normal as well as atypical acinar pancreatic cells remain present and responsive to CCK stimulation even during chronic treatment. The

absence of a promoting effect of CCK on development of pancreatic acinar carcinomas does not support the hypothesis that an enhancing effect on growth of AACN is indicative of promotion of pancreatic carcinogenesis and thus will ultimately lead to an increase in malignant tumours. In the present study, CCK enhanced growth of AACN, but not of carcinomas, which suggests that (a) carcinomas do not always develop according to an ordered evolutionary sequence starting from initiated acinar cells, which grow out to acidophilic foci, via nodules and adenomas to carcinomas as suggested previously (18) and (b) growth factors that stimulate the growth of altered cell foci, thus speeding up the process of carcinogenesis, need one or more extra steps (second hits?) to give rise to malignant tumours. Consequently, it seems justifiable to conclude from the present results that CCK alone does not play a decisive role in the development of adenocarcinomas in exocrine pancreas of rats treated with azaserine. It is also possible that the populations of AACN that ultimately lead to the formation of carcinomas have lost their receptors for CCK and hence do not respond to CCK treatment.

It has been established that the gut hormone CCK plays an important role in the effect of trypsin inhibitors on pancreatic growth (19). It is, however, not clear how the stimulation of growth leads to neoplasia. The discrepancy between our results and those of Green et al. (20, 21), McGuinness et al. (22) and Morgan et al. (23), all of whom found an increase in pancreatic carcinomas in rats treated with RSF or TI, may be attributable to a prolonged CCK plasma level elevation by RSF or TI, or to gastrointestinal polypeptides other than CCK, the secretion of which is stimulated by RSF and TI, or other unknown factors due to RSF or TI.

The present findings with bombesin point in the same direction. In contrast to CCK, bombesin enhanced the development of all azaserine-induced pancreatic lesions in rats, including carcinomas. Interestingly, the effects obtained with bombesin were more pronounced than those seen with CCK alone. Moreover, bombesin enhanced not only the development of AACN but also of carcinomas. This is the more important since the dose of bombesin used caused slightly supraphysiological plasma CCK levels which were almost equal to those obtained with the dose of CCK-8 used in the present study (Table 2, 11).

Concomitant administration of CR-1409 in a dose that significantly inhibited the effect of CCK on development of AACN demonstrated a consistently lower number of pancreatic lesions than for animals treated with bombesin alone. This observation supports our previous suggestion that the promoting effect of bombesin on pancreatic carcinogenesis may be partly mediated by stimulation of plasma CCK secretion. The observation that CR-1409 did not inhibit significantly the effect of bombesin-stimulated development of carcinomas supports our hypothesis that CCK-enhanced pancreatic growth alone does not cause the development of carcinomas. Apparently apart from pancreatic growth, factors such as gastrointestinal polypeptides or others, which are stimulated by feeding of RSF or TI and bombesin, are needed for the development of pancreatic adenocarcinomas. This interpretation is also supported by the observation that CR-1409 did not have a significant effect on bombesin-stimulated pancreatic growth. From this observation it may be concluded

that the effect of bombesin on pancreatic growth is rather due to a direct effect on the acinar cells via bombesin receptors, which have been identified in most species (24–28), rather than via CCK only. It is, however, not known whether altered acinar cell foci contain bombesin receptors, hence the mechanism by which bombesin induces the development of pancreatic adenocarcinomas in azaserine-treated rats needs further elucidation. The finding that CR-1409 administration induced a slight decrease in development of all types of pancreatic lesions during BBS treatment reaching statistical significance for adenomas only, may suggest that CCK plays a contributing role in the tumour development by bombesin.

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CHAPTER 7

Effects of castration, alone and in combination with aminoglutethimide, on growth of (pre)neoplastic lesions in exocrine pancreas of rats and hamsters

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Summary

We studied the effects of hormonal manipulation by orchiectomy, alone or in combination with the aromatase inhibitor aminoglutethimide, and by luteinizing hormone-releasing hormone (LHRH) agonist (goserelin) treatment on the development of early putative (pre)neoplastic lesions induced in the pancreas of rats and hamsters by azaserine and BOP, respectively. Treatment of the animals started one week after the (last) injection with carcinogen and continued for 4 months. Orchiectomy caused a significant inhibition of growth of acidophilic atypical acinar cell nodules in the rat model, whereas surgical castration did not show an effect in the hamster model. In rats, but not in hamsters, orchiectomy resulted in a significant decrease in body weight and in absolute, but not relative, pancreatic weight. Treatment of the animals with aminoglutethimide or goserelin did not have a significant effect on the development of either putative preneoplastic acinar lesions in rat pancreas or early ductular lesions in hamster pancreas. Hamsters showed clearly higher plasma EGF and IGF-1 concentrations than rats, while plasma testosterone levels were significantly lower. Plasma EGF and IGF-1 levels decreased with increasing age in both control and treatment groups. Compared to controls there were no unequivocal effects of treatment on EGF, IGF-1 and gastrin levels. Plasma testosterone levels were decreased by orchiectomy or LHRH agonist treatment. In rats hormone-induced effects on food intake and altered nutritional status might be important with respect to the development of carcinogen-induced preneoplastic pancreatic lesions.

Introduction

Epidemiological studies have demonstrated that in most countries, including the Netherlands, the age-adjusted incidence of pancreatic cancer is higher in men than in women (1–3). Although life-style factors such as cigarette smoking may be involved there is increasing evidence that steroid hormones play a role in this sex difference. In azaserine-treated rats (model for acinar adenocarcinomas) the growth of putative precancerous lesions is stronger in males than in females (4–9) resulting in a higher number of tumours in males than in females 12–15 months after injection of carcinogen. Moreover, transplanted acinar cell carcinomas grow more rapidly in syngeneic male recipients than in females (6), and (6-D-tryptophan)-luteinizing hormone-releasing hormone agonist (LHRH-A) inhibits growth of transplanted acinar carcinomas in rats (10–12). Recently, it was demonstrated that oestrogen treatment and surgical castration inhibits the development and growth of putative precancerous acinar lesions induced in rat pancreas by azaserine (7, 8). In addition, the presence of intracellular receptors for oestrogen (ER), progesterone (PR) and androgen (AR) in pancreatic cells indicates that sex hormones exert some influence on the growth of normal and malignant pancreatic cells (13–15).

It is obscure, however, whether these effects are due to an inhibitory effect of oestrogen or to a promotive effect of androgen. Testosterone may act directly via AR or indirectly via ER after having been metabolized to oestrogen by the enzyme aromatase (16). In vitro decrement of testosterone concentrations to less than 2.5 nM (comparable with plasma concentrations in castrated men) caused a striking decrease in aromatase activity in foetal as well as in malignant pancreatic tissue (17). In hamsters LHRH-A was found to inhibit growth of transplanted ductular carcinomas (10). Redding and Schally (10) have also reported a slight inhibitory effect of LHRH-A, either alone and in combination with the somatostatin analogue RC-160, on growth, but not on the incidence of ductular adenocarcinomas in hamsters repeatedly injected with BOP (18–23). Interestingly, in the BOP-hamster model a difference between males and females in the development of ductular adenocarcinomas, as seen in the azaserine-rat model, has not been reported. It is important to note that orchiectomy and chronic treatment with oestrogen has a significant inhibitory effect on body weight gain in rats (7, 8). Therefore, it cannot be excluded that the inhibitory effects of castration and oestrogen treatment on pancreatic carcinogenesis in azaserine-treated rats might be partly due to a non-specific mechanism such as reduced energy intake (24). Studies performed in our Institute with cholecystokinin, bombesin and a synthetic trypsin inhibitor demonstrated that the effects of hormonal manipulation of pancreatic carcinogenesis differ mearkedly between rats and hamsters (25–27). This difference is most probably related to the different types of tumours that develop in these animal models: ductular adenocarcinomas, closely resembling those occurring in man, in BOP-treated hamsters and acinar adenocarcinomas in rats.

Although the histogenesis of the ductular tumours in hamsters and their putative precancerous precursor lesions has not yet been established beyond doubt (28–32), it is not illogical to assume that hormones interact with atypical acinar cells in another manner than with centroacinar or ductular cells. Furthermore, most studies dealing with hormonal manipulation of pancreatic carcinogenesis have either used the incidence of pancreatic tumours evoked in experimental animals by multiple weekly injections of carcinogen or the growth of subcutaneously transplanted pancreatic tumours as parameters (10, 18–23). Less attention has been paid to the effects of hormones on development of early, putative precancerous acinar or ductular pancreatic lesions.

In the present study the effects were studied of orchiectomy, alone or in combination with aminoglutethimide, an aromatase inhibitor, and of LHRH agonist treatment on pancreatic carcinogenesis in rats and hamsters using number and size of (pre)neoplastic foci as parameters.

Materials and methods

Seventy-five male weanling SPF albino Wistar Bor rats (WISW; Cpb) were obtained from F. Winkelmann, Borchen, FRG, and 100 male weanling Syrian golden hamsters from SAVO, Kissleg, FRG. The rats were kept in stainless steel cages, fitted with wire-mesh floors and fronts, and the hamsters in macrolon cages on softwood bedding. All animals were housed under standard laboratory conditions, five animals per cage. They were fed a diet high in unsaturated fatty acids (20% maize oil). The diet was compounded from natural feed ingredients. The composition of the diet has been reported previously (33).

All rats were given a single i.p. injection of 30 mg azaserine/kg body wt at 19 days of age. The hamsters were each injected s.c., once weekly, with 20 mg BOP/kg body wt at 6, 7 and 8 weeks of age according to an injection protocol as previously described (34). BOP (Ash Stevens, 5861 John C. Lodge Freeway, Detroit, MI 48202) and azaserine (Calbiochem-Behring, La Jolla, CA) were dissolved freshly in a 0.9% NaCl solution.

The animals were allocated by a computerized randomization procedure to five different groups each of which consisted of either 15 rats or 20 hamsters. The animals of the five groups for both species were treated as follows: (A) controls, injected s.c. twice daily on week days and once daily during the weekend with saline (0.9% NaCl); (B) orchiectomy directly after the (last) treatment with carcinogen; (C) the LHRH agonist goserelin (one depot preparation, Zoladex, ICI, one s.c. injection every 4 weeks); (D) the aromatase inhibitor amino-glutethimide (AGT), 2 mg injected s.c. twice daily on week days and once daily during the weekend; (E) treated as group D in combination with orchiectomy. Treatment of the animals with goserelin and AGT started one week after the last injection with carcinogen and lasted 17 weeks (rats) or 15 weeks (hamsters).

Body weights were recorded weekly and food intake during one week per month. The general condition and behaviour of the animals were checked daily. Terminal autopsy of rats was on days 128 or 129 after azaserine treatment and of hamsters on days 115 or 116 after the last injection with BOP. The animals were anaesthetized with ether, exsanguinated by cannulating the abdominal aorta, autopsied and then examined for gross pathological changes. From each animal the pancreas, liver, testes (if present), adrenals and pituitary were excised and weighed. These organs were fixed in 10% buffered formalin. The pancreata were completely processed for microscopy by conventional methods, step-sectioned at 5 μm (about 10 sections per rat pancreas and about 5 per hamster pancreas), stained with haematoxylin and eosin (H&E) and examined by light microscopy. In rats quantitative determination of the number of foci per cm^2 of pancreas and of the area of focus transections was carried out using a grid inside the ocular as described previously (33). In hamsters, major attention was directed to intermediate and tubular ductal complexes showing dysplastic or anaplastic cellular changes, desmoplasia and/or inflammation, suggestive of progression to malignancy (classified as 'borderline lesions') and (early) carcinomas. These putative preneoplastic and (early) neoplastic lesions were evaluated as described previously (34, 35, 27). The ductular carcinomas induced by BOP in hamster pancreas were identified and classified according to Pour (35).

Assay of growth factors (EGF- and IGF-1-like activities) and of hormones (gastrin and testosterone)

Blood sampling for hormone estimations were performed 8, 12 and 16 weeks after start of the treatment. EGF- and IGF-1-like activities were measured in acid/ethanol-extracted EDTA-plasma by radioreceptor assays using human placental membrane preparations as receptor source. All procedures were exactly as described for determination of EGF- and IGF-1-like activities in human breast tumour cytosols (36). Gastrin was measured directly in EDTA-plasma with a radioimmunoassay (RIA) kit (Cambridge Medical Diagnostic, Billerica, USA). Testosterone levels were estimated as described by Verjans et al. (37).

The treatment effects on plasma hormone and growth factor levels were analysed by ANOVA (analysis of variance) on the pooled data of all treatment groups and controls at the end of treatment. Small *P* values from these analysis, performed for each growth factor or hormone in rats and hamsters, are indicative of a difference in effect, either a decrease or increase of plasma concentrations.

Body and organ weight data were evaluated statistically by two-way ANOVA with initial body weight as covariable. Food intake figures were subjected to Kruskal-Wallis analysis of variance and least statistical difference (LSD) tests. In rats, the calculated volumetric data of foci were evaluated by one-way ANOVA. In hamsters, the number of lesions was evaluated statistically by a generalized linear model with a Poisson error distribution. Evaluated sources of variation were: orchiectomy alone; aminoglutethimide alone; their interaction and treatment with LHRH agonist alone.

Results

Growth and food intake

Growth of surgically castrated rats decreased ($P < 0.01$) throughout the experimental period as compared to intact rats (Figure 1). This decrease in weight gain was accompanied with a decrease ($P < 0.001$) in mean food and energy intake (Table 1). Growth and mean food and energy intake of animals of the other groups, including those treated with medical castration by a LHRH agonist, was comparable with the controls (Figure 1 and Table 1).

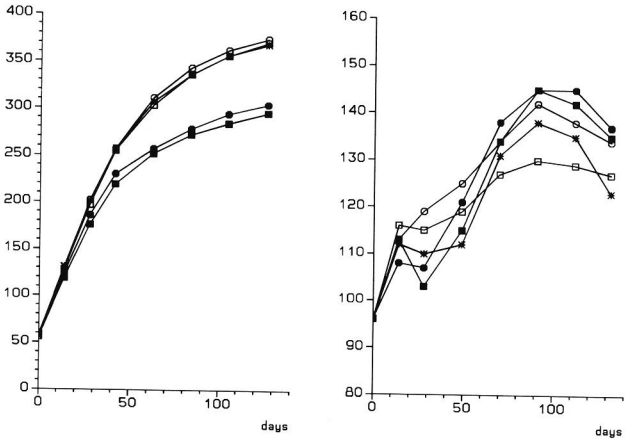

Fig. 1. Mean body weight. Left: rats; right: hamsters.
Open circles: control; solid dots: castration; open squares: AGT; solid squares: castration + AGT; asterisks: LHRH agonist.

Table 1. Mean food and energy intake.

Post-initiation treatment group	Rats		Hamsters	
	food g/animal per week	energy kJ/animal per week	food g/animal per week	energy kJ/animal per week
Control	89.6	1518.7	46.2	783.1
Castration	76.1***	1289.9	48.3	818.7
LHRH agonist	92.3	1564.5	43.4	735.6
AGT	91.6	1552.6	48.3	818.7
AGT + castration	75.8***	1284.8	45.5	771.2

Statistics: analysis of variance + LSD test; *** $P < 0.001$.

Table 2. Effects of the various treatments on body weight and the absolute weight of different organs (in g). [1]

	Control	Castration	LHRH agonist	AGT	AGT + Castr.
Rats(*n*)	(15)	(15)	(15)	(15)	(14)
Body weight	373.6 ± 10.2	303.5 ± 9.5**	367.8 ± 9.4	370.4 ± 8.1	295.1 ± 6.8**
Pancreas	1.145 ± 0.047	0.928 ± 0.035**	1.007 ± 0.039*	1.045 ± 0.026	0.895 ± 0.030**
Liver	10.67 ± 0.46	7.48 ± 0.27**	10.52 ± 0.30	11.04 ± 0.30	7.30 ± 0.24**
Testes	3.13 ± 0.07	—	2.50 ± 0.09**	3.25 ± 0.06	—
Adrenal	0.055 ± 0.002	0.049 ± 0.002	0.047 ± 0.001*	0.045 ± 0.002**	0.046 ± 0.003**
Pituitary	0.015 ± 0.001	0.017 ± 0.001	0.013 ± 0.001	0.013 ± 0.000	0.015 ± 0.001
Hamsters(*n*)	(18)	(15)	(18)	(18)	(12)
Body weight	134.1 ± 3.8	137.2 ± 6.5	122.8 ± 4.6	127.0 ± 4.2	135.4 ± 5.1
Pancreas	0.441 ± 0.021	0.518 ± 0.026	0.449 ± 0.026	0.441 ± 0.022	0.534 ± 0.036
Liver	5.373 ± 0.186	5.758 ± 0.391	4.986 ± 0.269	5.285 ± 0.171	5.541 ± 0.318
Testes	1.021 ± 0.206	—	0.538 ± 0.089*	0.425 ± 0.033**	—
Adrenal	0.026 ± 0.001	0.019 ± 0.002**	0.023 ± 0.001	0.026 ± 0.001	0.019 ± 0.001**
Pituitary	0.007 ± 0.000	0.009 ± 0.000**	0.007 ± 0.001	0.006 ± 0.000	0.010 ± 0.000**

[1] All values are means ± SEM.
Statistics: analysis of variance + Dunnett's test; compared to controls: $*P < 0.05$, $**P < 0.01$.

Table 3. Effects of the various treatments on body weight (g) and organ weight relative to body weight (g/kg) of different organs. [1]

	Control	Castration	LHRH agonist	AGT	AGT + Castr.
Rats (*n*)	(15)	(15)	(15)	(15)	(14)
Body weight	373.6 ± 10.2	303.5 ± 9.5**	367.8 ± 9.4	370.4 ± 8.1	295.1 ± 6.8**
Pancreas	3.08 ± 0.12	3.08 ± 0.12	2.74 ± 0.10	2.83 ± 0.08	3.03 ± 0.09
Liver	28.4 ± 0.6	24.6 ± 0.3**	28.7 ± 0.6	29.8 ± 0.7	24.7 ± 0.3**
Testes	8.43 ± 0.24	—	6.85 ± 0.30**	8.79 ± 0.16	—
Adrenal	0.149 ± 0.009	0.162 ± 0.006	0.130 ± 0.005	0.122 ± 0.004**	0.154 ± 0.007
Pituitary	0.040 ± 0.002	0.055 ± 0.002**	0.036 ± 0.002	0.035 ± 0.001	0.051 ± 0.002**
Hamsters (*n*)	(18)	(15)	(18)	(18)	(12)
Body weight	134.1 ± 3.8	137.2 ± 6.5	122.8 ± 4.6	127.0 ± 4.2	135.4 ± 5.1
Pancreas	3.33 ± 0.14	3.78 ± 0.11	3.66 ± 0.18	3.50 ± 0.17	3.99 ± 0.30
Liver	40.1 ± 0.8	41.9 ± 1.8	40.4 ± 1.1	41.7 ± 0.7	40.7 ± 1.1
Testes	7.22 ± 1.26	—	4.59 ± 0.80	3.36 ± 0.26**	—
Adrenal	0.191 ± 0.006	0.141 ± 0.014**	0.187 ± 0.007	0.206 ± 0.014	0.144 ± 0.008**
Pituitary	0.050 ± 0.003	0.071 ± 0.004**	0.059 ± 0.004	0.053 ± 0.003	0.076 ± 0.005**

[1] All values are means ± SEM.
Statistics: analysis of variance; compared to controls: $**P < 0.01$.

In hamsters, food and energy intake was comparable among all groups (Table 1). Neither orchiectomized nor LHRH-A-treated hamsters showed a significantly decreased growth rate as compared to untreated controls (Figure 1).

Body and organ weights

Absolute but not relative pancreas weights were significantly lower in orchiectomized and LHRH-treated rats; this effect was more pronounced in orchiectomized ($P < 0.01$) than in LHRH-A-treated animals ($P < 0.05$) (Tables 2 and 3).

Absolute and relative liver weights were significantly lower in the orchiectomized groups. Mean absolute adrenal weight was significantly lower in the groups treated with LHRH agonist and AGT. Mean relative, but not absolute, pituitary weight was higher ($P < 0.01$) in the orchiectomized groups (Tables 2 and 3). Neither LHRH-A nor AGT influenced absolute or relative pituitary weight. LHRH agonist, but not AGT, decreased absolute and relative testis weights ($P < 0.01$).

In contrast to rats, mean absolute pancreas weight of hamsters was comparable among all groups including the groups exposed to surgical or chemical castration. Orchiectomized hamsters exhibited increased absolute and relative pituitary weight ($P < 0.01$). In addition, lower ($P < 0.01$) mean absolute and relative adrenal weights were observed compared to intact hamsters.

Absolute testis weights were significantly lower in the groups treated with LHRH or AGT. In the group treated with AGT alone also relative testis weight had decreased. The absence of a significant decrease in relative testis weight in the group treated with LHRH seems mainly due to the relatively large variation in testis weight of the controls.

Plasma growth factor and hormone levels (Table 4)

In rats plasma EGF concentrations decreased during the treatment period in both control and treatment groups. Although plasma EGF levels tended to be lower in the rats treated with the LHRH agonist or AGT, there were no significant differences among groups at the end of the experiment. Also plasma IGF-1 levels decreased in all groups, with the exception of the group treated with surgical castration plus AGT. However, this group showed already lower levels after 8 weeks of treatment, so it cannot be concluded that AGT treatment does increase IGF-1 levels. No unequivocal effects of treatment or age on plasma gastrin levels were observed although plasma gastrin levels were lower in the castrated group. As expected, surgical castration caused very low plasma testosterone levels. Medical castration by goserelin also decreased plasma testosterone levels significantly, but to a lesser extent than surgical castration. On the other hand, AGT tended to increase plasma testosterone levels.

Hamsters showed clearly higher plasma EGF and IGF-1 concentrations than rats, while plasma testosterone levels were significantly lower in hamsters (Table 4). Treatment did not decrease EGF, IGF-1 and gastrin levels, although IGF-1 levels were slightly higher in the LHRH agonist treatment group. As expected, surgical and medical castration caused significantly lower plasma testosterone concentrations. In contrast to rats, also treatment with AGT decreased plasma testosterone levels.

Table 4. Effects of the respective treatments on plasma growth factor and hormone levels. [1]

	Controls	Castration	LHRH-A	AGT	AGT + castration	P[2]
Rats						
EGF (ng/ml)						
8 weeks	5.7 ± 1.1	4.1 ± 1.1	7.3 ± 1.3	8.5 ± 1.0	5.2 ± 2.1	
12 weeks	3.6 ± 1.4	1.0 ± 0.5	3.2 ± 0.8	2.6 ± 1.9	3.3 ± 1.3	
16 weeks	2.7 ± 0.7	2.5 ± 1.0	1.4 ± 0.6	1.4 ± 0.5	3.4 ± 1.0	0.30
n[3]	(15)	(15)	(15)	(15)	(15)	
IGF-1 (ng/ml)						
8 weeks	265 ± 41	216 ± 32	203 ± 18	213 ± 39	130 ± 15	
12 weeks	175 ± 14	193 ± 16	200 ± 17	128 ± 53	248 ± 18	
16 weeks	117 ± 6	97 ± 9	133 ± 14	148 ± 10	175 ± 7	< 0.0001
n	(15)	(15)	(15)	(15)	(15)	
Gastrin (ng/ml)						
8 weeks	92 ± 5	100 ± 3	106 ± 12	122 ± 20	111 ± 10	
12 weeks	101 ± 7	103 ± 7	126 ± 10	140 ± 10	128 ± 24	
16 weeks	114 ± 9	98 ± 4	120 ± 6	100 ± 8	74 ± 7	0.0005
n	(15)	(15)	(15)	(11)	(9)	
Testosterone (nmol/l)						
8 weeks	12.8 ± 5.2	0.4 ± 0.3	28.7 ± 5.8	3.5 ± 0.6	0.06 ± 0.02	
12 weeks	13.5 ± 5.1	0.4 ± 0.4	5.5 ± 0.8	14.6 ± 5.6	0.06 ± 0.02	
16 weeks	7.5 ± 1.3	0.17 ± 1.0	3.0 ± 0.4	10.7 ± 2.0	0.12 ± 0.04	< 0.0001
n	(15)	(14)	(15)	(15)	(14)	
Hamsters, 16 weeks						
EGF (ng/ml)	15 ± 2	17 ± 1	21 ± 2	18 ± 1	18 ± 2	< 0.03
n	(9)	(7)	(9)	(9)	(6)	
IGF-1 (ng/ml)	245 ± 17	264 ± 37	333 ± 32	286 ± 16	314 ± 20	0.10
n	(9)	(6)	(8)	(9)	(6)	
Gastrin (ng/ml)	106 ± 3	129 ± 19	117 ± 7	130 ± 5	134 ± 4	0.22
n	(9)	(7)	(6)	(6)	(6)	
Testosterone (nmol/l)	3.2 ± 1.1	0	0.17 ± 0.07	0.69 ± 0.40	0.02 ± 0.02	< 0.002
n	(9)	(8)	(9)	(10)	(6)	

[1] All values are means ± SEM.
[2] P values associated with test of difference between the five groups after 16 weeks of treatment.
[3] Number of animals at 16 weeks; at 8 and 12 weeks only 5 animals were used.

Microscopy

The results of the quantitative analyses of rat pancreatic H&E-stained paraffin sections are presented in Table 5. Statistical analyses of the calculated volumetric data of rats revealed that surgical castration caused a decrease ($P < 0.01$) in the total numbers of acidophilic foci which was not accompanied with a decrease in the total area affected (% of pancreas occupied by acidophilic focus tissue). There was no interaction between castration and AGT. AGT caused a slight decrease in number of basophilic foci which only reached the level of statistical significance ($P < 0.05$) in the category with a mean diameter of 96 μm. Treatment of the rats with LHRH agonist did not have a significant effect on the number or growth of acidophilic or basophilic foci.

In hamsters (Table 6), none of the presently studied potential therapies for pancreatic cancer in man caused a significant inhibitory effect on the development of putative precancerous or cancerous ductular lesions.

Table 5. Effects of treatments on growth of putative preneoplastic lesions in azaserine-treated rats. [1]

	Control (n = 15)	Castration [2] (n = 15)	LHRH agonist (n = 15)	AGT [3] (n = 15)	AGT + castr [2] [3] (n = 14)
Acidophilic foci					
Observed transection data					
Total number of foci per cm^2	5.84 ± 0.99	3.49 ± 0.99**	7.39 ± 1.28	6.69 ± 1.53	3.11 ± 0.79**
Transection area (cm^2)	3.49 ± 0.39	3.84 ± 0.56	4.34 ± 0.40	4.09 ± 0.44	3.30 ± 0.51
Calculated volumetric data					
Foci per cm^3 with mean diameter of					
136 µm	178 ± 42	100 ± 31	187 ± 44	124 ± 31	93 ± 26
192.5 µm	159 ± 34	89 ± 25	185 ± 45	152 ± 45	77 ± 17
272.5 µm	59 ± 12	33 ± 12	82 ± 22	90 ± 31	41 ± 20
385 µm	19 ± 11	14 ± 6	27 ± 7	28 ± 9	7 ± 5
545 µm	2 ± 2	5 ± 4	10 ± 5	8 ± 6	1 ± 1
total	417 ± 64	241 ± 58**	491 ± 71	402 ± 69	219 ± 49**
Mean diameter of foci (µm)	193 ± 11	191 ± 9	200 ± 7	205 ± 9	187 ± 12
Area of foci as % of pancreas	0.22 ± 0.05	0.18 ± 0.07	0.35 ± 0.08	0.35 ± 0.12	0.12 ± 0.04
Basophilic foci					
Observed transection data					
Total number of foci per cm^2	11.00 ± 1.33	7.64 ± 1.37	11.13 ± 1.64	8.35 ± 0.73	8.24 ± 1.42
Transection area (cm^2)	1.41 ± 0.09	1.50 ± 0.18	1.31 ± 0.08	1.52 ± 0.09	1.18 ± 0.12
Calculated volumetric data					
Foci per cm^3 with mean diameter of					
68 µm	379 ± 94	437 ± 88	465 ± 73	266 ± 51	380 ± 97
96 µm	357 ± 75	315 ± 100	347 ± 71	136 ± 45*	244 ± 50*
136 µm	340 ± 64	136 ± 29	259 ± 57	325 ± 46	277 ± 47
192.5 µm	128 ± 26	85 ± 32	165 ± 32	107 ± 20	55 ± 18
272.5 µm	27 ± 11	19 ± 6	23 ± 10	25 ± 5	27 ± 16
total	1230 ± 150	991 ± 157	1258 ± 158	858 ± 85	982 ± 170
Mean diameter of foci (µm)	114 ± 4	101 ± 4	108 ± 4	122 ± 5	108 ± 5
Area of foci as % of pancreas	0.15 ± 0.02	0.12 ± 0.03	0.15 ± 0.03	0.13 ± 0.01	0.11 ± 0.02

[1] All values are means ± SEM.
[2] Castration vs. no castration.
[3] AGT vs. no AGT; no interaction between AGT and castration.
*$P < 0.05$; **$P < 0.01$ (analysis of variance).

Table 6. Effects of various treatments on development of (pre)neoplastic lesions in exocrine pancreas of BOP-treated hamsters

	Control (n = 18)	Castration (n = 15)	LHRH agonist (n = 18)	AGT (n = 18)	AGT + Castr. (n = 12)
Borderline lesion	19	16	9	13	7
Carcinoma *in situ*	7	7	5	6	3
Microcarcinoma	4	4	7	6	4
Carcinoma	1	1	0	1	0

Statistics: analysis of variance: $^*P < 0.05$.

Discussion

The results of the present study indicate that castration does not have a consistent effect on the development of early putative (pre)neoplastic lesions induced in pancreas of rats and hamsters by azaserine and BOP, respectively. We found a significant inhibitory effect of surgical castration on putative precancerous acidophilic foci in the rat model, whereas surgical castration did not have any effect in the hamster model. In rats, the effect of surgical castration on the development of precancerous foci was accompanied with a significant decrease in body weight gain of the animals.

Remarkably, such an effect on body weight gain was neither seen in orchiectomized hamsters nor in rats medically castrated by the LHRH agonist Zoladex. Moreover, neither in rats nor in hamsters medical castration by Zoladex modulated development of early lesions. These observations might be partly related to the fact that plasma testosterone levels were not completely suppressed to castrate levels by goserelin.

From studies with azaserine-treated rats, it appeared that both orchiectomy and oestradiol treatment resulted in inhibition of the growth of acidophilic atypical acinar cell foci, whereas no effect was seen on growth of basophilic foci (7, 8). Although it has been concluded that testosterone stimulates the growth of azaserine-induced AACN, findings with respect to such an effect of testosterone have not yet been demonstrated to be very consistent. On the one hand, orchiectomy resulted in a decreased burden of AACN whereas, on the other hand, administration of testosterone to orchiectomized rats did not restore the number of AACN to the level observed in control rats (7, 8). In these studies orchiectomy resulted in remarkably lower pancreas and body weights of the treated animals as compared to controls.

In contrast to other authors reporting lower numbers of foci/cm^3 in females than in males (6, 7, 8), Bax et al. did not find such a sex difference using adenosine triphosphatase as a marker (4). Since acidophilic foci in females are consistently

smaller than in males, this discrepancy is most probably related to the difficulty to detect small acidophilic foci in the H&E-stained sections. The lower number of atypical acinar cell foci observed in H&E-stained pancreata of castrated males as compared to those found in intact controls is most probably attributable to the same phenomenon: the pancreas of castrated rats contains more foci with a diameter below the measurement limit than the pancreas of intact animals.

Based on the aforementioned observations and considerations, it is not illogical to assume that in a large pancreas the number of detectable foci will be higher than in a small pancreas. Our findings in rats treated with the synthetic trypsin inhibitor camostate are in accordance with this hypothesis (26). The significantly increased weight of the pancreas after treatment with camostate, as compared to controls, was accompanied with a much larger mean diameter of the observed acinar lesions. We suggest, therefore, that there exists a close relationship between pancreatic growth and the number of measurable atypical acinar cell foci. In the present experiment, orchiectomy in rats reduced the yield of lesions, but this effect is accompanied with a lower pancreatic weight. Statistical correction for pancreas weight brings tumour yield almost back to normal values. Therefore, we feel that the substantial decrease in growth rate as observed in orchiectomized or oestradiol-treated rats is a confounding factor, which has been reported in the various papers on this subject without influencing the final conclusions and thus leading to an overestimation of the direct enhancing potency of testosterone in pancreatic carcinogenesis. The results obtained with LHRH-A-treated rats indicate that a decrease in testis weight accompanied with a decrease in plasma testosterone level does not result in an inhibitory effect on development of putative preneoplastic pancreatic lesions when such an effect is not accompanied with a decrease in growth rate. It has to be noted that, although plasma testosterone levels were clearly suppressed during goserelin treatment, these plasma testosterone concentrations were slightly higher than in castrated rats and hamsters, indicating that in rats plasma testosterone levels slightly above castration levels suffice to prevent decrease of body weight gain. Our hypothesis that, besides testosterone, pancreatic growth plays a highly significant role in the development of putative preneoplastic lesions is supported by the observation that in hamsters orchiectomy did not cause diminished growth rate and did not have any effect on development of putative precancerous ductular lesions.

The present findings in hamsters are in accordance with those of Zalatnai and Schally (19, 20) who found that the agonistic LHRH analogue D-Trp-6-LH-RH, given twice daily or injected in the form of microcapsules for constant controlled release, significantly decreased tumour weight and volume but did not cause a decrease in incidence of ductular adenocarcinomas in female Syrian golden hamsters injected with BOP once a week at a dose of 10 mg/kg body wt for 18 weeks; histological regression was seen in only 35% of the specimens.

Szende et al. demonstrated receptors for epidermal growth factor and insulin-like growth factor 1 (IGF-1) in nitrosamine-induced pancreatic cancer in hamsters (22). These growth factors can stimulate pancreatic tumour growth (9). Szende et al. (22) report down-regulation of IGF-1-R by D-Trp-6-LH-RH treatment, but not for EGF-R. However, data on a change of plasma levels of these growth factors were lacking. Our study shows the important role of age with respect to plasma EGF and IGF-1 levels. LHRH-A- treated animals had slightly higher plasma IGF-1 levels, which might explain such down-regulation of tumour IGF-1-R levels. However, the precise role of growth factors in the development and differentiation of pancreatic tumours is still unclear. Since pancreatic lesions can be subject to hormonal manipulation from the moment they contain receptors, it is quite conceivable that, in contrast to plain carcinomas, the acinar or ductular cells that form an atypical acinar cell focus or a (pseudo)ductular complex in rat or hamster pancreas do not contain steroid receptors.

Also Zalatnai and Schally (19) found only effects of LHRH-A treatment on some malignant tumours, but minor effects were seen on preneoplastic lesions. They concluded that the hormone sensitivity of the experimental pancreatic cancer develops gradually during the course of the disease and that altered hormone receptor levels may play a role. In agreement with this hypothesis is their observation that [D-Trp-6]-LHRH receptors were absent in the pancreas of normal hamsters but appeared after the carcinoma had been induced with BOP (20).

Therefore, further research is needed to find out whether early acinar and/or ductular lesions have steroid receptors. The presently found absence of a distinct effect of testosterone and LHRH on the development of early acinar and ductular pancreatic lesions induced in rats and hamsters by azaserine and BOP, respectively, suggests that these lesions, in contrast to acinar and ductular carcinomas, do not contain enough steroid receptors to respond to hormonal manipulation.

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CHAPTER 8

Effects of orchiectomy, either alone or in combination with testosterone, and cyproterone acetate on exocrine pancreatic carcinogenesis in rats and hamsters

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Summary

The results of a previous 4-month study in azaserine-treated rats and BOP-treated hamsters indicated that orchiectomy inhibited pancreatic growth and development of putative preneoplastic lesions in the exocrine pancreas of rats, but not of hamsters. The present 12-month study was carried out to investigate the effects of orchiectomy, alone or in combination with testosterone, and of treatment with cyproterone acetate on pancreatic carcinogenesis in azaserine-treated rats and BOP-treated hamsters. Treatment started four months after injection of the carcinogen.

In orchiectomized rats, pancreatic weight was lower than in controls, whereas pancreatic weight of orchiectomized treated with testosterone was similar to that of controls. Both orchiectomy and cyproterone acetate caused a decrease in body weight gain and had an inhibitory effect on pancreatic carcinogenesis. Testosterone treatment did not influence the inhibitory effects of orchiectomy on body weight gain and on pancreatic carcinogenesis.

In hamsters, neither orchiectomy, alone or in combination with testosterone, nor cyproterone acetate affected pancreatic growth or pancreatic carcinogenesis.

The present study indicates that testosterone plays a minor role in the development of pancreatic tumours induced in rats by azaserine, but not of pancreatic tumours induced in hamsters by BOP.

Introduction

In most countries, the age-adjusted incidence of pancreatic cancer is higher in men than in women (1, 2). Besides life-style factors, hormonal factors have been suggested to be responsible for this sex difference.

A higher number of atypical acinar cell lesions (AACN) and a higher incidence of pancreatic neoplasms have been found in male than in female rats treated with azaserine in comparison with female animals (3). Using ATPase staining Bax et al. (4) found that acidophilic foci in male rats were consistently larger (1.75-fold) than in females and concluded that the sex-related difference in the numbers of acidophilic AACN induced in rat pancreas by azaserine may be attributed to the difficulty to detect small acidophilic foci in H&E-stained sections. In rats, the inhibitory effect of castration on growth of azaserine-induced AACN has been ascribed to decreased serum testosterone levels (5, 6, 7). This inhibitory effect of castration, however, was not influenced by treatment with testosterone (5, 6, 7). In a previous 4-month study with azaserine-treated rats we also found an inhibitory effect of orchiectomy on the development of AACN, which was accompanied with a significant decrease in absolute but not relative pancreatic weight. From the results of that study we concluded that, besides testosterone, nutritional status and pancreatic growth may play a highly significant role in the development of putative pancreatic preneoplastic lesions (8).

No consistent variation has been found in the incidence of pancreatic (pre)neoplastic lesions in male and female hamsters induced by s.c. injections with nitrosamine derivatives. Pour et al. (9, 10) did not find a difference between male and female hamsters in the incidence of pancreatic tumours after injection with N-nitrosobis(2-oxopropyl)amine (BOP). Kokkinakis et al. (11), on the contrary, found that incidence, tumour multiplicity and tumour size were generally larger in female than in male hamsters after administration of N-nitroso(2-hydroxopropyl)(2-oxopropyl)amine (HPOP). Pancreatic (H2T) cells transplanted in hamster cheek pouches developed faster into ductal adenocarcinomas in intact females and castrated males than in ovariectomized females and intact males, respectively, pointing to an inhibitory effect of testosterone or an enhancing effect of oestrogen (12). Schally and co-workers (13–18), however, found that the development of pancreatic tumours induced in male hamsters by BOP was inhibited by medical or surgical castration. Furthermore, in a previously conducted 4-month study, we did not find any effect of castration or LHRH treatment on the development of early (pre)neoplastic lesions induced in hamster pancreas by BOP (8).

The present experiment was performed to evaluate the effects of castration, either alone or in combination with testosterone treatment, and of treatment with the antiandrogen cyproterone acetate (CA) on pancreatic carcinogenesis in hamsters and rats during a long-term treatment period of 8 months starting 4 months after tumour induction.

Materials and methods

Animals and tumour induction

Two hundred male weanling SPF albino Wistar Bor rats (WISW, Cpb) were obtained from F. Winkelmann (Borchen, FRG). They were injected i.p. four times with 30 mg

azaserine per kg body wt at 19, 26, 33 and 103 days of age. One hundred and forty male Syrian golden hamsters (Charles River Wiga, Sulzfeld, FRG) were each injected s.c., once weekly, with 20 mg BOP per kg body wt at 6, 7 and 8 weeks of age according to an injection protocol described previously (19). BOP (Ash Stevens, 5861 John C. Lodge Freeway, Detroit, MI 48202) and azaserine (Calbiochem-Behring, LaJolla, CA) were dissolved freshly in 0.9% NaCl solution. The animals were allocated by a computerized randomization procedure to four different groups each of which consisted of either 50 rats or 35 hamsters. The rats were housed in stainless steel cages, fitted with wire-mesh floors and fronts, and the hamsters in macrolon cages on softwood bedding. The animals were kept under standard laboratory conditions, five animals per cage, and fed a high-fat/high-protein diet during the first 4 months of the study to enhance the development of ductular lesions in the exocrine pancreas.

Treatment

To mimic the human situation, treatment started when early (pre)neoplastic lesions had already developed in the pancreas, i.e. four months after the last injection with carcinogen. The animals received one of the following treatments: Group A: saline (controls), Group B: surgical castration, Group C: surgical castration plus testosterone, and Group D: CA. Testosterone was administered as testosterone propionate by Silastic implants as described previously (20). Rats received two 3 cm long implants per animal and hamsters, because of their lower body weight, one implant per animal at the start of treatment. CA was used as Androcur tablets (50 mg CA per tablet) dispersed in tap water. The animals were given CA by gavage daily at a dose of 50 mg/kg as described previously (21).

During treatment the animals were fed the Institute's basal diet, which is low in fat (5%) and compounded from natural feed ingredients. 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 and histological analysis

In rats an interim kill was performed on 20 animals of each group after four months of treatment. Terminal autopsy on rats and hamsters was performed after eight months of treatment on days 371 (rats), and 363 or 364 (hamsters) after the last injection with carcinogen. 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, liver, testes (if present), adrenals and pituitary were excised and weighed. These organs were 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.

About 200 mm^2 of pancreatic tissue of each rat was screened microscopically for the number and size of azaserine-induced atypical acinar cell nodules (AACN) using

a grid inside the ocular as described before (22). Only acidophilic AACN with a transection area >0.5 mm^2 were counted and classified. Atypical acinar cell nodules, acinar cell adenomas (AACN with a transection area larger than 3 mm^2), and (localized) carcinomas were identified and classified according to criteria of Longnecker et al. (3, 23).

In the hamster pancreas, major attention was paid to tubular ductal complexes showing dysplasia or anaplastic changes, desmoplasia, inflammation, with or without apoptosis, suggestive of progression to malignancy. Tubular ductal complexes exhibiting one or more of the characteristics were classified as 'borderline' lesions (24). The ductular carcinomas induced by BOP were identified and classified according to Pour and Wilson (25).

Body and organ weight data were evaluated statistically with one-way analysis of variance using initial body weight as covariable. The number of borderline and other (pre)neoplastic lesions were evaluated statistically with a generalized linear model (26). With such a model, the effects of type of treatment (control, surgical castration, surgical castration plus testosterone, CA) were assessed. For rats the influence of time of sacrifice (interim kill, terminal autopsy) and possible interaction between type of treatment and time of sacrifice were assessed as well. A Poisson error distribution was used for the evaluation of the lesions.

Evaluation of the prevalence (incidence) of tumours (terminal autopsy animals only) was done with a generalized linear model. The difference with the model used for lesions was the error distribution. With respect to prevalence, the binomial distribution was appropriate.

Plasma growth factor and hormone levels

Blood sampling for hormone estimations was performed at the start of the treatment period (n = 19–20), after 4 months (n = 10) and after 8 months of treatment (n = 10) at the end of the experiment by exsanguination. In groups of 5 animals, also blood sampling was performed after 2 and 6 months by orbital punctions. Table 3 only lists the data obtained at 0, 4 and 8 months after treatment. EGF- and IGF-1-like activities were measured in acid/ethanol-extracted EDTA-plasmas by radioreceptor assays using human placental membrane preparations as receptor source. All procedures were exactly as described before for the determination of EGF- and IGF-1-like activities in human breast tumour cytosols (27). Growth hormone (GH) levels were determined by radio-immunoassay (28). Testosterone levels were estimated as described by Verjans et al. (29). The effects of treatment on plasma hormone and growth factor levels were analysed by analysis of variance (ANOVA) on the pooled data of all treatment groups and controls at the end of treatment. Small P values resulting from these analyses, performed for each growth factor or hormone in rats and hamsters, are indicative of a difference in effect, either a decrease or increase of plasma concentrations. In addition, to prevent the influence of outliers also the Wilcoxon test was applied whenever useful.

Results

Body weights (Fig. 1, Tables 1 and 2)
In rats, but not in hamsters, orchiectomy inhibited body growth by 19% ($P < 0.01$). Body weight of orchiectomized rats treated with testosterone was also significantly lower (15%) than that of controls.

Rats treated with CA showed also a lower body weight ($P < 0.01$) than controls, whereas body weight of hamsters treated with CA increased by 10% ($P < 0.05$) compared to controls.

Organ weights (Tables 1 and 2)
In rats, absolute but not relative pancreas weight was decreased ($P < 0.01$) by orchiectomy and by treatment with CA ($P < 0.05$) as compared with controls. Supplementation restored largely the loss of absolute pancreas weight caused by orchiectomy resulting in the absence of a significant difference with the control group. In orchiectomized rats the absolute and relative pituitary weight increased ($P < 0.01$). Supplementation with testosterone prevented the absolute increase, but not the relative increase in pituitary weight. Adrenal weight was not significantly affected by any of the treatment modalities. In rats, absolute liver weights were lower ($P < 0.01$) in all treated groups, whereas relative liver weight was only lower in orchiectomized rats not treated with testosterone ($P < 0.01$).

In hamsters, apart from an increase of pituitary weight caused by castration, none of the treatments influenced significantly absolute or relative organ weights.

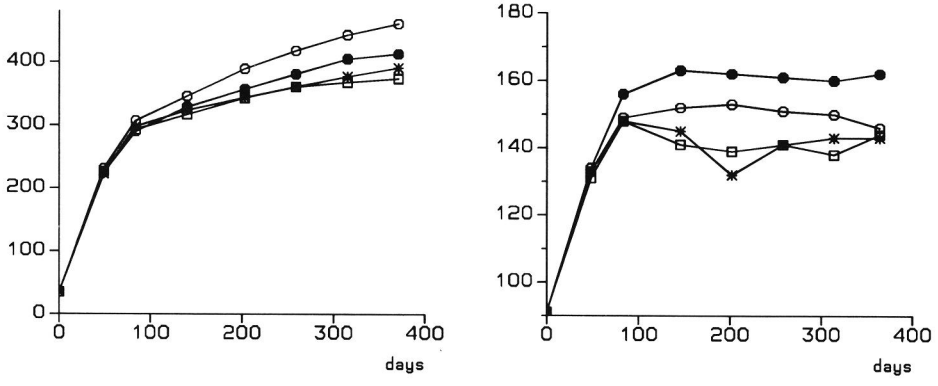

Fig. 1. Mean body weight. Left: rats; right: hamsters.
Open circles: control; open squares: castration; asterisks: castration + testosterone; solid dots: cyproterone acetate.

Table 1. Effects of treatments on body weight (in g), absolute organ weights (in g) and organ weights relative to body weight (g/kg) of rats after 8 months of therapy (means ± SEM)

	Control (n = 27)	Castration (n = 24)	Castration + testosterone (n = 19)	Cyproterone acetate (n = 26)
Body weight	460.7 ± 10.9	373.7 ± 6.9**	391.4 ± 8.0**	413.1 ± 8.0**
Absolute organ weight(g)				
Pancreas	1.40 ± 0.05	1.14 ± 0.04**	1.28 ± 0.06	1.22 ± 0.04*
Testes	3.55 ± 0.06	–	–	3.37 ± 0.06
Pituitary	0.014 ± 0.000	0.018 ± 0.001**	0.015 ± 0.001	0.013 ± 0.000
Adrenals	0.054 ± 0.002	0.047 ± 0.002	0.054 ± 0.002	0.051 ± 0.002
Liver	13.01 ± 0.37	9.33 ± 0.25**	11.69 ± 0.33*	11.52 ± 0.23**
Relative organ weight (g/kg)				
Pancreas	3.06 ± 0.12	3.07 ± 0.11	3.26 ± 0.11	2.96 ± 0.10
Testes	7.77 ± 0.15	–	–	8.18 ± 0.13
Pituitary	0.031 ± 0.001	0.048 ± 0.001**	0.038 ± 0.001**	0.031 ± 0.001
Adrenals	0.118 ± 0.005	0.128 ± 0.007	0.138 ± 0.004	0.125 ± 0.005
Liver	28.3 ± 0.7	25.1 ± 0.7**	29.9 ± 0.6	27.9 ± 0.3

Statistics: one-way analysis of variance followed by Student's t-test (two-tailed) for comparison with control group; * $P < 0.05$; ** $P < 0.01$.

Table 2. Effects of treatments on body weight (in g), absolute organ weights (in g) and organ weights relative to body weight (g/kg) of hamsters after 8 months of therapy (means ± SEM).

	Control (n = 24)	Castration (n = 19)	Castration + testosterone (n = 16)	Cyproterone acetate (n = 23)
Body weight	145.8 ± 4.3	144.2 ± 4.3	142.9 ± 2.3	162.3 ± 5.8*
Absolute organ weight (g)				
Pancreas	0.531 ± 0.034	0.578 ± 0.030	0.541 ± 0.037	0.497 ± 0.040
Testes	2.12 ± 0.19	–	–	2.67 ± 0.14
Pituitary	0.007 ± 0.000	0.010 ± 0.000**	0.009 ± 0.001*	0.007 ± 0.000
Adrenals	0.024 ± 0.001	0.021 ± 0.001	0.023 ± 0.001	0.026 ± 0.002
Liver	12.10 ± 1.06	15.58 ± 2.19	12.79 ± 1.51	12.40 ± 1.15
Relative organ weight (g/kg)				
Pancreas	3.67 ± 0.27	3.99 ± 0.20	3.81 ± 0.28	3.05 ± 0.22
Testes	14.33 ± 1.14	–	–	16.60 ± 0.92
Pituitary	0.05 ± 0.00	0.07 ± 0.00**	0.06 ± 0.00*	0.05 ± 0.00
Adrenals	0.17 ± 0.01	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.01
Liver	83.42 ± 7.27	105.33 ± 12.14	87.85 ± 8.88	78.25 ± 7.99

Statistics: one-way analysis of variance followed by Student's t-test (two-tailed) for comparison with control group; * $P < 0.05$; ** $P < 0.01$.

Table 3. Effects of of treatments on plasma growth factor and hormone levels. [1]

	Controls	Castration	Castration + testosterone	Cyproterone acetate	P[2]
Rats					
EGF (ng/ml)					
0 months	29.0 ± 4.5				
4 months	53.4 ± 6.3	68.2 ± 8.7	46.7 ± 10.1	15.2 ± 6.5	
8 months	47.6 ± 31.2	43.4 ± 8.8	31.0 ± 5.1	45.2 ± 28.7	0.97
n[3]	(12)	(10)	(9)	(12)	
IGF-1 (ng/ml)					
0 months	131 ± 9				
4 months	200 ± 11	191 ± 16	161 ± 19	145 ± 11	
8 months	101 ± 14	94 ± 11	107 ± 14	127 ± 16	0.35
n	(12)	(10)	(10)	(12)	
GH (ng/ml)					
0 months	11.7 ± 4.1				
4 months	2.4 ± 0.8	5.7 ± 1.8	11.3 ± 3.2	24.5 ± 14.1	
8 months	16.9 ± 6.2	3.0 ± 0.7	20.8 ± 9.1	14.8 ± 6.6	0.29
n	(12)	(10)	(10)	(12)	
Testosterone (nmol/l)					
0 months	11.6 ± 3.2				
4 months	2.7 ± 0.5	0.1 ± 0.0	16.9 ± 1.2	5.3 ± 1.3	
8 months	4.6 ± 0.6	0.1 ± 0.0	10.2 ± 1.2	11.6 ± 3.1	0.0003
n	(10)	(10)	(10)	(12)	
Hamsters					
EGF (ng/ml)					
0 months	22.2 ± 1.8				
8 months	62.8 ± 4.5	60.8 ± 5.5	60.0 ± 5.5	76.5 ± 5.6	0.09
n	(12)	(10)	(10)	(13)	
IGF-1 (ng/ml)					
0 months	255 ± 14				
8 months	144 ± 7	244 ± 19	194 ± 15	206 ± 18	0.0005
n	(12)	(10)	(10)	(13)	
GH (ng/ml)					
0 months	5.5 ± 1.6				
8 months	2.8 ± 0.7	4.3 ± 0.6	2.0 ± 0.5	3.3 ± 0.5	0.09
n	(12)	(10)	(9)	(12)	
Testosterone (nmol/l)					
0 months	15.3 ± 4.2				
4 months	6.3 ± 0.8	0.1 ± 0.0	3.8 ± 1.3	10.8 ± 1.5	
8 months	17.0 ± 5.1	0.1 ± 0.0	2.2 ± 0.5	13.0 ± 2.2	0.0000
n	(10)	(10)	(10)	(7)	

[1] All values are means ± SEM.
[2] P values associated with test of difference between the four groups after 8 months of treatment.
[3] Number of animals used at 8 months; at the start of treatment (0 months, T_0) and after 4 months 10 animals were used.

Plasma growth factor and hormone levels (Table 3)
In rats significant differences between groups were observed at the end of the experiment with respect to EGF and IGF-1-like activity and plasma GH levels. Plasma IGF-1 levels increased until 4 months of treatment and subsequently decreased to adult values when the growth curves (almost) plateaued. Plasma GH levels tended to decrease after surgical castration and to increase in case of testosterone substitution, but the differences were not significant compared to controls (both by ANOVA and Wilcoxon test analysis). During the treatment period plasma testosterone concentrations were lower than before in the control group, possibly as a consequence of late damage of testicular function by carcinogens. As expected surgical castration caused very low plasma testosterone levels. Substitution with testosterone implants caused significant plasma testosterone concentrations comparable with those present in the control group before treatment. CA did not decrease plasma testosterone as could be expected. Hamsters showed higher plasma EGF and IGF-1 concentrations, but lower GH levels than rats. In contrast to our previous study (8) plasma testosterone levels were not lower, but tended to be higher compared to rats. No differences were found between groups with respect to EGF-like activity, while IGF-1 levels were higher in the treatment groups compared with

Table 4. Effects of treatments on number of (pre)neoplastic lesions in the exocrine pancreas of azaserine-treated rats. [1]

	Control	Castration testosterone	Castration + acetate	Cyproterone
Number of lesions after 4 months of treatment				
Number of animals	*(14)*	*(13)*	*(11)*	*(13)*
Nodules, TA[2] 0.5–1.0 mm[2]	4	1	1	1
Nodules, TA 1.0–3.0 mm[2]	1	0	2	0
Adenomas (nodules, TA >3.0 mm[2])	1	0	0	0
Carcinomas *in situ*	0	0	0	0
Microcarcinomas	0	0	1	0
Carcinomas	0	0	0	1
Total carcinomas	0	0	1	1
Number of lesions after 8 months of treatment				
Number of animals	*(29)*	*(26)*	*(24)*	*(29)*
Nodules, TA 0.5–1.0 mm[2]	89	27**	8**[3]	23**
Nodules, TA 1.0–3.0 mm[2]	39	6**	5**	11**
Adenomas (nodules, TA >3.0 mm[2])	3	4	2	2
Carcinomas *in situ*	17	5	4	7
Microcarcinomas	5	1	1	1
Carcinomas	4	3	3	6
Total carcinomas	26	9*	8*	14*

[1] Values are totals per group.
[2] TA = transection area.
[3] Castration + testosterone vs castration or cyproterone acetate: $P < 0.05$.
Statistics: log-linear model with a Poisson distribution; * $P < 0.05$, ** $P < 0.01$ compared to controls.

those of controls (all $P < 0.01$, Wilcoxon test). Although plasma GH tended to be higher in castrated animals, overall there were no clear differences between groups.

As expected, after castration plasma testosterone levels were very low. Testosterone substitution caused a significant raise of plasma testosterone concentrations, but these remained lower than in rats, possibly owing to the lower dose used.

Microscopy (Tables 4, 5 and 6)

In rats the total number of lesions increased significantly in time (Table 4). After eight months of treatment the number of AACN with transection areas larger than 0.5 mm^2 was lower ($P < 0.01$) in each of the treatment groups than in the controls. The number of adenomas (AACN with transection areas larger than 3 mm^2) was similar in all groups, controls included.

The total number (Table 4) but not the incidence of carcinomas (Table 6) was lower ($P < 0.05$) in all treatment groups after eight months of treatment.

In hamsters none of the treatments had a significant effect on the total number of borderline lesions (Table 5) or on the total number (Table 5) or incidence of carcinomas (Table 6). Surgical castration decreased both the number and the incidence of total carcinomas by about 50%, but this difference was not statistically significant.

Table 5. Effects of treatments on number of (pre)neoplastic lesions in exocrine pancreas of BOP-treated hamsters[1]

Advanced ductular lesions	Number of lesions			
	control (n = 26)	castration (n = 26)	castration + testosterone (n = 26)	cyproterone acetate (n = 28)
Borderline lesions	227	214	204	221
Carcinomas *in situ*	5	0	0	2
Microcarcinomas	5	1	3	0
Carcinomas	5	6	8	11
Total carcinomas	15	7	11	13

[1] Values are totals per group. Statistics: a log-linear model with a Poisson error distribution; there were no significant differences between the treatments.

Table 6. Effects of castration, either alone or in combination with testosterone, and treatment with cyproterone acetate on incidence of carcinomas in exocrine pancreas of azaserine-treated rats and BOP-treated hamsters.

	Incidence of pancreatic carcinomas			
	control	castration	castration + testosterone	cyproterone acetate
Rats				
Effective number of animals	29	26	24	29
Number of animals bearing a lesion	11	6	7	13
Hamsters				
Effective number of animals	26	26	26	28
Number of animals bearing a lesion	14	7	10	10

Statistics: generalized linear model with a binomial error distribution; there were no significant differences between the treatments.

Discussion

The results of the present 12-month study indicate that both orchiectomy and treatment with cyproterone acetate cause: (1) a decrease in body weight gain and of absolute pancreatic weight in rats, but not in hamsters; (2) inhibition of pancreatic carcinogenesis in azaserine-treated rats, but not in BOP-treated hamsters. Moreover, treatment of orchiectomized rats with testosterone did not affect the inhibitory effect of surgical castration on body weight gain in spite of the presence of normal plasma testosterone levels, while pancreas weight of testosterone-treated castrated rats increased in comparison with orchiectomized rats not treated with testosterone.

The notion that testosterone has an enhancing effect on pancreatic carcinogenesis in azaserine-treated rats has been based on the observation that orchiectomy and oestradiol treatment inhibited the development of AACN (6, 7). In a previously performed 4-month study with azaserine-treated rats (8) we also found that orchiectomy reduced the yield of AACN significantly, but this effect was accompanied with a significant decrease in absolute pancreatic weight. After correction for pancreas weight the number of AACN in the orchiectomized rats was almost similar to that in intact controls (8). These findings made us conclude that a substantial decrease in body growth rate as observed in surgically castrated rats has to be considered a confounding factor in such experiments which, if not taken into account, may have led to an overestimation of the direct, enhancing effect of testosterone on pancreatic carcinogenesis. The results of the present study support this conclusion since it appeared that castrated rats treated with testosterone developed even less AACN than castrated rats not treated with testosterone (Table 4). Treatment of orchiectomized rats with testosterone did not result in a tumour yield similar to that of intact controls, notwithstanding pancreatic weight in the

testosterone-treated group was almost similar to that of controls. This observation indicates that changes in body weight during different treatments are at least as important as changes in plasma testosterone concentrations. On the other hand, plasma testosterone concentrations might be of importance, i.e. high and castration levels, inhibitory and intermediate levels, stimulatory. In agreement with this hypothesis are the findings of Greenway et al. (30) who have shown low levels, albeit not as low as in the case of castration, of testosterone in patients with carcinoma of the pancreas. Furthermore, relative loss of body weight might decrease (intratumoral) aromatase activity resulting in a decreased conversion of androgens to estrogens. The present findings in hamsters are in agreement with those in our previous 4-month study (8). In BOP-treated hamsters orchiectomy did not influence body weight gain and inhibited not significantly pancreatic carcinogenesis. The present findings support partly those of Zalatnai and Schally (13–18) who have found a significant decrease in tumour weight and tumour volume but no effect on the incidence of ductular adenocarcinomas in BOP-treated hamsters chemically castrated by LHRH treatment.

The aforementioned findings suggest that early putative preneoplastic acinar and ductular lesions which develop in the pancreas of rats and hamsters after treatment with azaserine and BOP, respectively, might not contain steroid receptors and hence cannot be modulated by testosterone. It may be concluded, therefore, that manipulation of pancreatic carcinogenesis by steroid hormones is only relevant for the treatment of existing carcinomas. On the basis of the findings described in this paper, it may be concluded that the observed inhibitory effects of orchiectomy and cyproterone acetate on pancreatic carcinogenesis in azaserine-treated rats may, at least partly, be attributed to a significant decrease in body growth. However, the mechanism of action of a relative delay in body growth on pancreatic carcinogenesis is still unclear. Especially, we did not observe a significant decrease in GH and growth factor secretion, as in our previous study (8) on gastrin secretion. Therefore, studies on the effect of relative weight loss on other factors potentially involved in pancreatic carcinogenesis are needed.

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CHAPTER 9

General discussion

Pancreatic cancer in man has been characterized in 90% of the cases to be of ductular type referring to the histopathological appearance which is dominated by dysplastic ducts and only a few acinar cells. Rats treated with azaserine and hamsters treated with propylated nitrosamines are the most frequently used experimental models to study pancreatic carcinogenesis (1).

There are marked differences between the two animal models with respect to the histomorphology of the induced pancreatic tumours, being of ductal/ductular type in the hamster and of acinar cell type in the rat (1, 2). Because of the similarity of the induced tumours to those occurring in man, the hamster model is considered to be more relevant to the human situation than the rat model. However, the assumption that a ductular appearance is indicative of a ductular origin has been a matter of debate in the literature. Several investigators have postulated that ductular adenocarcinomas may originate from proliferating, dedifferentiated (duct-like) cells.

We observed that acinar cells, being part of pseudoductular lesions in hamsters, either are pushed away into the lumen of the pseudoductule or are completely surrounded by processes of adjacent centroacinar cells (Chapter 2). Moreover, most ductal/ductular cells of the BOP-induced pseudoductular lesions stained positively for cytokeratins specific to ductal/ductular cells. Acinar cells were negative and, moreover, those lining the pseudoductular lesions were frequently surrounded by cytoplasmic processes of adjacent cells that stained strongly with the cytokeratin antibody. Based on these findings we concluded that proliferating ductal/ductular/centroacinar cells are mainly involved in the formation of pseudoductular lesions and ductular adenocarcinoams in the exocrine pancreas of hamsters treated with BOP or its analogues. Using the monoclonal antibody PC-10 directed against proliferating cell nuclear antigen, it has recently been demonstrated that, besides tubular ductal complexes and ductular tumours, large areas of acinar tissue show a high staining index (3). Moreover, focal ductular/ductal proliferations containing acinar cells are frequently observed within PCNA-positive acinar tissue, which suggests that acinar cells may also be involved in the formation of ductal complexes, and hence in the development of ductular adenocarcinomas. These observations and considerations, and the knowledge that AACN do occur in human pancreas, lead us to recommend to use simultaneously both the azaserine-rat and the BOP-hamster model for studying factors modulating pancreatic carcinogenesis.

In the aetiology of human pancreatic cancer a relationship with life-style factors has been suggested. Epidemiological studies have demonstrated a positive correlation between diets rich in protein and fat (macronutrients known to induce

CCK release) and the occurrence of pancreatic cancer in man (4–7). Other evidence for a role of CCK in the pathogenesis of pancreatic cancer has been derived from animal experiments. Rat fed raw soya flour (RSF) for 2 years developed significantly more pancreatic cancer than rats fed heated soya flour. It has been suggested that the effects of TI are mediated by an increased CCK level in plasma, stimulated through feedback mechanisms designed to regulate pancreatic function (8). In rats treated with azaserine, TI indeed enhanced growth of both the pancreas and acidophilic AACN. These effects of TI were prevented by pre-treatment of the rats with CR-1409, a highly specific CCK-receptor antagonist, which strongly suggests that the effects of TI in rats are mediated by CCK (9). Since the effects of TI and the possible mediation by CCK had not been studied in hamsters, we decided to examine the effects of these peptides and of bombesin (the amphibian analogue of the gastrin-releasing peptide in mammals) on pancreatic carcinogenesis in the BOP-hamster model. These studies are described in Chapters 3 (CCK), 4 (TI) and 5 (bombesin). It appeared that CCK enhanced the growth of the pancreas but, unlike in rats, CCK treatment did not lead to an increased incidence of pancreatic (pre)neoplastic ductular lesions, and CR-1409 did not influence the promoting effect of CCK on pancreatic growth. Furthermore, it was found that camostate (a synthetic TI) and bombesin caused an increase in growth of the hamster pancreas accompanied with a decrease in the number of (pre)neoplastic ductular pancreatic lesions. CR-1409 did not influence the effects of these compounds. Comparison of the present results in hamsters with those previously obtained in rats (9, 10) indicates that the effects of short-term administration of CCK, TI and bombesin on pancreatic growth is similar in rats and hamsters, whereas the effects of these peptides on the development of early putative preneoplastic pancreatic lesions induced in hamsters by BOP is completely different from those on putative preneoplastic lesions induced in rat pancreas by azaserine. From the observations in the rat model it may be inferred that CCK, TI and bombesin have a promoting effect on growth of normal and putative preneoplastic (AACN) tissue (10, 11). The results obtained with hamsters (Chapters 3, 4 and 5) demonstrate trophic as well as inhibitory responses on the pancreas suggesting that the actions of these peptides are site-specific and interact differently in normal and (pre)neoplastic pancreatic tissue. The differences between rat and hamster with respect to the carcinogenic effects of CCK, bombesin and TI may be attributed to a difference in cell of origin of the pancreatic tumours (Chapter 2).

As the acinar cell is the main target cell for CCK and bombesin, it seems crucial to the sensitivity to CCK and bombesin of the putative (pre)neoplastic ductular lesions induced in hamster pancreas by BOP whether they originate from acinar cells. The observation that TI and bombesin inhibit pancreatic carcinogenesis in hamsters is highly relevant to man because of the morphological similarity of the tumours induced in hamster pancreas by BOP to those occurring in man.

It is of paramount importance to know whether the rat is unusually susceptible to the development of pancreatic AACN and tumours when the pancreas is stimulated through feedback mechanisms meant to regulate pancreatic function such as in case of TI. This is the more important because TI are widely distributed in man's food

supply, especially in legumes (12). TI occur particularly in soya beans, which are an increasingly important nutritional source of protein. The safety of soya beans and TI was questioned when rats developed adenomas and carcinomas in the pancreas after being fed high-fat raw soya flour for 2 years (13). It has been established that the effects of TI on pancreatic growth and pancreatic carcinogenesis in rats (9, 14) is mediated by gastrointestinal hormones such as CCK. It is, however, not clear how the stimulation of growth leads to neoplasia. Our findings with CCK in the hamster model (Chapter 3) contradict those of other workers. Enhancing as well as inhibitory effects, and also absence of an effect, of CCK on the development of ductular pancreatic lesions induced in hamsters by nitrosamines have been reported (15–19). Enhancing effects of CCK have been found only in studies in which supraphysiological doses of CCK (20–30 μg/kg body wt) were used in combination with repeated injections of the carcinogen. Such high doses repeatedly injected may lead to pancreatitis (20). Since pancreatitis is a pathological condition generally accompanied with recurrent tissue damage and repair it is not illogical to assume that such high doses of CCK given simultaneously with a pancreatic carcinogen may induce more lesions than experiments in which slightly supraphysiological doses of CCK are given *after* initiation (Chapter 3). The results of the study described in Chapter 4 revealed that administration of TI *after* initiation with BOP significantly inhibited the development of putative preneoplastic ('borderline') lesions in hamster pancreas, indicating that CCK has the potential to inhibit the development of pancreatic tumours not only in the initiation phase, as has been demonstrated by Pour et al. (19), but also in the promotion phase.

In previously performed short-term experiments in rats it was found that both CCK and bombesin enhanced pancreatic growth as well as the development of AACN (10). To verify the presumptive role of both compounds in pancreatic carcinogenesis in rats, it was found worth-while to carry out a long-term study in azaserine-treated rats (Chapter 6). It appeared that growth of the pancreas and the number of acidophilic AACN was enhanced siginificantly by CCK as well as bombesin, but the number of acinar adenocarcinomas was increased only in the group treated with bombesin. The observation that CCK enhanced growth of AACN, but not of carcinomas, suggests that carcinomas do not always develop according to an ordered evolutionary sequence starting from initiated acinar cells, which grow out to acidophilic foci, via nodules and adenomas to carcinomas. Moreover, this observation indicates that growth factors that stimulate the growth of altered cell foci, and thus speed up the process of carcinogenesis, need one or more extra steps (second hits?) to give rise to the development of malignant tumours. It was concluded from the long-term study that CCK does not play a decisive role in the development of adenocarcinomas in exocrine pancreas of rats treated with azaserine.

The discrepancy between these results and those of Green et al. (21, 22), McGuinness et al. (13) and Morgan et al. (23), who found an increase in pancreatic carcinomas in rats after long-term treatment with RSF or TI, may be attributable to gastrointestinal polypeptides other than CCK, the secretion of which may be stimulated by RSF and TI. Our finding that CR-1409 did not inhibit significantly the

effect of bombesin-stimulated development of pancreatic adenocarcinomas supports our hypothesis that CCK-enhanced pancreatic growth alone does not cause the development of carcinomas. Apart from pancreatic growth, factors such as gastrointestinal peptides, which are released by RSF, TI as well as bombesin, are needed for the development of adenocarcinomas. It is not known whether AACN contain bombesin receptors. Therefore, the mechanism by which bombesin induces the development of pancreatic adenocarcinoams in azaserine-treated rats needs further elucidation. Moreover, research into the chronic effects of CCK and bombesin on the development of ductular adenocarcinomas induced in hamster pancreas by BOP is recommended in order to elucidate the relevance to man of the findings described in Chapter 6.

Besides life-style factors and gastrointestinal hormones, steroid hormones such as testosterone and oestradiol have been implicated as factors that may play a role in the development of pancreatic cancer in man (24, 25). Recently, it has been demonstrated that oestrogen treatment and surgical castration inhibit the development and growth of putative precancerous acinar lesions induced in rat pancreas by azaserine (26, 27). In addition, the presence of receptors for oestrogen, progesterone and androgen in pancreatic cells indicates some influence of sex hormones on the growth of normal and neoplastic pancreatic cells (28–30). Testosterone may act directly via the androgen receptor or indirectly via the oestrogen receptor after having been metabolized to oestrogen by the enzyme aromatase (31).

Therefore, we decided to use aminoglutethimide, an aromatase inhibitor, to influence the production of oestrogen, and thus the balance between testosterone and oestrogen. In fact, we studied the effects of manipulation of the sex steroid metabolism on the development of induced (pre)neoplastic lesions in rat and hamster pancreas both in short-term (Chapter 7) and long-term (Chapter 8) studies.

The results of the studies described in Chapters 7 and 8 indicate that orchiectomy does not have a consistent effect on pancreatic carcinogenesis in rats and hamsters. In rats, orchiectomy caused a significant inhibitory effect on the development of putative preneoplastic acidophilic AACN as well as of acinar adenocarcinomas. In the hamster model, however, surgical castration had no noticeable effect on pancreatic carcinogenesis. The inhibitory effect of orchiectomy in azaserine-treated rats was accompanied with a significant decrease in growth of the animals and in pancreatic weight. After correction for pancreas weight the incidence of AACN and carcinomas was almost similar to that in intact controls. The substantial decrease in growth rate as observed in orchiectomized or oestradiol-treated rats is a confounding factor leading to an overestimation of the (direct) enhancing potential of testosterone in pancreatic carcinogenesis.

The data presented in Chapters 7 and 8 lead to the conclusion that testosterone has only a minor enhancing effect, or no effect at all, on the development of acinar adenocarcinomas induced in rat pancreas by azaserine. Our hypothesis as to the important role of pancreas weight is supported by the observation that in hamsters orchiectomy did not influence pancreatic growth and at the same time did not have

any effect on pancreatic carcinogenesis. These findings make it conceivable that early putative preneoplastic acinar and ductular lesions that develop in the pancreas of rats and hamsters after treatment with azaserine and BOP, respectively, do not contain steroid receptors and hence cannot be modulated by hormones. Apparently, manipulation of pancreatic carcinogenesis by steroid hormones is only relevant for the treatment of existing carcinomas. Moreover, it may be concluded that testosterone does not play an important role in pancreatic carcinogenesis in BOP-treated hamsters, while the observed inhibitory effects of ochiectomy on pancreatic carcinogenesis in azaserine-treated rats may, at least partly, be attributed to a significant growth retardation (Chapters 7 and 8). Finally, the results indicate that the usefulness of orchiectomy in the treatment of pancreatic cancer is not reflected in effects of orchiectomy on the early stages of pancreatic carcinogenesis in the two different animal models used.

The studies on the effects of sex steroid hormone manipulation on pancreatic carcinogenesis have shown that both in hamsters and rats short-term effects on the pancreas are by and large in line with the long-term effects, demonstrating the usefulness of short-term studies in experimental pancreatic carcinogenesis. Moreover, the studies described in this thesis, once more, showed the remarkable differences in mechanism of (pancreatic) carcinogenesis that may exist between species, emphasizing the possible difficulties one may meet in translating data obtained in one species to another species such as man.

In summary, the studies described in this thesis allow the following overall conclusions.

1. We confirmed that the ductular lesions induced in hamster pancreas of BOP-treated hamsters originate from proliferating ductal/ductular/centroacinar cells rather than from dedifferentiated acinar cells.
2. Gastro-intestinal hormones such as bombesin and CCK enhance the development of pancreatic lesions in azaserine-treated rats, but not in BOP-treated hamsters.
3. Testosterone plays only a minor role, if any, in the development of pancreatic tumours in azaserine-treated rats and BOP-treated hamsters.
4. Adenocarcinomas induced in azaserine-treated rats do not always develop according to an ordered evolutionary sequence starting from initiated acinar cells via foci, nodules and adenomas to carcinomas.

If the data obtained in animals in these studies can be extrapolated to man, it may be concluded that exposure of man to trypsin inhibitors, as present in soya beans, seems to constitute a negligible risk for the development of pancreatic cancer, and that sex hormones do not play an important role in the development and growth of pancreatic adenocarcinomas.

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Samenvatting

Alvleesklierkanker komt in de meeste westerse landen relatief vaak voor. Na longkanker, borstkanker en tumoren van het maag/darmkanaal veroorzaakt alvleesklierkanker het grootste aantal jaarlijkse sterfgevallen door kanker.

De alvleesklier of pancreas is een orgaan gelegen in de buikholte. Men onderscheidt in de pancreas een endocrien gedeelte, dat hormonen zoals insuline produceert, en een exocrien gedeelte, dat spijsverteringsenzymen produceert. Alvleesklierkanker betreft voor het grootste deel kanker van de exocriene pancreas. Dit proefschrift beperkt zich daartoe.

De incidentie van pancreaskanker is de laatste decennia snel gestegen. In Nederland is in de periode 1950–1972 de sterfte aan pancreaskanker met een factor 2,7 toegenomen bij mannen en met een factor 2,0 bij vrouwen; de gemiddelde jaarlijkse sterfte aan pancreassterfte steeg in die periode van 5 tot 12 per 100.000 inwoners. Vroegtijdige vaststelling van de ziekte is moeilijk vanwege het gebrek aan symptomen, hetgeen veelal een slechte prognose tot gevolg heeft. De eenjaars-overlevingskans is ongeveer 8% en de vijfjaars-overlevingskans slechts 1–2%.

Voor het bestuderen van pancreaskanker bij proefdieren wordt hoofdzakelijk gebruik gemaakt van twee diermodellen, te weten het azaserine-rattemodel en het BOP-hamstermodel. Pancreastumoren kunnen bij de rat worden geïnduceerd door i.p. injectie van azaserine en bij de Syrische goudhamster door s.c. injectie van BOP. Het is opmerkelijk dat bij rat en hamster verschillende typen pancreastumoren ontstaan. Bij de rat ontstaat het acinaire en bij de hamster het ductulaire pancreascarcinoom, zo genoemd omdat de cellen lijken op respectievelijk de acinaire en de ductulaire cellen van de exocriene pancreas. Aangezien bij de mens voornamelijk het ductulaire carcinoom voorkomt wordt wel gesuggereerd dat het hamstermodel voor de mens het meest relevant is. Daarentegen wordt ook gesteld dat de ductulaire tumoren bij de hamster ontstaan uit acinaire cellen na dedifferentiatie van deze cellen, zodat de oorsprong van beide tumortypen dezelfde zou zijn. Een belangrijk aspect van deze diermodellen is het voorspellen van de omvang van de uiteindelijke tumorvorming aan de hand van de voorstadia, de preneoplastische laesies. Zo kan in een vroeg stadium al een uitspraak worden gedaan over de carcinogene potentie van een stof waaraan de dieren zijn blootgesteld. Dit gaat op voor het hamstermodel, waarin preneoplastische laesies op grond van consistente criteria zijn te identificeren. Bij de rat evenwel kunnen weliswaar atypische acidofiele acinaire noduli (AACN's) in een vroeg stadium worden vastgesteld, maar hun relatie met de echte tumoren is nog steeds vaag.

Voor wat betreft het BOP-hamstermodel bestaat er dus verschil van mening over de vraag of de geïnduceerde ductulaire carcinomen ontstaan uit ductulaire dan wel uit acinaire cellen. Daarom werden in het eerste onderzoek (hoofdstuk 2) de vroege voorstadia bestudeerd met behulp van histochemische kleuringen en elektronenmicroscopie. De conclusie luidt dat deze voorstadia bij de hamster

vermoedelijk ontstaan uit ductulaire cellen en niet uit gededifferentieerde acinaire cellen.

Ondanks de sterk toegenomen incidentie van pancreaskanker in de laatste decennia is tot nu toe geen risicofactor gevonden die eenduidig verantwoordelijk is voor het ontstaan van pancreaskanker. Wel is aangetoond dat het voorkomen van pancreaskanker vaak samengaat met de opname van veel vet en eiwit; daarom lijkt voeding belangrijk bij het ontstaan van pancreaskanker. Met betrekking tot voeding speelt cholecystokinine (CCK), een hormoon dat in de darmwand wordt aangemaakt, een belangrijke rol. CCK speelt een belangrijke rol bij de stimulering door voeding van de galblaascontractie, pancreasenzymsecretie en mobiliteit van maag en darmen. Daarnaast heeft CCK een groeibevorderend effect op de alvleesklier. Toediening van CCK aan dieren leidt tot vergroting van de pancreas en bevordert het optreden van pancreaskanker. Men veronderstelt dat CCK ook een belangrijke rol speelt bij de effecten van consumptie van onverhit sojameel via de daarin voorkomende trypsineremmers (TI). Voeding met onverhit sojameel veroorzaakt namelijk een verhoogde afscheiding van CCK en heeft dezelfde effecten op de pancreas als toediening van CCK. Ook zou het effect van andere maag/darmhormonen, zoals bombesine, tot stand kunnen komen door middel van CCK. CCK, bombesine en TI bleken bij de rat ook de ontwikkeling van door azaserine geïnduceerde AACN's te stimuleren. Uit de literatuur blijkt dat de invloed van CCK op de pancreas hoofdzakelijk is onderzocht bij de rat. In de hoofdstukken 3, 4 en 5 worden studies beschreven naar de effecten van CCK in het hamstermodel. CCK, TI en bombesine werden daartoe gedurende 4 maanden aan hamsters toegediend en hun effecten op de pancreas werden bestudeerd. Er werd geconstateerd dat zowel CCK, TI als bombesine bij de hamster, evenals bij de rat, vergroting van de pancreas veroorzaken. CCK stimuleert echter niet de ontwikkeling van preneoplastische (ductulaire) laesies in de pancreas van de hamster. Toediening van TI en bombesine hebben zelfs een remmend effect op het ontstaan van voorstadia van ductulaire pancreastumoren. Bovendien bleek CCK voor het ontstaan van pancreastumoren onder invloed van TI en bombesine bij de hamster slechts van ondergeschikte betekenis. CCK, TI en bombesine blijken dus op de pancreas van hamster en rat nogal verschillende effecten te hebben.

In hoofdstuk 6 worden de lange-termijneffecten van CCK en bombesine in het rattemodel beschreven. Het blijkt dat na toediening van CCK of bombesin gedurende 8 maanden, evenals na 4 maanden, de pancreas groter is dan bij controledieren. Ook het aantal AACN's blijkt na zowel 4 als 8 maanden groter te zijn dan bij controledieren. Het is echter opmerkelijk dat alleen na behandeling met bombesine (en dus niet na behandeling met CCK), het aantal tumoren is toegenomen. Dit wijst erop dat er geen duidelijk verband bestaat tussen het aantal AACN's en het aantal pancreastumoren. Omdat een soortgelijke constatering ook is gedaan in enkele eerdere chronische studies met het rattemodel, kan worden gesteld dat bij de rat het aantal AACN's een onzekere parameter is voor de ontwikkeling van pancreaskanker.

Naast een rol van voedingsfactoren bij het ontstaan van pancreaskanker zijn er aanwijzingen dat geslachtshormonen een rol spelen bij het ontstaan van pancreaskanker. Allereerst komt pancreaskanker meer voor bij mannen dan bij vrouwen. Bovendien zijn er receptoren voor geslachtshormonen aangetoond in normaal pancreasweefsel en in pancreastumoren. Het onderzoek beschreven in de hoofdstukken 7 en 8 was gericht op het beantwoorden van de vraag of het ontstaan van pancreaskanker bij de hamster en de rat te beïnvloeden is met behulp van geslachtshormonen. De dieren werden daartoe gecastreerd en/of behandeld gedurende 4 maanden (hoofdstuk 7) en 8 maanden (hoofdstuk 8) met middelen die de geslachtshormoonhuishouding beïnvloeden. Het bleek dat deze behandelingen bij de hamster noch effect hadden op de normale pancreas noch op het ontstaan of de ontwikkeling van pancreaskanker. Bij de rat bleek castratie remmend te werken op het ontstaan van pancreaskanker en op de groei van AACN's. Deze effecten bij de rat gingen echter gepaard met een geremde groei van de pancreas, zodat na correctie voor het verschil in pancreasgewicht de effecten nagenoeg verdwenen.

De belangrijkste conclusies uit het onderzoek beschreven in dit proefschrift zijn als volgt:

(1) vroegtijdige laesies in het BOP-hamstermodel ontstaan waarschijnlijk uit ductulaire cellen
(2) als de gegevens verkregen met het BOP-hamstermodel kunnen worden geëxtrapoleerd naar de mens, dan lijkt blootstelling aan trypsine inhibitors, zoals bijv. aanwezig in soyabonen, een verwaarloosbaar risico voor de ontwikkeling van pancreaskanker bij de mens te vormen
(3) een (specifiek) effect van geslachtshormonen op het ontstaan van pancreaskanker is noch bij de rat noch bij de hamster vastgesteld
(4) stimulatie van de ontwikkeling van AACN bij de rat kan, maar hoeft niet noodzakelijkerwijs een indikatie van een carcinogeen effect op de pancreas te zijn.

Nawoord

Het in dit proefschrift beschreven onderzoek is het resultaat van een samenwerking van de afdeling Biologische Toxicologie van ITV-TNO, de afdeling Maag-, darm- en leverziekten van het Academisch Ziekenhuis Leiden en de afdeling Endocriene Oncologie van de Dr. Daniel den Hoed Kliniek.

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Curriculum vitae

Michiel Meijers werd geboren op 1 juli 1950 te Pladju (Indonesië). Hij behaalde het HBS-B-diploma in 1968 in 's-Gravenhage en studeerde daarna diergeneeskunde aan de Rijksuniversiteit te Utrecht. Na het behalen van het dierenartsexamen in 1978 werkte hij verscheidene jaren in de kleine-huisdierenpraktijk. Hij begon in 1982 de studie biologie aan de Rijksuniversiteit te Utrecht, die hij in 1988 afsloot met het doctoraaldiploma. Vanaf 1988 was hij verbonden aan het ITV-TNO, voorheen het CIVO, te Zeist, waar hij werkte aan het in dit proefschrift beschreven onderzoek.


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