Dietary fat and carcinogenesis of the exocrine pancreas in rats and hamsters. Effects of ω-6 and ω-3 polyunsaturated fatty acids.

Voedingsvet en kanker van de exocriene pancreas bij ratten en hamsters.
Effecten van ω-6 en ω-3 meervoudig onverzadigde vetzuren.
(met een samenvatting in het Nederlands)

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Marko Jan Appel

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Promotores: Prof. Dr. V.J. Feron
(Research Institute of Toxicology, University of Utrecht)

Prof. Dr. R.J.J. Hermus
(Faculty of Medicine, State University of Limburg)

Co-promotor: Dr. R.A. Woutersen
(TNO Nutrition and Food Research Institute, Division of Toxicology)

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Contents

Abbreviations

AA	Arachidonic acid
AACF	Atypical Acinar Cell Focus/Foci
AACN	Atypical Acinar Cell Nodule(s)
AIN	American Institute of Nutrition
Aza	Azaserine
BLL	Borderline lesion
BrdU	Bromodeoxyuridine
BHP	N-nitrosobis(2-hydroxypropyl)amine
BOP	N-nitrosobis(2-oxopropyl)amine
CCK	Cholecystokinin
CDC	Cystic Ductal Complex
CIS	Carcinoma *in situ*
CO	Corn oil
DHA	Docosahexaenoic acid
DMBA	7,12-Dimethylbenz(a)anthracene
DMH	Dimethylhydrazine
EFA	Essential Fatty Acid
EPA	Eicosapentaenoic acid
FABP	Fatty Acid Binding Protein
FCE	Food Conversion Efficiency
GLC	Gas-Liquid Chromatography
GST	Glutathione S-transferase
4-HAQO	4-Hydroxyaminoquinoline-1-oxide
H&E	Haematoxylin and Eosin
HF	High Fat
HPLC	High Performance Liquid Chromatography
HPOP	N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine
LA	Linoleic acid
LF	Low Fat
LI	Labeling Index
LnA	Linolenic acid
MNU	N-methylnitrosurea
MO	Menhaden oil
NMU	N-nitroso-N-methylurea

OA	Oleic acid
PBS	Phosphate Buffered Saline
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid(s)
SA	Safflower oil
SO	Sunflower oil
RSF	Raw Soya Flour
TDC	Tubular Ductal Complex
TPA	12-O-tetradecanoylphorbol-13-acetate
TX	Thromboxane
VLDL	Very Low Density Lipoproteins

General Introduction

Epidemiology and etiology of pancreatic cancer

Pancreatic cancer is the 5th leading cause of death from cancer in the US (1) and the 7th in the European Community (2). In the Netherlands it rates 8th in men and 11th in women (3). Diagnosis of pancreatic cancer is usually late due to lack of symptoms and treatment at this late stage of the disease is virtually impossible. Prognosis is poor, mainly due to metastatic spread. The median survival time is about 2-3 months, the 1 year survival about 8% and the 5 year survival is only 3% (4). Until better diagnostic tools and/or more effective treatments have been developed, primary prevention is the most effective way to deal with this fatal form of cancer.

In order to come to effective prevention of this disease one needs to know its etiology. There are several demographic and geographic differences in the occurrence of pancreatic cancer. In the US the incidence of pancreatic cancer is higher in black people than in whites, whereas Japanese Americans show the highest incidence (1). Generally low incidences are observed in native Japanese, whereas high incidences are seen in New Zealand Maoris and female native Hawaiians (1). In the European countries the highest incidence for pancreatic cancer is observed in the Netherlands, Denmark, Germany and Ireland, while the lowest incidences are seen in France, Italy, Greece and Portugal (5). Religion is also a source of variation: Jews show higher cancer rates than Catholics or Protestants, while (non-smoking, non-drinking) Mormons or Seventh-Day Adventists show very low incidences of pancreatic cancer (1).

Several other factors have been implicated to be predisposing for pancreatic cancer. There is some evidence that familial and genetic factors are involved in pancreatic cancer, but the data are limited (1). The data on a relationship between diabetes mellitus or pancreatitis with pancreas cancer remain inconclusive. An association of both factors with pancreatic cancer is often observed, but in both cases this may be ascribed to an early manifestation of the tumour (1). From the life-style factors that are implicated in the development of pancreatic cancer, tobacco smoking shows a consistent association. In most studies the relative risk of pancreatic cancer in smokers compared to non smokers is higher than 2 (6,7). Dietary factors like coffee (8), alcohol (9) and fat (10) have also been

implicated in the etiology of pancreatic cancer. However, recent epidemiological research demonstrated no clear association between alcohol consumption and the occurrence of tumours of the exocrine pancreas (11). The same holds true for coffee consumption (12). The influence of dietary fat is discussed further on in this chapter.

Experimental models

Animal models.

To determine environmental factors that are involved in the development of tumours, epidemiological cancer studies are essential. However, the causality of the associations of factors with increased or reduced cancer risk are often difficult to establish by epidemiological studies. Putative causal associations that emerge from epidemiological research provide the basis for experimental research, in which conditions can be kept constant and the mechanisms of action can be addressed. Pancreatic carcinogenesis has mainly been studied in animal models with chemically induced pancreatic tumours. A total of 15 carcinogens have been reported to cause pancreatic tumours in several laboratory animal species, mainly rodents. Carcinogens of the exocrine pancreas included dimethylbenz(a)anthracene (DMBA), N-nitroso-N-methylurea (NMU), 4-hydroxyamino-quinoline-1-oxide (4-HAQO), azaserine and several propylnitrosamines (13). The two models that are most extensively characterized and most commonly used are the azaserine-rat model (14) and the N-nitrosobis(2-oxopropyl)amine (BOP)-hamster model (15).

The azaserine-rat model. Azaserine (O-diazoacetyl-L-serine) is a naturally occurring compound, produced by Streptomyces Fragilis. Azaserine is moderately toxic in mice and rats (LD_{50} of 150 mg/kg/day and 170 mg/kg/day, respectively) and was initially used as an anti-cancer drug in medicine. Evidence has been provided that azaserine is specifically mutagenic and carcinogenic to pancreatic acinar cells in rats. L-azaserine caused DNA adducts that persisted for at least 4 weeks. When isolated pancreatic acinar cells are incubated with azaserine, DNA damage has been identified by HPLC in the form of N^7-carboxymethylguanine (16). D-azaserine did not damage DNA, nor did it induce pancreatic atypical acinar cell nodules (AACN), suggesting an enzyme-mediated stereo-specific step in the uptake and/or activation of azaserine (17). Furthermore, Zurlo *et al.* (18) found less azaserine-induced DNA damage and AACN in pyridoxal-deficient rats in comparison with control rats. These data point to an activation of azaserine by formation of an azaserine-pyridoxal complex followed by an enzymatic cleavage of the ester bond by an α,β-elimination reaction, resulting in formation of diazoacetate, ammonia, pyruvate and

phosphate. Diazoacetate is highly reactive and can form adducts with DNA and other macromolecules.

A single i.p. dose of azaserine is sufficient to induce putative preneoplastic AACN in the pancreas of all treated rats after 2-4 months. Two types of foci have been identified in haematoxylin and eosin (H&E) stained pancreatic tissue slides: basophilic and acidophilic foci (19,20). The basophilic foci demonstrate a low growth potential, as measured by mitotic index or incorporation of ^{3}H-thymidine (19) or incorporation of BrdU (21). The low proliferative capacity of the basophilic lesions suggests a minor involvement, if any, of these foci in development of pancreatic tumours. Acidophilic AACN proliferate rapidly and show a high frequency of mitoses, which is rarely seen in normal cells or basophilic focus cells. ^{3}H-thymidine Labeling Index (LI) was 2.3 in cells of acidophilic AACN in contrast to 0.1 in basophilic AACN (19). Studies with incorporation of BrdU showed a 15-20 fold increase in LI in AACN in comparison with normal acinar cells. LI in basophilic AACN resembled that of normal tissue (21). Both acidophilic AACN, adenomas and carcinomas (*in situ*) stain positive for ATP-ase activity in frozen tissue sections, whereas basophilic AACN are negative (22). Based on these findings the acidophilic AACN, but not the basophilic AACN are considered to be putative preneoplastic. It is estimated that about 1% of the pancreatic proliferative lesions develops into acinar cell carcinomas 6-15 months after carcinogen treatment.

In the present thesis pancreatic (pre)neoplasms in rats are classified in H&E stained tissue slides according to the criteria of Rao *et al.* (19), Longnecker *et al.* (23) and Woutersen *et al.* (24) as follows:

Atypical Acinar Cell Focus (AACF): A small group of acinar cells showing increased nuclear size, irregular nuclear form, nucleolar prominence, reduced cytoplasmic zymogen content, pale cytoplasm and reduced cytoplasmic mass.

Atypical Acinar Cell Nodule (AACN)/Acinar Cell Adenoma: Arbitrary a distinction between AACF and AACN has been made on the basis of size: hyperplastic nodules larger than islets are classified AACN. AACN show similar cellular changes as AACF. An AACN with a diameter of over 3 mm has been designated acinar cell adenoma. An adenoma is by concept a benign neoplasm. Recent experiments have shown that adenomas have normal diploid DNA content, explants from adenomas failed to grow in soft agar and transplants of adenomas failed to grow in recipient animals (25). Lacking these characteristic malignant changes, the azaserine-induced pancreatic acinar cell adenoma has to be classified as a putative preneoplastic lesion.

Carcinoma *in situ*/localized carcinoma: Lesions with degrees of anaplasia and/or desmoplasia, suggestive of neoplastic transformation, but without local invasion or metastasis are called carcinoma *in situ*.

Acinar adenocarcinoma: Acinar adenocarcinoma is diagnosed on evidence of local invasion in adjacent tissue or metastatic spread.

The BOP-hamster model. BOP is one of several and the most specific propyl-derivative nitrosamine, capable of inducing cancer of the pancreas in hamsters. The metabolism of BOP *in vivo* involves reduction to N-nitroso(2-hydroxypropyl)(2-oxopropyl) amine (HPOP) with subsequent reduction to N-nitrosobis(2-hydroxypropyl)amine (BHP) or conjugation with sulphate or glucuronic acid (26). After activation, BOP can cause DNA alkylation in both acinar and ductular pancreatic cells in rats and hamsters (27). The most abundant DNA adducts were N^7-methylguanine and O^6-methylguanine (28,29). HPOP also yields 7- and O^6-hydroxypropylguanines in addition to the methylguanine adducts and causes a five-fold increase in pancreatic DNA synthesis in hamster pancreas. No significant repair of O^6-hydroxypropylguanine was observed for at least 8 days. The persistence of the adducts and the mitogenic effect may contribute to the pancreatropic carcinogenesis of BOP (30).

Single or multiple s.c. injections of BOP result in proliferative cystic and ductular lesions in the pancreas of hamsters, which can be classified by grade of malignant phenotype. Adenocarcinomas in BOP-treated hamsters are invariably of the ductal/ductular type. Pancreatic (pre)neoplasms in hamsters are classified according to the criteria of Pour and Wilson (31), Woutersen *et al.* (32) and Meijers *et al.* (33) as follows:

Cystic ductular complex: a lesion composed of one or more luminal spaces lined by flat or cuboidal epithelium, which usually does not contain zymogen.

Tubular ductal complex: a lesion composed of several small lumina lined with cuboidal or columnar epithelium.

Three major changes, suggestive of malignant transformation, were taken into account when lesions were classified neoplastic: (i) dysplastic or anaplastic cellular changes, (ii) desmoplasia and (iii) inflammation.

Borderline lesion: tubular ductal complex showing one or more of the aforementioned characteristics suggestive of progression to malignancy, with emphasis on the epithelial changes.

Carcinoma *in situ*: a lesion showing all three characteristics of neoplastic change, but not showing evidence of infiltration in adjacent tissue.

Ductular adenocarcinoma: a lesion showing all three characteristics of neoplastic change, with clear infiltration in adjacent tissue.

Transgenic mice. Transgenic mice are made by introduction of foreign DNA into the germline of mice by directly injecting DNA sequences containing the genes of interest into the pronucleus of the zygote. The transfected fertilized eggs are subsequently transferred to foster mothers. The transgenic offspring carry one to several hundred copies of the

foreign DNA in their chromosomes. Recent developments in transgenic science rendered it possible to target the expression of known oncogenes to specific tissue sites. This has resulted in several animal models for transgenic carcinogenesis (34,35), including a number of strains of transgenic mice that are predisposed to develop acinar cell carcinomas in the exocrine pancreas. These mice have been provided with sequences of the elastase I, the trypsin I or the amylase gene fused to early region DNA sequences of the simian virus 40 genome. This results in targeted expression of both large and small T-antigen in the acinar cells of the pancreas. A first stage in the ontogenic process is believed to be T-antigen induced preneoplasia characterized by hyperplasia, dysplasia, increased number of tetraploid cells and arrest of acinar cell differentiation. In the second stage putative monoclonal tumour nodules develop, containing less total RNA, less pancreas-specific mRNA and more T-antigen mRNA in comparison with normal pancreas. The fact that numerous nodules develop in the hyperplastic pancreas of these transgenic mice and only a few develop to ultimate carcinomas, suggests that this model may be useful in studying modulating effects of exogenous factors.

***In vitro* models.**

Organ culture. An elegant model for organ culture of rodent embryonic pancreatic budds was developed by Parsa and coworkers (36), which was also applicable in carcinogenesis research. In this model rat embryonic pancreases could be cultured for 10 weeks, with rapid growth of the tissue combined with morphogenesis and cytodifferentiation as well as occurrence of specific pancreatic enzymes like amylase, lipase and chymotrypsin.

Transplantable pancreatic tumours. Several azaserine-induced acinar cell carcinomas have been serially transplanted and cryopreserved. These transplantable tumours have been used in a number of studies to investigate the effects of hormones on tumour growth (37,38). Transplantable hamster carcinomas induced by BOP have also been developed and used to study modulation of tumour growth (39,40).

Cell lines. From transplantable pancreatic tumours of the rat several continuous cell lines have been established. The AR42J cell line is widely used and extensively characterized (41). Cell lines derived from BOP-induced hamster tumours are the PC-1 and the PC-1-0 cell lines, which share the characteristic of an activated *ras* oncogene with the majority of human pancreatic tumours (42).

Dietary fat

Digestion and uptake. The digestion of dietary lipids starts in the stomach with hydrolysis by lingual or gastric lipase. This process takes place at low pH and in the absence of bile salts (43). In the duodenum lipids are emulsified with the help of bile salts. Micelles are formed, which contain triglycerides, cholesterylesters and phospholipids. In the proximal small intestine these mixed micelles are further digested by pancreas lipase with the help of trypsin-activated colipase, which binds to lipase at the oil-water interphase and digests triglycerides into 2-monoglycerides and free fatty acids. Absorption of the lipids takes also place at this proximal part of the small intestine. The micelles are dissociated and the lipid hydrolysis products are taken up in an energy-independent way. This passive permeation increases with the lipophilicity of the fatty acids: increase in uptake with chain length and decrease with degree of unsaturation (44). Linoleic acid and arachidonic acid can be taken up by a facilitated diffusion mechanism without energy requirement (45,46). Additionally, a specific fatty acid binding protein (FABP) is involved in the uptake of long chain fatty acids (47). Inside the enterocyte, cytosolic FABPs bind the long chain unsaturated fatty acids. Short chain fatty acids are transported to the blood bound to albumin. Long chain polyunsaturated fatty acids (PUFA) are reesterified by acyltransferases into triglycerides and delivered to the lymph in lipoproteins: VLDL and chylomicrons. In rats, menhaden oil and fish oil concentrate are less well absorbed than corn oil (48), but equally well as olive oil (49).

Metabolism of polyunsaturated fat. There are 2 families of PUFA: the ω-6 family, derived from linoleic acid (LA; C18:2ω-6) and the ω-3 family, derived from α-linolenic acid (LnA; C18:3ω-3). Both families are known as essential fatty acids, because they can not be synthesized by the human body and must be obtained from the diet (50). ω-6 PUFA are derived from seeds and leaves, while ω-3 PUFA predominate in chloroplasts of green plants (LnA) and in phytoplankton (eicosapentaenoic acid: EPA, C20:5 and docosahexaenoic acid: DHA, C22:6). Via the marine food chain, all forms of marine life, in particular cold water fish, become enriched with PUFA from the ω-3 family (51). The metabolism of PUFA in warm blooded animals involves desaturation and elongation in which both PUFA families compete for metabolism via the same elongase and δ^6-, δ^5- and δ^4-desaturases. LA is desaturated and elongated to dihomo-γ-LnA (C20:3) and subsequently desaturated to arachidonic acid (AA, C20:4). LnA is desaturated and elongated to EPA and DHA. The enzymatic processes of desaturation and elongation of fatty acids are located mainly in the microsomal fraction of the liver, with δ^6-desaturation being the rate-limiting step (52). The desaturation of ω-3 fatty acids is slightly more effective than that of ω-6 fatty acids (53).

The loss of δ^6-desaturase, as observed in cats, characterizes the obligatory carnivore. Eskimos seem to be obligatory carnivores among humans because they seem to lack both δ^6- and δ^5-desaturases and hence have to obtain AA from dietary meat (54). Apart from differences between human populations, it should be taken into account that fatty acid metabolism in rodents differs considerably from that in humans. In rats and mice, δ^6- and δ^5-desaturases are highly active, while both desaturases are much less active in humans, resulting in differences in AA formation (50).

Dihomo-γ-LnA, AA and EPA are the direct precursors of the eicosanoids, the oxygenated metabolites of these 20-carbon PUFA (55). Dihomo-γ-LnA, AA and EPA are converted via cyclooxygenase to the 1-, 2- and 3-series prostaglandins and via lipoxygenase to the 3-, 4- and 5-series leucotrienes, respectively (56). Most eicosanoids are biologically highly active compounds that are assumed to be involved in inflammatory processes, immunologic-allergic reactions, atherosclerosis and cancer (56). The desaturation, elongation and cyclooxygenase pathways of PUFA metabolism are presented in figures 1 and 2.

Fatty acids are stored in the adipose tissue in the form of triglycerides or used to form cellular phospholipids. They can be liberated from cellular membranes by phospholipase A_2.

Modulation of tissue fatty acid composition by dietary fat. Most tissue fatty acid compositions are a reflection of the dietary intake of fatty acids. When rats are fed diets rich in ω-9, ω-6 or ω-3 fatty acids, the levels of these respective fatty acids are high in blood plasma as well as in erythrocytes, liver, small intestine and colonic phospholipids (57) and also in pancreatic microsomes (21,58,59). An exception seems to be brain tissue. Rat brain phospholipids, which contain low levels of LA (ω-6) and high levels of DHA (ω-3) and oleic acid (OA, ω-9), are relatively irresponsive to changes in dietary fatty acid composition (60). Fatty acid profiles can be modulated by fatty acids from a single family in a dose-dependent way. For example, Reddy and Sugie (61) showed a dose-related increase in the incorporation of ω-3 fatty acids into the microsomes of colonic mucosal cells, when feeding increasing levels of menhaden oil to rats.

A diet high in LA resulted in high blood plasma levels of this fatty acid in humans (62) and dietary supplementation of fish oil to humans resulted in an increase in EPA and DHA in blood plasma lipids (62,63). These data lead to the conclusion that both ω-6 and ω-3 PUFA are efficiently taken up and distributed through the body.

Modulation of prostaglandin production by dietary fat. The conversion of LA to AA is a highly regulated process. AA is not produced in direct proportion to dietary LA, and studies reporting stimulation, depression and no change in eicosanoid production are equally documented (53). Recently, Blair *et al.* (64) fed human volunteers diets containing

either 3.0 en% or 8.3 en% of LA and measured several urinary eicosanoids. PGE_2 was elevated and 2,3-dinor-TXB_2 was decreased in the 8.3% LA diet group, whereas no changes were observed in the urinary excretion of 6-oxo-$PGF_{1\alpha}$, 2,3-dinor-6-oxo-$PGF_{1\alpha}$ or TXB_2. With a high intake of LA the total urinary prostaglandin excretion had initially increased, but decreased again after 3-4 days (65). This indicates, that the role of dietary ω-6 PUFA in accelerated prostaglandin production is not conclusively elucidated.

The metabolic pathway of LA to AA and PGs can be significantly influenced by ω-3 PUFA, which compete with ω-6 PUFA for conversion by the elongase and desaturases involved in this cascade. It is well accepted that the desaturation and elongation of ω-3 PUFA is slightly more efficient than the conversion of ω-6 PUFA (53). Clear evidence has been provided for the inhibiting effects of dietary fish oil, rich in EPA and DHA, on eicosanoid production. The concentration of PGE_2 in plasma was significantly lower in menhaden oil fed mice than in corn oil fed mice (56). Boudreau *et al.* (66) observed that the potential to synthesize TXB_2 and 6-keto-$PGF_{1\alpha}$ was significantly reduced in lung homogenates and platelet suspensions from rats fed 10% menhaden oil diets as compared with those from rats fed diets without fish oil. Similarly, Chanmugam *et al.* (67) observed a decreased 6-keto-$PGF_{1\alpha}$-synthesizing ability in testes homogenate from rats fed diets containing menhaden oil. Alam *et al.* (68) observed a significant reduction of PGE_2- and TXB_2-levels in gingiva and submandibular salivary glands of rats fed a 10 wt% menhaden oil in comparison with a 10 wt% corn oil diet.

In humans supplementation of fish oil also influences eicosanoid production. Ferretti *et al.* (69) observed a 14% reduction in urinary PGE excretion after supplementation of male subjects with fish oil capsules for 10 weeks. In a 5-month study, in which human volunteers ingested daily doses of 40 ml of cod liver oil, TXB_2 in serum decreased significantly (70). Knapp *et al.* (71) fed 50 ml of fish oil to humans for 30 days, and found a significant reduction of excretion of TXB_2-metabolites. Large amounts of dietary ω-3 PUFA will also produce a marked depression in platelet-derived TXA_2 and PGI_2 (53).

The results of the aforementioned studies clearly indicate that dietary ω-6 PUFA and ω-3 PUFA are readily taken up and distributed through the body and that ω-3 PUFA, but not ω-6 PUFA, is likely to influence eicosanoid production.

Dietary fat and pancreatic cancer

From ecological, population-based studies it can be concluded that the total fat intake is positively related to mortality from pancreatic cancer in selected countries (10). The most convincing evidence that environmental factors are involved in the occurrence of

cancer can be derived from migratory studies. The migrant retains the genetic characteristics he had in his country of origin, but he is exposed to much of the environment of his adoptive country. The relatively low mortality from pancreatic cancer in Japan in comparison to the Western countries, as well as the relatively higher rates observed among Japanese who have migrated to the United States suggest that dietary factors may play an etiologic role in the occurrence of pancreatic cancer (4,8,72). Cancer rates in Greenland Eskimos are relatively low when their total fat intake is considered (73). Like Japanese, Eskimos consume large quantities of marine foodstuffs, resulting in a high intake of long chain ω-3 PUFA, like EPA (C20:5) and DHA (C22:6). The fat consumed by Greenland Eskimos contains 5.9% DHA, 4.6% EPA, 5.0% LA, 0.4% AA and 22.8% saturated fatty acids (74), whereas the dietary fat in Great Britain contains 0.4% DHA, 0.1% EPA, 9.8% LA, 0.5% AA and 45.0% saturated fatty acids (65). Obviously, PUFA ingested by Eskimos are dominated by the ω-3 class, in contrast to the dominance of the ω-6 class in the Western world.

Other epidemiological studies indicate a positive association with caloric intake and pancreatic cancer (75,76), but the investigators ascribe this effect to the increased intake of carbohydrates. Gold *et al.* (77) and Farrow and Davis (12) did not find an association between dietary fat and pancreas cancer, but recent other case-control studies demonstrated increased risks for pancreas cancer with saturated fat and cholesterol and reduced risk with intake of polyunsaturated fat (78-80). Japanese investigators found a relation between pancreatic cancer and western type of diet, the risk being increased among Japanese who consumed meats (81). Moreover, in Japan the number of annual deaths from pancreatic cancer increased from 1948 to 1978, over the same period the Japanese diet became more akin to that of the Western countries (82). The results from these epidemiological studies point to an association between total amount as well as the type of fat available for consumption and incidence of and/or mortality due to pancreatic cancer.

In experimental pancreatic carcinogenesis an involvement of dietary fat has been well established. Roebuck *et al.* (83,84) and Birt *et al.* (85) demonstrated that high levels of fat in the diets of rats and hamsters, respectively, promoted the development of chemically induced pancreatic tumours. The enhancement of neoplasia in rat pancreas was seen with high levels of dietary polyunsaturated fat but not with similar quantities of saturated fat (84). In the hamster model, high dietary polyunsaturated fat also stimulated tumour growth relative to low levels of fat, but high levels of largely monounsaturated fat (beef tallow) had an even larger stimulatory effect (85).

The involvement of polyunsaturated fat in tumour promotion in rats led to the implication of LA as the responsible fatty acid. One of the postulated mechanisms by which a diet high in LA can be linked to a high cancer risk is an acceleration of

prostaglandin production. LA can be desaturated and elongated via γ-linolenic acid and dihomo-γ-linolenic acid to yield AA. AA is a substrate for cyclooxygenase and lipoxygenases, which convert it into prostaglandins and leucotrienes, respectively. A number of experimental findings indicate that modulation of this pathway may influence carcinogenesis. When high fat diets with increasing amounts of LA up to 4.4 wt% were fed to DMBA-treated rats, the incidence of mammary gland tumours increased proportionally (86). Similar results were obtained in the azaserine-rat model, where the promoting effect of a high fat diet was modulated by the LA-content of the fat: an increase in number of AACN was observed with an increase in dietary LA level to 4.4 - 8.5 wt% (87).

The other main family of PUFA, the ω-3 fatty acids, have also been implicated to be involved in carcinogenesis. Long chain ω-3 fatty acids (abundant in fish oil) have been shown to inhibit tumour development in mammary, colon and pancreatic tumour models. Fish oil supplemented to a HF diet inhibited mammary gland carcinogenesis in MNU- (88,89) and DMBA-treated rats (90,91) and colon carcinogenesis in azoxymethane- (92) and dimethylhydrazine-treated rats (93). ω-3 PUFA as such (4.7% EPA + 0.3 % LA as sole lipid source) also had a chemopreventive effect on colon carcinogenesis (94). O'Connor *et al.* (73) observed that in azaserine-treated rats maintained on a 20% fish oil diet for 4 months, the number and size of pancreatic preneoplastic AACN were significantly reduced as compared to rats fed a 20% CO diet. In a subsequent 4-month study they found a decrease of the number of AACN with increasing levels of fish oil in a 20% fat diet (95).

Prostaglandins and carcinogenesis. Prostaglandins (PGs) are highly active substances that play vital roles in many aspects of human physiology. An altered production of these eicosanoids is often associated with pathological processes, including cancer. PGs are elevated in several tumours in both humans and experimental animals (96). PGE_2 levels were elevated in carcinomas of the thyroid gland (97), the lungs (98), the kidney (99), the mammary gland (100,101) and the colon (102). $PGF_{2\alpha}$ levels were elevated in renal (99) and thyroid tumours (103).

In human breast cancer PGE_2 has been associated with metastatic spread, hypercalcemia and survival time and increased TXB_2 metabolism was related to tumour size and metastasis. The plasma concentrations of 6-keto-$PGF_{1\alpha}$ and the urinary concentrations of PGE_2 and $PGF_{2\alpha}$ correlated with tumour growth and clinical outcome of prostate and renal cancer (96).

Several prostaglandins of the 2-series stimulate DNA synthesis, and hence cell proliferation *in vitro* (104). High levels of PGE_2 in tumours may cause inhibition of the immune system, rendering the tumour less susceptible to the immunologic defence of the

Figure 1. The desaturation and elongation pathway of PUFAs

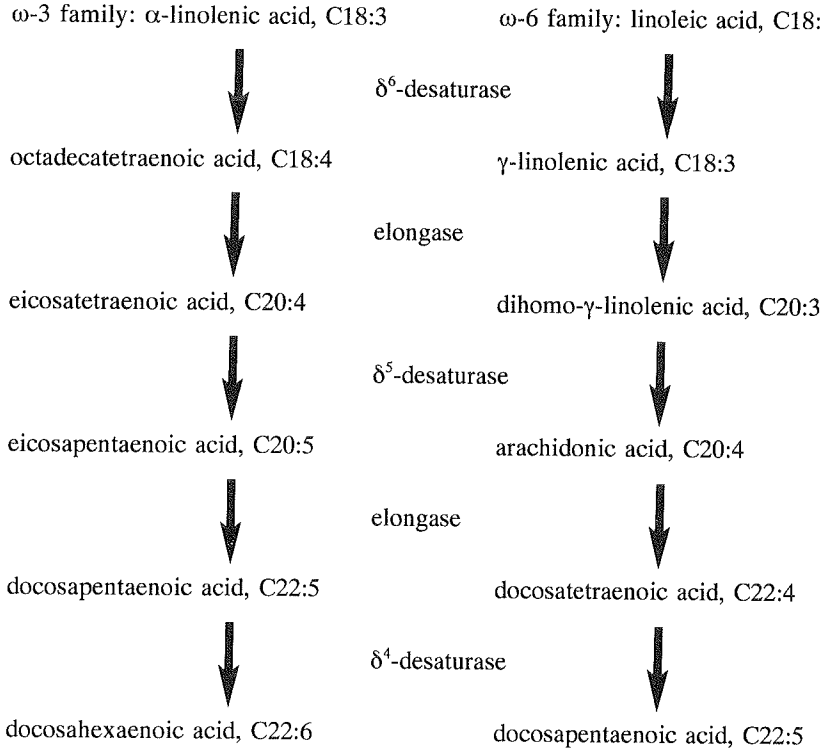
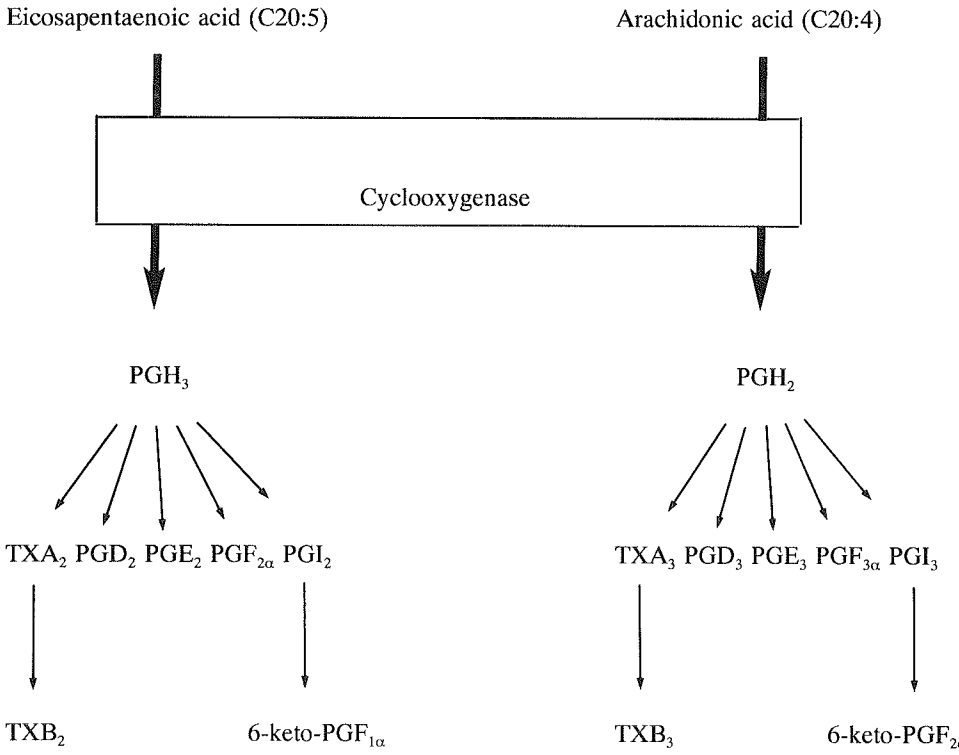


Figure 2. The cyclooxygenase pathway of PUFA



host (105). The fact that the well known skin tumour promotor 12-O-tetradecanoylphorbol-13-acetate (TPA) induces the production of PGE_2 (106) and that malondialdehyde, which is formed as an end product of the AA-cascade, is carcinogenic in Swiss mice (107), provide further indications that PGs are involved in carcinogenesis .

Cyclooxygenase inhibitors. When specific inhibitors of cyclooxygenase, like indomethacin, aspirin or carprofen, are added to the diet or the drinking water, the tumour growth is inhibited in several animal models, including the BOP-hamster model. Carter *et al.* (108) found that 0.004% indomethacin in the diet blocked the promoting effect of a high fat diet on the development of mammary tumours induced in rats by DMBA. In the same model they reported inhibition of carcinogenesis by dietary indomethacin in rats fed high fat diets containing 4% or 12% LA, but not in rats fed a diet containing 0.5% LA (109). In BOP-treated hamsters Takahashi *et al.* (110) found that the cyclooxygenase inhibitor phenylbutazone caused a decrease in incidence and multiplicity, while indomethacin caused a decrease in multiplicity of pancreas tumours.

Glutathione S-transferases. Glutathione S-transferases (GSTs) are a family of multifunctional dimeric proteins, which are predominantly located in the cytosol. A number of isoenzymes have been described, which are grouped into 3 classes: α, μ and π. Both homo and hetero dimers can be formed from a number of subunits of different molecular weight. These subunits are numbered 1, 2 (α-class), 3, 4, 5, 6, (μ-class) and 7 (π-class; Ref. 111).

Table 1 Mean GST subunit content of pancreas of saline- and azaserine-treated rats (μg/g).

Class	Subunit	Saline-treated[1]	Azaserine-treated
α	-	n.d.	n.d.
μ	3	1.81±0.37	0.27±0.18
	4	0.87±0.46	2.10±0.32*
π	7	1.89±0.21	6.08±0.66***

[1]Values are means± S.D; n.d., not detected

GSTs are involved in the AA-cascade because all isoenzymes have cyclooxygenase activity (112), which means they all can accelerate prostaglandin production. GSTs are

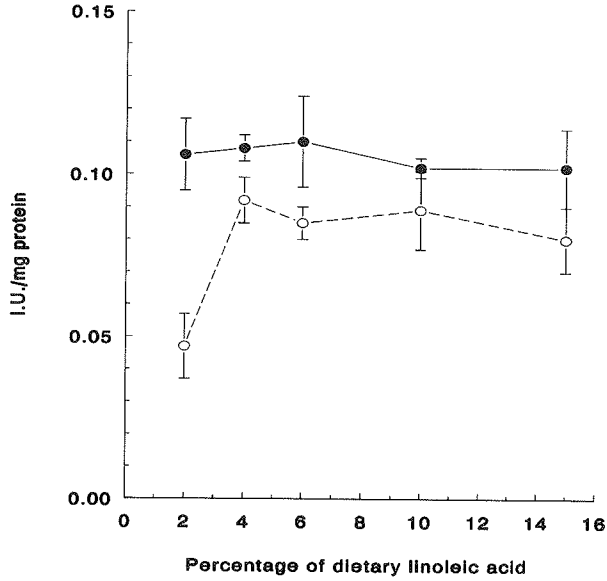


Figure 3. Glutathione S-transferase activity in pancreatic cytosol from saline-treated (○) and azaserine-treated (●) rats. Values are means ± SD (*N*=3).

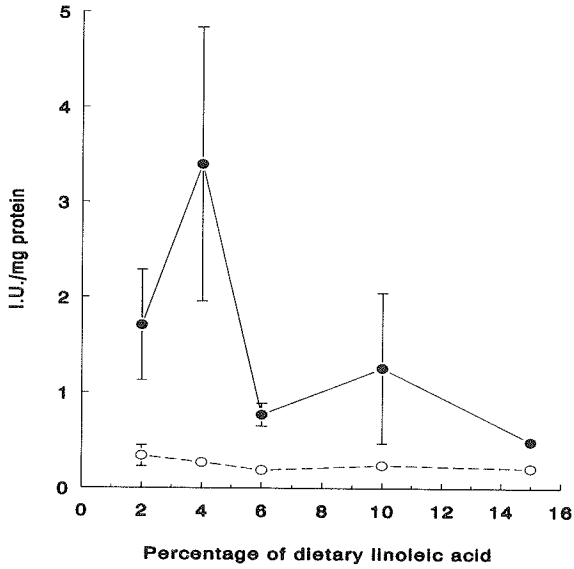


Figure 4. Glutathione S-transferase activity in pancreatic cytosol from saline-treated (○) and BOP-treated (●) hamsters. Values are means ± SD (*N*=3).

found in virtually all tissues, but they are frequently elevated in human tumours and tumour cell lines. In some tumours GSTs can comprise as much as 3% of all cytosolic protein (113). GST-π is generally associated with neoplastic change. GST-π has been found to be increased in hyperplastic hepatocellular foci and hepatocellular carcinomas in rats (111), as well as in hyperplastic ductular lesions and ductular adenocarcinomas induced in hamster pancreas by BHP (114,115). In rat pancreas HAQO-induced basophilic AACF were found to be strongly positive for GST-π, whereas in acidophilic AACF GST-π binding was limited to a portion of the nuclei (116). Daly *et al.* (117) reported that early AACF induced in rat pancreas by azaserine stained positive for GST-μ.

Several GST isoenzymes have been purified from rat pancreas. Singhal *et al.* (118) found that all 3 GST classes were present in the pancreas of rats. N-terminal amino acid sequencing of the isoenzymes suggested that subunits 3, 4, 6, and 7 were expressed in rat pancreas.

Affinity purification followed by HPLC analysis of pancreatic homogenate from 4-month old azaserine- or saline-treated rats, revealed a significant increase in subunits 4 ($P<0.05$) and 7 ($P<0.001$) as a result of carcinogen treatment (Table 1).

We also found GST activity to be significantly elevated as a result of carcinogen treatment in both azaserine-treated rats and BOP-treated hamsters (Figures 3 and 4). Total GST activity was measured spectrophotometrically in cytosolic fractions of pancreatic homogenates and expressed as μmol/min CDNB converted per mg cytosolic protein (I.U./mg protein).

The aforementioned data suggest that GSTs are induced in the course of the carcinogenic process and that induction of GST-π and GST-μ may be associated with carcinogen-induced preneoplastic changes in both rat and hamster pancreas. The relevance of these findings to the metabolism of fatty acids and prostaglandins remains uncertain, but may be of minor importance since the PG-isomerase activity of GST-π and GST-μ were very much lower than that of GST-α (112).

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Objectives of this thesis

Dietary fat has been found to enhance pancreatic carcinogenesis in azaserine-treated rats and BOP-treated hamsters. However, the mechanism by which dietary fat exerts this effect is unknown. The gut hormone cholecystokinin (CCK) is an important regulatory hormone in pancreatic function and growth and has also been found to promote azaserine-induced pancreatic carcinogenesis in rats. It has been demonstrated that ω-6 polyunsaturated fat has stronger tumour promoting properties than saturated fat. This led to the hypothesis that linoleic acid (LA; C18:2ω-6), the major ω-6 fatty acid in frequently used vegetable oils, is responsible for this effect. It has also been hypothesized that dietary ω-3 polyunsaturated fat (fish oil) is able to inhibit carcinogenesis, by counteracting the enhancing effects of linoleic acid on tumour development. The inhibitory effects of fish oil have been attributed to eicosapentaenoic acid (EPA; C20:5ω-3) and docosahexaenoic acid (DHA; C22:6ω-3). One of the postulated mechanisms by which a diet high in LA is able to promote tumour development is an acceleration of the production of the 2-series prostaglandins, whereas, on the contrary, EPA and DHA have been shown to inhibit the production of the 2-series prostaglandins.

The main objective of the studies described in the present thesis is to elucidate the mechanism by which dietary fat promotes experimental pancreatic carcinogenesis in rats and hamsters. In order to reach this goal the following specific questions were formulated and studies were designed and performed to answer these questions.

1. Does tumour promotion by dietary fat and by CCK take place via the same mechanism? In order to answer this question azaserine-treated rats were treated with CCK or maintained on a high fat diet, either with or without pretreatment with the CCK-receptor antagonist lorglumide.

2. What is the biological role of dietary LA in pancreatic tumour promotion? In order to answer this question azaserine-treated rats and BOP-treated hamsters were maintained on a well defined high fat diet, containing increasing levels of LA. The LA concentration was varied by mixing high-linoleic safflower oil with high-oleic sunflower oil, resulting in dietary lipids with 92%-94% 18-carbon fatty acids.

3. What are the chemopreventive and/or anti-tumour effects of ω-3 polyunsaturated fatty acids? In order to find out these effects azaserine-treated rats and BOP-treated hamsters were maintained on a well defined high fat diet, containing a fixed LA level and including increasing levels of fish oil. The LA level was kept constant and the fish oil level was varied by mixing high-linoleic safflower oil, high-oleic sunflower oil and fish oil.
4. What are the effects of the type of fat on fatty acid metabolism and on the conversion of arachidonic acid (AA, C20:4ω-6) to prostaglandins? In order to find out the role of these eicosanoids in tumour promotion the fatty acid profiles were determined in blood plasma and pancreatic microsomes and the levels of certain prostaglandins were measured in pancreas of rats and hamsters maintained on diets containing variable levels of LA or fish oil.

Cell proliferation in the exocrine pancreas of azaserine-treated rats and N-nitrosobis(2-oxopropyl)amine-treated hamsters

Marko J. Appel, Corjan J. T. Visser and Ruud A. Woutersen, TNO Nutrition and Food Research Institute, Division of Toxicology, Department of Pathology, P.O. Box 360, 3700 AJ, Zeist (The Netherlands)

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Abstract

Cell proliferation in normal pancreatic tissue of saline treated rats and hamsters and in putative preneoplastic pancreatic tissue of azaserine-treated rats (Atypical Acinar Cell Foci; AACF) and of N-nitrosobis(2-oxopropyl)amine (BOP)-treated hamsters (atypical ductular lesions) was determined at 2, 6, 12, 26 and 52 weeks post-treatment. The BrdU Labeling Index (LI) in normal, non tumorous acinar pancreatic cells showed a similar time-related decrease in saline- and azaserine-treated rats. In ductular pancreatic cells the LI was also similar in saline- and azaserine-treated rats. The LI in AACF was significantly higher in comparison with normal acinar tissue ($P<0.001$).

Up to 26 weeks after treatment the LI in pancreatic acinar cells was significantly higher in BOP-treated hamsters in comparison with saline-treated animals ($P<0.001$). The LI in ductular cells was significantly higher in BOP-treated animals compared to saline-treated animals 2 weeks post-treatment ($P<0.001$), but similar, thereafter. The LI in centroacinar cells had significantly increased in BOP-treated hamsters 2 and 26 weeks post-treatment ($P<0.01$ and $P<0.05$, respectively).

In hamsters, cell proliferation was higher in putative preneoplastic lesions with a high potential for malignant transformation (Tubular Ductal Complexes) compared to those with a low potential to develop to ductular adenocarcinomas (Cystic Ductal Complexes). Cell proliferation was highest in putative preneoplastic lesions characterized by atypia, desmoplasia and inflammatory cells (Borderline Lesions). It is concluded that determination of cell proliferation provides an easily quantifiable parameter to discriminate

between putative preneoplastic lesions with a high or low growth potential, hence with a high or low potential to develop into ultimate carcinomas.

Introduction

To study experimental pancreatic cancer two animal models are most frequently used. One is the azaserine-rat model, which leads to acinar adenocarcinomas (1), and the other is the N-nitrosobis(2-oxopropyl)amine (BOP)-hamster model which leads to ductular adenocarcinomas (2). Most human carcinomas of the exocrine pancreas are classified as ductular tumours (>90%). Apart from similar histology, pancreatic tumours from hamster and man share mutations in the K-ras oncogene (3,4), in contrast to rat pancreatic tumours, which lack K-ras mutations (5). Although adenocarcinomas in the hamster have ductular phenotype, it is not clear whether the ductular cell is the actual cell of origin of the ultimate tumour. Longnecker *et al.* (6) observed atypical acinar cell foci (AACF), similar to those seen in the pancreas of azaserine-treated rats, in 'normal' pancreatic tissu of humans with a ductular adenocarcinoma of the pancreas. Spontaneously occurring pancreatic tumours in dogs are ductular of phenotype and similar to those observed in ma (7), but in aging dogs with no pancreatic cancer, nodular acinar cell hyperplasia is frequently observed (8). The possible involvement of the acinar cell in the development o pancreatic ductular tumours is also a matter of debate in experimental carcinogenesis in the hamster. Evidence for the involvement of both acinar cells (9) and centroacinar/ductular cells (10,11) in the development of ductular tumours in the pancreas of BOP-treated hamsters has been reported.

In the azaserine-rat model it is generally accepted that the acinar cell is the sole progenitor of the acinar adenocarcinoma.

Most of our knowledge on replication and regeneration of the pancreas is based on studies with rats (12). Therefore, additional data on pancreatic cell proliferation in other species are warranted. In the present paper comparative data are presented on pancreatic cell proliferation in both rats and hamsters. Basal cell-turnover was quantitated in phenotypically normal exocrine pancreas, in azaserine-induced acinar lesions (rat pancrea and in BOP-induced ductular lesions (hamster pancreas) at various time intervals over a period of 52 weeks.

Materials and Methods

Rats. Twelve one-week pregnant female SPF Wistar rats were obtained from Harlan-CPB, Austerlitz, The Netherlands. After partus, 50 male pups were selected from the litters. At 14 and 21 days of age, twenty five rats received 2 i.p. injections of 30 mg azaserine (Calbiochem-Behring Corp., La Jolla, CA) per kg body wt, which was dissolved freshly in 0.9% NaCl-solution. Twenty five control animals received injections with 0.9% NaCl-solution only. Directly after the second injection the animals were weaned. The animals were kept in stainless steel cages, with wire-mesh floors and fronts, 5 animals per cage, under standard laboratory conditions. One week after carcinogen treatment the rats were fed an AIN^{76}- based purified diet containing 5 wt% lard (Best Food, The Netherlands).

Hamsters. Fifty male Syrian golden hamsters (4 weeks old) were obtained from Harlan-CPB, Austerlitz, The Netherlands. The hamsters were kept in macrolon cages, 5 animals per cage, on a softwood bedding and under standard laboratory conditions. At 5, 6 and 7 weeks of age 25 hamsters received a s.c. injection of 20 mg N-nitrosobis(2-oxopropyl)amine (BOP; Nacalai Tesque Inc., Kyoto, Japan) per kg body wt, which was dissolved freshly in 0.9% NaCl-solution. Twenty five control animals received injections with 0.9% NaCl-solution only. During the initiation phase the hamsters were fed a standard laboratory chow. One week after carcinogen treatment the hamsters were fed an AIN^{76}-based purified diet containing 5 wt% lard (Best Food, The Netherlands).

Autopsy. Autopsy of 5 randomly chosen carcinogen-treated and 5 randomly chosen control animals was performed at 14, 42, 84, 182 and 364 days after the last injection with carcinogen. Three days before autopsy, all animals had an osmotic mini-pump (Alza Corp., Palo Alto, USA, model 2001) implanted subcutaneously, containing 200 μl of a BrdU solution (Sigma Chemie, Brussels, Belgium; conc. 25 mg/ml). The release rate of this pump was 1 μl/h. The animals were killed after ether anaesthesia by exsanguination via the abdominal aorta. The pancreata were excised and fixed in 10% neutral formalin for 24 hours, followed by 72 hours in 70% ethanol. The pancreata were processed for microscopy by conventional methods, sectioned at 5 μm and collected on organosilane-coated slides.

Immunohistochemistry. Parallel sections were stained with haematoxylin and eosin (H&E) or with haematoxylin and a monoclonal antibody against BrdU (Beckton Dickinson, CA) and examined by light microscopy. The BrdU staining protocol included incubation of the slides in 1 N HCl at 37°C for 1 h, 0.05% pronase E (Sigma, Belgium) in phosphate buffered saline (PBS) pH 7.4, at 20°C for 10 min, 25% normal goat serum in PBS at 20°C for 10 min, anti-BrdU (diluted 1:60) at 20°C for 60 min, biotin-conjugated rabbit anti mouse antibody (diluted 1:400) at 20°C for 30 min and peroxidase-conjugated streptavidin (diluted 1:400) at 20°C for 30 min (Dakopatts, Glostrup, Denmark). To examine centroacinar cells, parallel sections of hamster pancreas were stained with haematoxylin, a monoclonal antibody against BrdU and a polyclonal antibody against cytokeratin filaments (Dakopatts, Glostrup, Denmark), which was made visible with an alkaline phosphatase-conjugated second antibody. The antibody against cytokeratins has been reported to bind specifically to ductular and centroacinar cells (11).

Microscopy. In the rat pancreas hyperplastic lesions were identified as acidophilic or basophilic atypical acinar cell foci (AACF) according to the criteria of Longnecker (13) and Rao *et al.* (14). In hamster pancreas cystic/ductular complexes (CDC) and tubular/ductular complexes (TDC) and borderline lesions (BLL) were classified according to the criteria of Woutersen *et al.* (15), Meijers *et al.* (16) and Pour and Wilson (17).

Labeling Index. In slides stained for BrdU the Labeling Index (LI) was expressed as the ratio of brown stained BrdU-positive cells to blue stained normal cells. To select a random sample of acinar cells, only

nuclei were counted that were located beneath the crossings of the horizontal and vertical lines in a 20x20 intra-ocular grid at high power magnification (400x).

Rats. In normal pancreatic acinar tissue from saline-treated rats and phenotypically normal acinar tissue from azaserine-treated rats, at least 1000 nuclei per pancreas were counted. The LI in normal ductal/ductular tissue was determined by counting all cells in a total of 152 transections through interlobular ducts. A mean of 3.0 ductular transections per rat (with a mean of 205 nuclei per transection) were evaluated.

In azaserine-treated rats the LI in a total of 90 acidophilic AACF (a mean of 4.5 AACF per animal) was determined by counting a mean of 91 nuclei per AACF. Because of insufficient yield the basophilic AACF were not scored.

Hamsters. In normal pancreatic acinar tissue from saline-treated hamsters and phenotypically normal acinar tissue from BOP-treated hamsters, at least 300 nuclei per pancreas were counted. The LI in normal ductal/ductular tissue was determined by counting all cells in a total of 91 transections through interlobular ducts. A mean of 2.3 ductular transections per hamster (with a mean of 189 nuclei per transection) were evaluated.

A total number of 32 CDC (a mean of 2.1 per hamster) and a total of 54 TDC (a mean of 3.6 per hamsters) were scored. Borderline lesions were observed at 26 weeks (N = 2) and at 52 weeks (N = 3) post initiation. At least 350 centroacinar cells per pancreas were counted (a mean of 429 cells per hamster).

The LI of pancreata obtained from hamsters autopsied 42 days post-initiation could not be evaluated, because of lack of staining with the antibody against BrdU. This was most probably due to a too prolonged formalin fixation.

All data were statistically analyzed by means of analysis of variance followed by Student's t tests.

Results

Rats. Normal acinar cells. Because labeling indices (LIs) of normal acinar cells in azaserine-treated rats were not significantly different from those in saline-treated animals, the data were pooled.

The mean percentage (± SEM) of BrdU-positive acinar cells in normal (non-focus) tissue dropped rapidly from 21.9±1.5% at 2 weeks to 5.2±0.7% at 6 weeks and leveled off at 1.1±0.2%, 0.7±0.1% and 1.5±0.2% at 12, 26 and 52 weeks post-treatment respectively. In Figure 1 the LI for both treatments are shown as separate curves.

Normal ductular cells. No differences in cell turnover of normal ductular cells were observed between saline or azaserine-treated rats. Therefore, the data were pooled. The mean LI (± SEM) of ductular cells was 17±1.0% at 2 weeks, 9.1±0.8% at 6 weeks, 5.1±0.4% at 12 weeks, 3.9±0.4% at 26 weeks and 7.0±0.4% at 52 weeks post-treatment. In Figure 2 the LIs of ductular cells are shown as separate curves.

AACF. Acidophilic AACF were observed in H&E stained slides of the pancreata of azaserine-treated rats 6 weeks post-treatment and onwards, whereas no AACF were seen

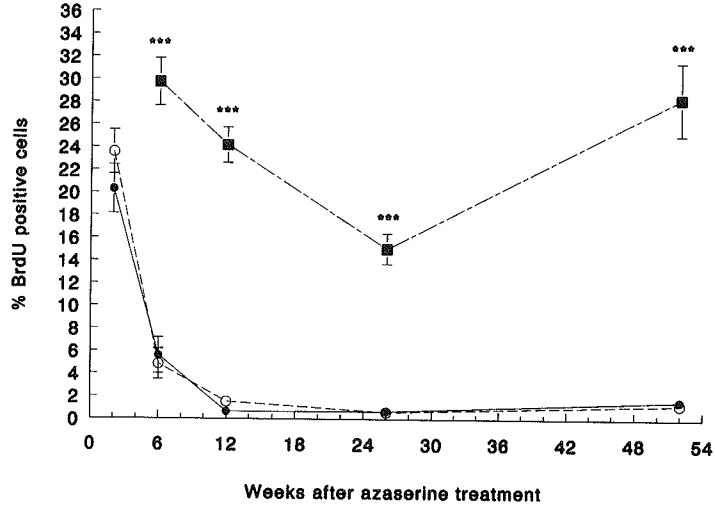

Figure 1. Labeling Index of acinar cells from normal and hyperplastic rat pancreas. ●, normal pancreas from saline-treated rats; ○, normal pancreas from azaserine-treated rats; ■, AACF from azaserine-treated rats. Statistics: analysis of variance followed by Student's *t* tests; ***P<0.001.

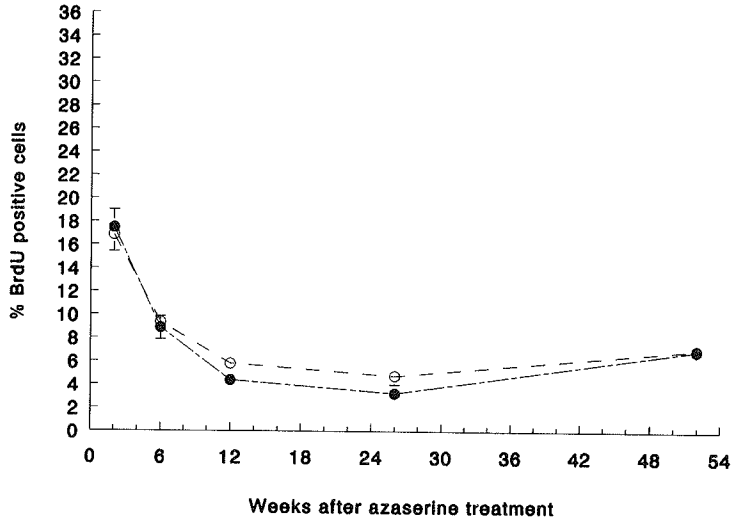

Figure 2. Labeling Index of ductular cells from normal rat pancreas. ●, saline-treated rats; ○, azaserine-treated rats. Statistics: analysis of variance followed by Student's *t* tests.

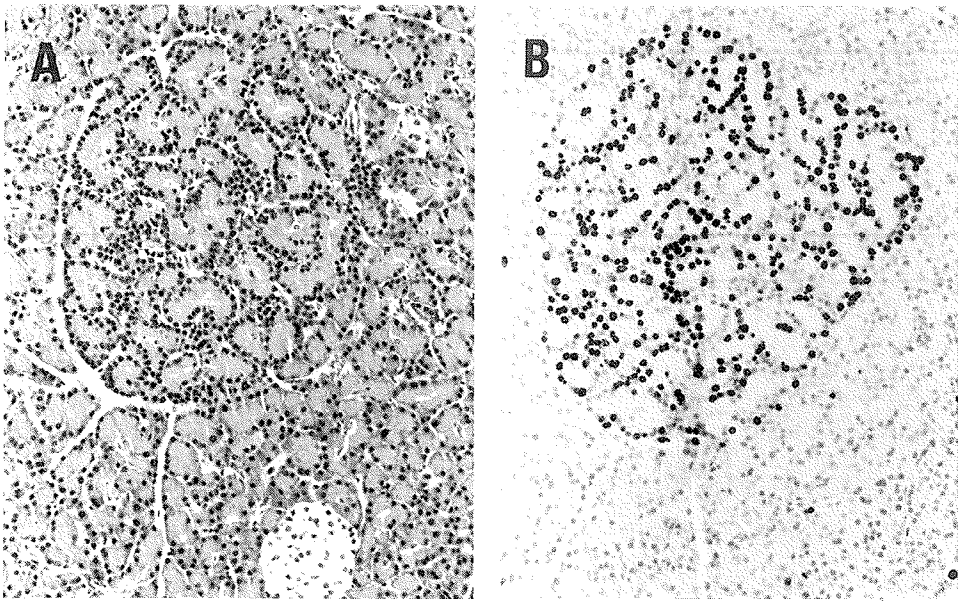

Figure 3. A. Normal acinar cells and AACF in rat pancreas (H&E; 100x); B. Normal acinar cells and AACF in rat pancreas (BrdU, haematoxyline counterstained; 100x). Note the extensive labeling in the AACF.

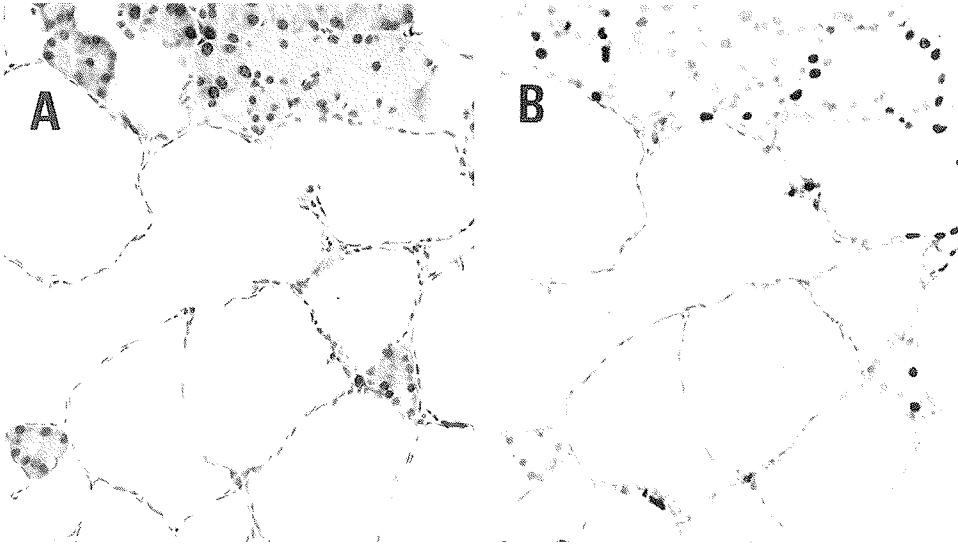

Figure 4. A. Normal acinar cells and Cystic Ductal Complex (CDC; H&E; 250x); B. Normal acinar cells and CDC (BrdU, haematoxyline counterstained; 250x). Note the pronounced labeling in the acinar cells relative to the epithelium in the CDC.

the pancreas of saline-treated rats at any time point. The LI in acidophilic AACF (Figure 3) was significantly higher ($P<0.001$) in comparison with normal acinar tissue at all time points. Six, 12, 26 and 52 weeks after azaserine-treatment the LIs in AACF were 29.7±2.1%, 24.2±1.6%, 15.1±1.4% and 28.4±3.2%, respectively (Figure 1). The LI in AACF at 26 weeks post-treatment was significantly lower ($P<0.01$) than the LIs in AACF at the other time-points.

Hamsters. Acinar cells. The LI of normal pancreatic acinar cells in BOP-treated hamsters was significantly higher in comparison to acinar cells in saline- treated animals (analysis of variance: $P<0.001$). In BOP-treated animals the mean LI (± SEM) of acinar cells was 13.9±3.0%, 18.1±3.9%, 13.8±0.9% and 5.8±1.2% at 2, 12, 26 and 52 weeks post-treatment, respectively. In saline- treated hamsters the LI of acinar cells was 3.8±0.7%, 2.8±0.6%, 1.1±0.2% and 0.7±0.1% at 2, 12, 26 and 52 weeks, respectively (Figures 4 and 5). Student's *t* tests following the analysis of variance revealed significantly higher LIs of acinar cells in BOP-treated hamsters than in saline-treated animals at 2 ($P<0.05$), 12 ($P<0.001$) and at 26 weeks ($P<0.01$) after treatment.

Hepatocyte-like cells. In a number of H&E stained pancreatic slides from BOP-treated hamsters, foci of hepatocyte-like cells were observed (Figure 6A). The LI of these cells was not quantitated, due to insufficient yield. After staining for BrdU some of these hepatocyte-like cells showed labeling (Fig 6B), pointing to a proliferative potential.

Ductular cells. Two weeks after treatment the LI of pancreatic ductular cells was significantly higher in BOP-treated hamsters than in saline-treated hamsters (12.6±1.0% *versus* 5.1±0.9%; $P<0.001$). The LI in BOP-treated animals was 4.1±1.0%, 8.2±1.3% and 8.9±1.6%, at 12, 26 and 52 weeks, respectively. The LI in saline-treated controls was 2.9±0.4%, 6.7±0.7% and 7.4±0.7% at 12, 26 and 52 weeks (Figure 7).

Centroacinar cells. LI of pancreatic centroacinar cells was significantly higher in BOP-treated hamsters than in saline-treated hamsters at 2 weeks (9.8±1.7% *versus* 2.9±0.7%; $P<0.01$) and at 26 weeks (7.2±2.6% *versus* 1.5±0.2%; $P<0.05$) post-treatment. The LI at 12 (3.3±0.4% *versus* 1.8±0.4) and 52 (4.0±1.1 *versus* 1.2±0.4) weeks was not significantly different (Figures 8 and 9).

Ductular lesions. All BOP-treated hamsters developed putative preneoplastic ductular lesions in the pancreas, which were sufficient in number to be evaluated at 12 weeks post-treatment and onwards. Lesions were scored and classified in H&E stained slides as Cystic Ductal Complexes (CDC; Figure 5), Tubular Ductal Complexes (TDC; Figure 9) or Borderline lesions (BLL; Figure 10). The LI of CDC was significantly lower ($P<0.05$) than LI of normal ductular cells at 52 weeks post-treatment. The LI of TDC and BLL was at all times significantly higher than the LI in normal ductular cells (at least $P<0.05$). The LI of CDC was 3.2±0.6%, 6.0±1.5% and 2.5±0.3%, whereas LI of TDC was 9.8±2.2%,

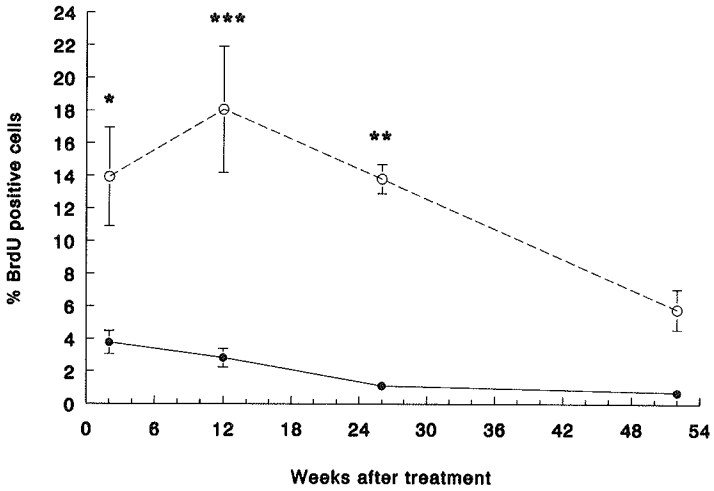

Figure 5. Labeling Index of acinar cells from hamster pancreas. ●, saline-treated; ○, BOP-treated. Statistics: analysis of variance followed by Student's *t* tests; *P<0.05; **P<0.01; ***P<0.001.

20.4±3.6% and 14.4±2.5%, at 12, 26 and 52 weeks post-treatment, respectively. BLL, which were occasionally observed, showed a mean LI of 57.0±7.5% and 39.1±12.4% at 26 and 52 weeks post-treatment, respectively. The LIs of TDC were significantly higher in comparison with the LIs of CDC at all time points investigated (P<0.01). The LIs of BLL were significantly higher than the LIs in CDC (P<0.01 at 26 wks; P<0.001 at 52 wks), and significantly higher than the LIs in TDC (P<0.01 at 26 wks; P<0.001 at 52 wks) (Figure 11).

In a large number of BOP-induced ductular lesions remnants of acini or one or more vital acinar cells were present. Figure 12 shows a pseudoductular structure comprised of cytokeratin-positive ductular cells and cytokeratin-negative acinar cells. The double stain for BrdU and cytokeratins shows that the ductular cells of TDC divide whereas acinar cells present in these ductular lesions do not.

Discussion

The results of the present study with azaserine-treated rats (model for acinar pancreatic tumours) and BOP-treated hamsters (model for ductular pancreatic tumours) demonstrate

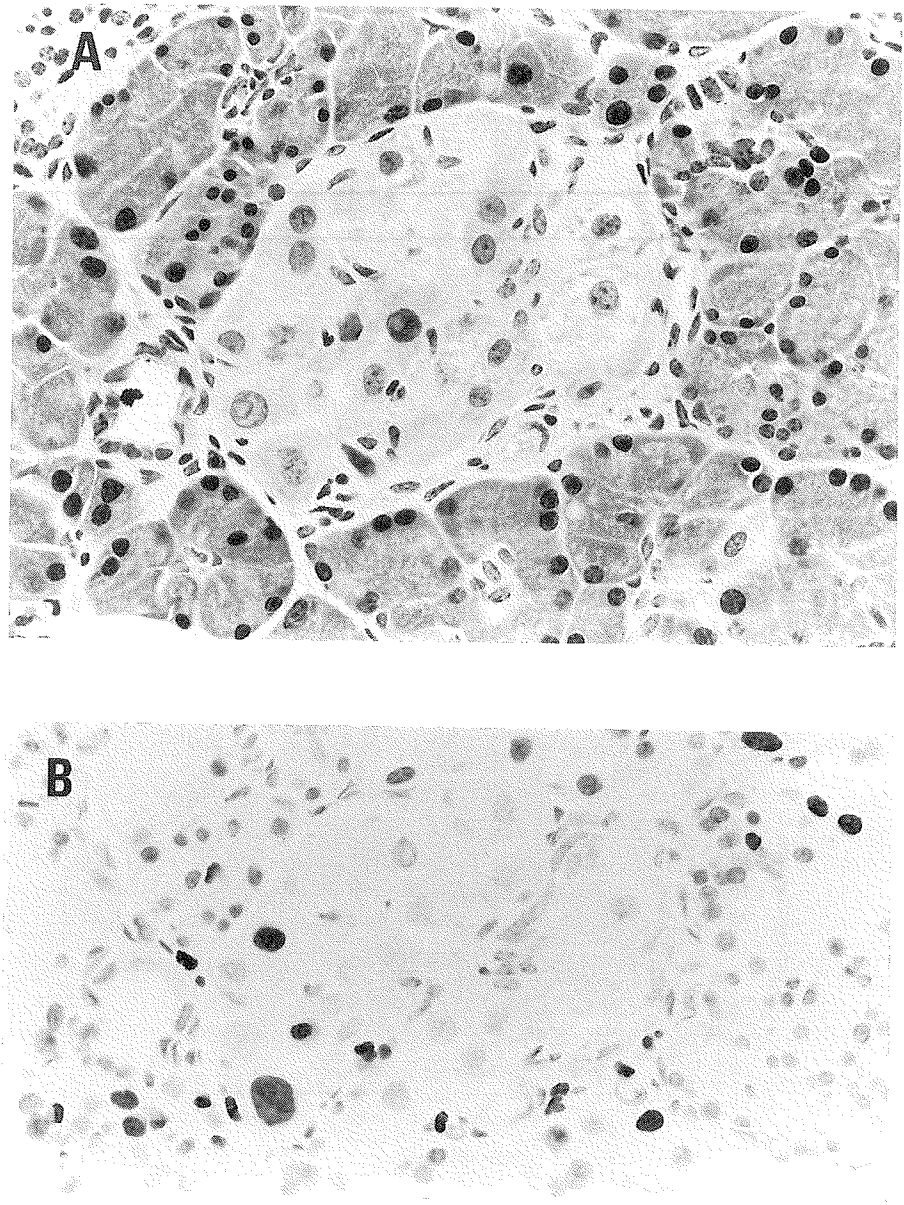

Figure 6. Hepatocyte-like cells in the pancreas of a BOP-treated hamster. A. H&E, 400x; B. BrdU, haematoxyline counterstained, 400x.

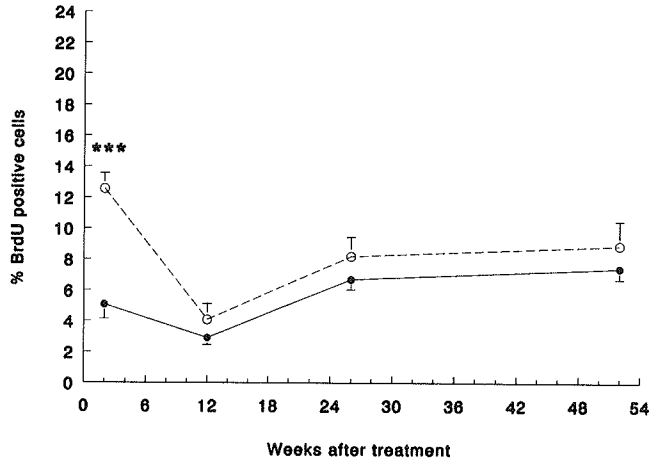

Figure 7. Labeling Index of ductular cells from hamster pancreas. ●, saline-treated; ○, BOP-treated. Statistics: Analysis of variance followed by Student's *t* tests; ***P<0.001.

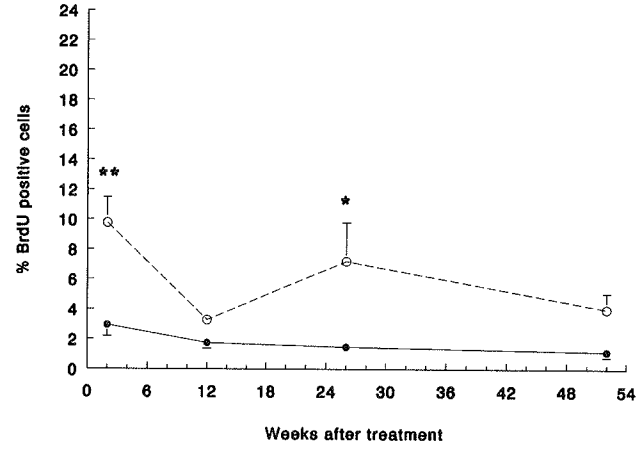

Figure 8. Labeling Index of centroacinar cells from hamster pancreas. ●, saline-treated; ○, BOP-treated. Statistics: Analysis of variance followed by Student's *t* tests; *P<0.05; **P<0.01.

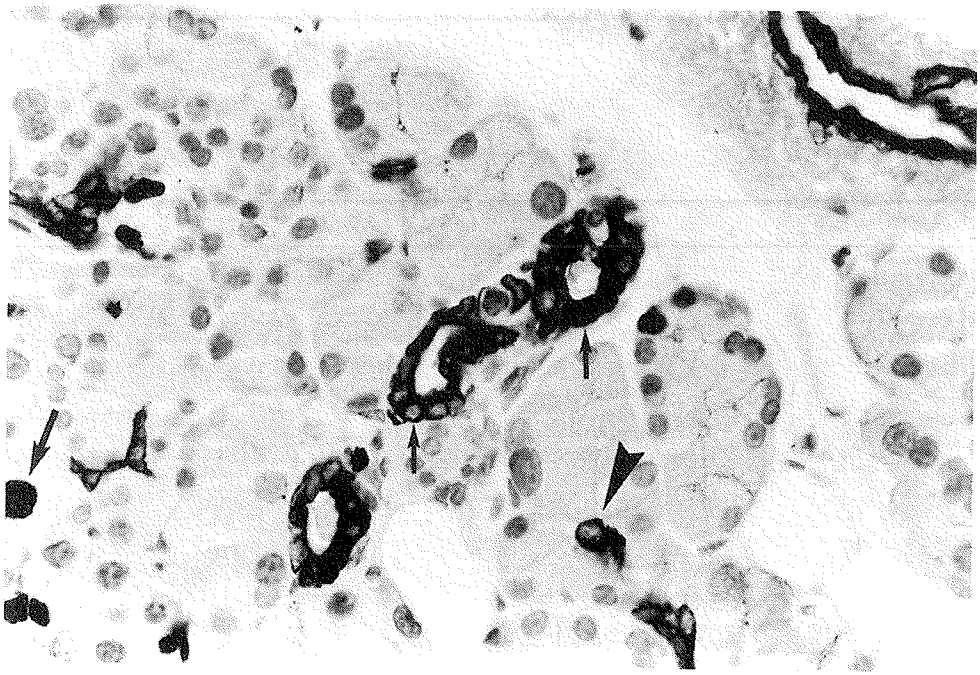

Figure 9. Tubular Ductal Complex (TDC) in the pancreas of a BOP-treated hamster. Double immunostain for BrdU and cytokeratins, counterstained with haematoxyline, 400x. Note the positivity for cytokeratins within the cytoplasm of the epithelial cells of the TDC and the light (BrdU-negative) and the dark (BrdU-positive) nuclei in the TDC (small arrows). Also note the BrdU-positive acinar cells (large arrow) and the cytokeratin-positive, BrdU-negative centroacinar cell (arrowhead).

that acidophilic AACF have a significantly higher cell proliferation than acinar cells in normal pancreatic tissue. This observation is in agreement with findings reported previously by Rao *et al.* (14). This increased growth potential of the AACF supports the hypothesis that some of these putative preneoplastic lesions have the potential to develop into malignant tumours, as a result of uncontrolled cell proliferation within a focal lesion, hence enhancing the chance of subsequent neoplastic changes ("second hits"). Inhibition of cell proliferation may, therefore, directly be related to inhibition of carcinogenic risk. A striking example of inhibition of cell proliferation within acidophilic AACF was reported by Daly *et al.* (18), who found a decrease in ^{3}H-thymidine LI from 2.5% to 0.07% in AACF of rats fed raw soya flour (RSF) for 20 weeks in comparison with rats fed RSF for 19 weeks followed by one week of normal chow. The LI of 0.07% was similar to that observed in normal pancreatic acinar tissue of chow fed rats and below

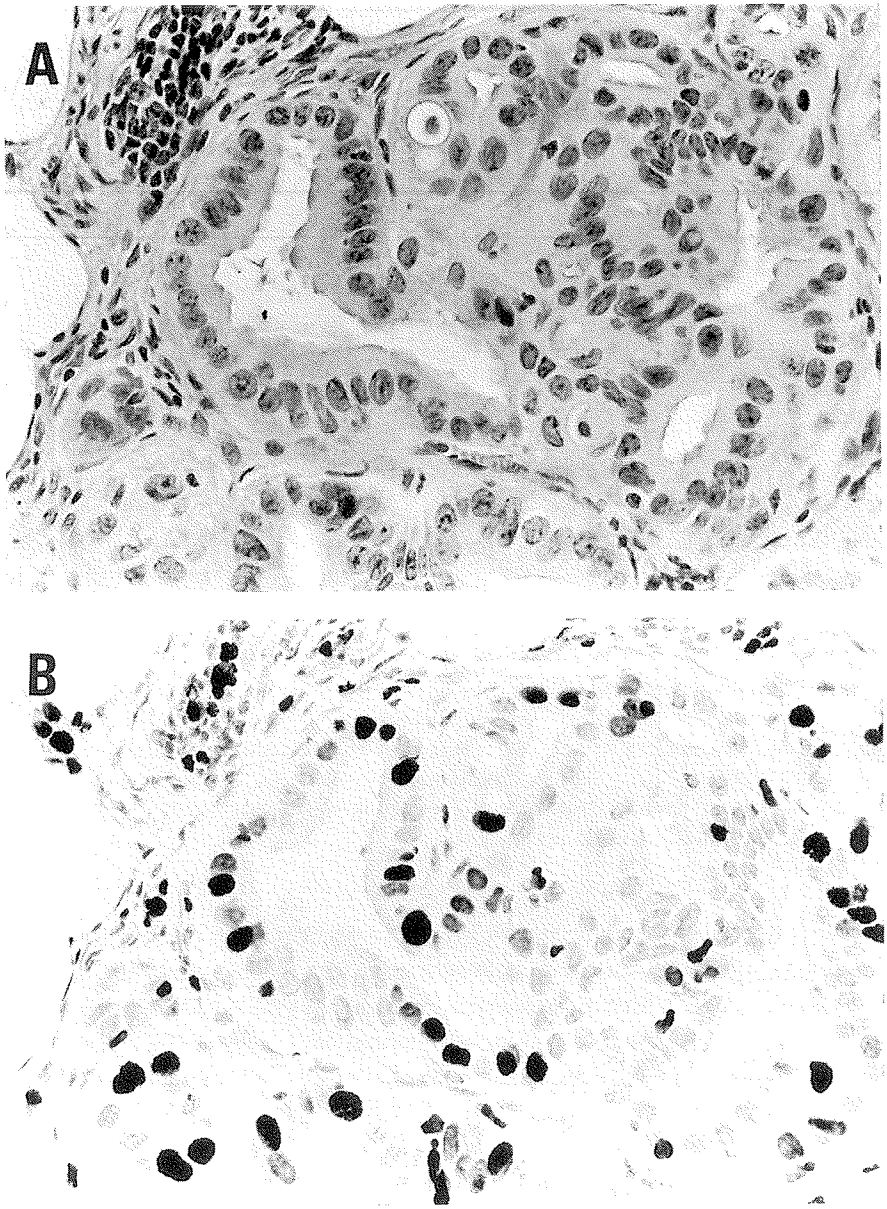

Figure 10. Borderline lesion in the pancreas of a BOP-treated hamster. A. H&E; B. BrdU, haematoxyline counterstained, 400x.

that observed in normal pancreatic tissue of the RSF fed rats. These findings indicate that dietary factors may dramatically influence cell proliferation. This observation suggests that cell proliferation is a useful parameter for determining the enhancing and inhibitory effects of life-style factors on pancreatic carcinogenesis.

The results of the cell proliferation studies with saline-treated hamsters demonstrate that the LIs in acinar (1-4%) and centroacinar cells (1-3%) were relatively low, in comparison with basal proliferation in ductular cells, which was relatively high (3-7%). Hamsters treated with BOP consistently exhibited an increased proliferation of all cell types present in the exocrine pancreas (acinar, ductular and centroacinar cells), but the most striking finding was the significant increase in proliferation of acinar cells in comparison with acinar cells of saline-treated hamsters, indicating a possible role of this cell type in the development of ductular adenocarcinomas. This observation is of particular interest, since it has been suggested that centroacinar and/or ductular cells are mainly involved in development of BOP-induced pancreatic lesions in this model (10,11), whereas Flaks *et al.* (9) suggested a possible involvement of acinar cells in formation of pancreatic tumours in the hamster. The labeling of the acinar cells may be ascribed to a regeneration of acinar tissue as a consequence of a cytotoxic insult. Undoubtedly, chronic tissue damage and repair, accompanied by hyperproliferation may play a role in the formation of cancer in both humans and experimental animals. An example of this phenomenon is the

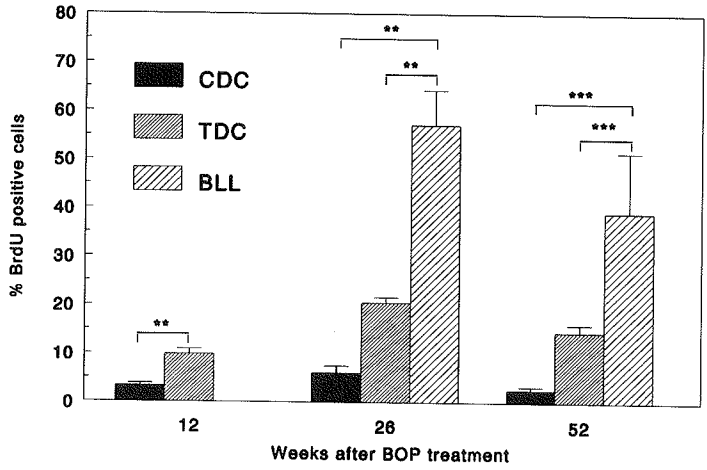

Figure 11. Labeling Index in (pre)neoplastic lesions induced by BOP in hamster pancreas. CDC, Cystic Ductal Complex; TDC, Tubular Ductal Complex; BLL, Borderline Lesion. Statistics: Analysis of variance followed by Student's *t* tests; **P<0.01; ***P<0.001.

sustained damage and repair caused by formaldehyde and acetaldehyde in the nasal epithelium of rats, which ultimately leads to nasal tumours (19). The prolonged increased LI of acinar cells in BOP-treated hamsters as compared to control values may be indicative for an increased risk of subsequent neoplastic transformation.

The presence of hepatocyte-like cells in pancreata of BOP-treated hamsters supports the concept of damage and regeneration of acinar tissue, because hepatocyte-like cells have been reported to develop in regenerating pancreata after a single dose of BOP (20). The origin of these pancreatic hepatocytes remains unclear. Evidence has been provided for acinar (21) or ductular (22,23) origin.

Flaks *et al.* (9) reported that the first ultrastructural changes after treatment with a carcinogen closely related to BOP, N-nitrosobis(2-hydroxypropyl)amine (BHP), could be observed in acinar cells. In view of the hypothesis of the involvement of a pluripotent stem-cell in pancreatic regeneration (12), it seems logical to assume that the presently observed BOP-induced sustained regenerative cell proliferation in acinar tissue which is accompanied with transdifferentiation to hepatocytes, may also lead to metaplasia of acinar cells to duct-like cells. BOP induces many pseudoductular structures, which contain both

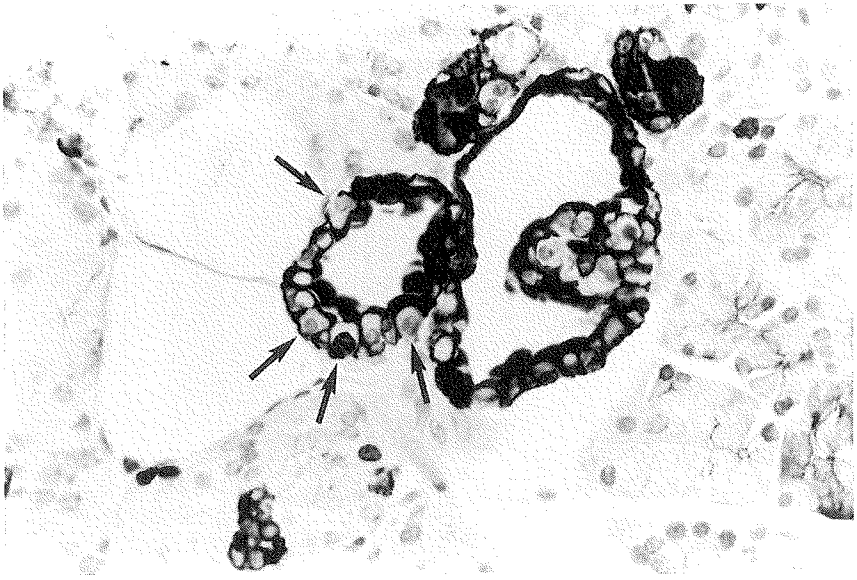

Figure 12. Pseudoductular lesion in the pancreas of a BOP-treated hamster. Double immunostain for BrdU and cytokeratins, counterstained with haematoxyline, 400x. Note the cytokeratin-negative acinar cells (arrows) within the cytokeratin-positive ductular lesion.

cytokeratin-negative acinar cells and cytokeratin-positive ductular cells. Autophagy of RER and zymogen granules in acinar cells of BHP- and BOP-treated hamsters (9,24), indicative for dedifferentiation of acinar cells (to e.g. the less differentiated ductular cell), is frequently observed in pseudoductules.

From the present results it can be concluded that the various BOP-induced cytokeratin-positive ductular lesions have different growth potential. CDC show a similar or decreased growth potential in comparison to normal ductular cells, whereas TDC and BLL show an increased growth potential relative to normal ducts. These results indicate that cell proliferation is a parameter that correlates well with (pre)neoplastic development in the hamster pancreas. In future studies routine assessment of cell proliferation in pancreatic tissue slides will facilitate diagnosis, and may prove to be a valuable parameter to monitor the course of neoplastic development. Moreover, measuring cell proliferation in the exocrine pancreas may be useful for monitoring both enhancing and inhibitory effects of dietary (and other) factors in carcinogenesis. Further research is needed to address the question whether pseudoductules are formed by preexistent ductular cells or transdifferentiated acinar cells, in order to definitively establish the cell of origin of the ductular lesions induced in hamster pancreas by BOP and hence the cell of origin of the ultimate ductular adenocarcinomas.

Acknowledgments

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Chapter 4

Modulation of azaserine-induced pancreatic carcinogenesis in rats: role of standard laboratory chow, saturated fat and cholecystokinin[1]

M.J. Appel[a], M. Meijers[a], A. van Garderen-Hoetmer[a], C.B.H.W. Lamers[b], L.C. Rovati[c], D. Sprij-Mooij[b], J.B.M.J. Jansen[b] and R.A. Woutersen[a,2]

[a]Department of Biological Toxicology, TNO Nutrition and Food Research Institute, P.O. Box 360, 3700 AJ, Zeist, The Netherlands; [b]Department of Gastroenterology and Hepatology, University Hospital, P.O. Box 9600, 2300 RC, Leiden, The Netherlands; [c]Rotta Research Laboratories, Monza, Milan, Italy.

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Abstract

In the present study the effects of a standard laboratory chow, a high lard, a low lard and a linoleic acid supplemented low lard diet on the development of azaserine-induced (pre)neoplastic lesions in rat pancreas were investigated. Furthermore, the role of cholecystokinin (CCK[3]) in dietary fat-promoted pancreatic carcinogenesis was investigated, using lorglumide, a highly specific CCK-receptor antagonist.

A high lard diet significantly enhanced and a linoleic acid supplemented low lard diet significantly inhibited the development of atypical acinar cell nodules (AACN) and adenocarcinomas in comparison with the low lard control group. Rats fed a standard laboratory chow developed significantly less pancreatic AACN and carcinomas than low

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: CCK, cholecystokinin; AACN, atypical acinar cell nodule(s); AACF, atypical acinar cell focus or foci; BOP, N-nitrosobis(2-oxopropyl)amine; HF, high fat; LF, low fat; LFsupp, linoleic acid supplemented low fat; H&E, haematoxylin and eosin; CIS, carcinoma *in situ*; FCE, food conversion efficiency; EFA, essential fatty acid; DMBA, 7,12-dimethylbenz(a)anthracene; Lab chow, laboratory chow.

lard controls.

CCK, but not dietary fat, increased pancreatic weight. Rats treated with CCK developed more AACN, adenomas and adenocarcinomas than control animals. Rats maintained on a high fat diet developed significantly more adenomas and adenocarcinomas than animals fed a low fat diet. Lorglumide largely inhibited the enhancing effects of CCK, but not of dietary fat, on pancreatic carcinogenesis.

It is concluded that (i) a diet high in saturated fat has a promoting and laboratory chow has an inhibitory effect on azaserine-induced pancreatic carcinogenesis in rats and (ii) it is unlikely that the promoting effect of dietary unsaturated fat is mediated via CCK.

Introduction

Dietary fat has been implicated in the etiology of various human cancers, including pancreatic cancer. Epidemiology has revealed a direct association between total fat consumption and mortality of pancreatic cancer (1-4). Studies with azaserine-treated rats and with N-nitrosobis(2-oxopropyl)amine (BOP)-treated hamsters have demonstrated that a diet high in unsaturated fat (corn oil) promotes the development of pancreatic tumours (5-10). In short-term studies we found an enhancing effect of saturated fat (20% lard) on development of BOP-induced putative preneoplastic ductular lesions in hamster pancreas and on growth of azaserine-induced basophilic atypical acinar cell foci (AACF) in rat pancreas (11,12). Birt *et al.* (13) recently showed a pronounced enhancing effect of saturated fat (beef tallow) on pancreatic carcinogenesis in BOP-treated hamsters. It has been proposed that a combination of both type and amount of dietary fat is responsible for the promoting effect on carcinogenesis (14). Roebuck *et al.* (15) found an enhancement of the promoting effect of fat on pancreatic carcinogenesis in rats when dietary levels of linoleic acid were increased. The objectives of the first experiment presented in this chapter were to investigate whether (i) saturated fat (lard) may have the potential to promote the development of pancreatic tumours induced in rats by azaserine and (ii) linoleic acid is responsible for the effect of high-fat on pancreatic carcinogenesis. For this purpose, a group maintained on a low-lard diet with an amount of linoleic acid similar to the high-lard group was incorporated. Furthermore, a group maintained on a standard laboratory chow (containing a level of linoleic acid similar to the high-lard diet) was added to confirm earlier studies which suggested that natural ingredient diets caused less neoplasms in chemically induced cancer models as compared to semi-purified diets (16-18).

Apart from the composition of the lipids within a high fat diet, the physiological effect of the dietary fat on the small intestine may be a modulating factor in pancreatic carcinogenesis. One of the postulated mechanisms involves an increase in intraduodenal

CCK release. The cells producing the gut hormone CCK are located in the small intestine and release CCK into the circulation upon ingestion of food. CCK is considered to be the most important hormonal regulator of pancreatic enzyme secretion, and it is assumed that those nutrients that stimulate pancreatic enzyme secretion do so by stimulating CCK release. Douglas *et al.* (19) have demonstrated that unsaturated fat as well as protein administered intragastrically to rats causes a rapid and significant rise in plasma CCK exceeding the threshold for pancreatic stimulation. This result suggests that CCK release induced by fat and protein may play a role in the postprandial stimulation of the pancreas in rats. In human volunteers ingestion of fat also causes a rise in plasma CCK levels, especially with an unsaturated fat such as corn oil (20).

Moreover, it has been demonstrated that lorglumide, a highly specific CCK receptor antagonist (21,22), inhibits the promoting effect of CCK on pancreatic growth (23,24) and on the development of putative preneoplastic acinar lesions induced in rat pancreas by azaserine (23,25). Roebuck *et al.* (26), however, did not find plasma CCK increments in rats maintained on a diet high in unsaturated fat, which was due to an inadequate time of blood sampling in combination with *ad libitum* feeding. We have demonstrated that plasma CCK concentrations increase almost instantly after ingestion of food and return to basal levels thereafter (19). This observation indicates that the time lag between ingestion of food and collection of plasma is highly critical with respect to the elucidation of the role of CCK in diet-promoted pancreatic carcinogenesis. In the second study presented in this chapter, the rats were gradually accustomed to eat only one 4-hour meal per day, either or not in combination with pre-treatment with lorglumide, to allow us to manipulate the food-induced release of CCK. Groups treated with CCK, alone or in combination with lorglumide, were incorporated for comparison.

Materials and Methods

Chemicals. Azaserine was purchased from the Calbiochem-Behring Corp., La Jolla, CA, USA. Cholecystokinin-octapeptide (CCK) was obtained from Cambridge Research Biochemical, Cambridge, UK and Lorglumide, a highly specific CCK-receptor antagonist was kindly provided by Rotta Research Laboratories, Milan, Italy.

Animals and diets. Experiment 1: One hundred and forty three male weanling SPF albino Wistar rats (WISW; Cpb) were obtained from F. Winkelmann (Versuchtiersucht GmBh, Borchen, Germany). They were housed in wire-mesh stainless steel cages, 5 animals per cage, under standard laboratory conditions. The animals were fed a high fat control diet (HF; 20% lard), a low fat diet (LF; 5% lard/safflower oil) or a linoleic acid supplemented low fat diet (LFsupp: 5% lard/corn oil). The semi-purified diets were prepared freshly each month in our institute and were stored at -20°C until use. A fourth group was fed a standard laboratory chow (Lab. chow; containing ca. 6% fat and ca. 3% linoleic acid). Food and drinking water were available *ad libitum*. The percentage composition of the three semi-synthetic diets and their energy content are summarized in Table 1.

Experiment 2: Two hundred and forty male weanling SPF Wistar rats (WISW; Cpb) were obtained from F. Winkelmann (Versuchtiersucht, GmBh, Borchen, Germany). The animals were housed in wire-mesh stainless steel cages, five animals per cage, under standard laboratory conditions. The semi-purified diets, either high or low in unsaturated fat (HF; 20% corn oil or LF; 5% corn oil, respectively) were prepared freshly each month in our institute and were stored at -20°C until use. The diets were composed of natural

Table 1 Weight % composition of the semi-synthetic diets (Experiment 1)[a]

Ingredients	HF	LF	LFsupp
Soya protein isolate[b]	7.9	6.9	6.7
Casein	15.6	13.3	13.3
DL-Methionine	0.24	0.2	0.2
Wheat starch	13.6	38.55	38.55
Pregelatinized starch	20.3	20.0	20.0
Cellulose	11.8	10.0	10.0
Jones Foster Minerals	5.3	4.5	4.5
KH_2PO_4	0.71	0.6	0.6
Vitamin ADEK prep.	0.53	0.45	0.45
Vitamin B mixture	0.35	0.3	0.3
Choline chloride	0.47	0.4	0.4
Corn oil	-	-	3.6
Safflower oil	-	0.48	-
Lard	20.0	4.52	1.4
Total	100.0	100.0	100.0
Linoleic acid	1.2	0.6	2.0
Energy content (MJ/kg)	17.7	15.1	15.1

[a]HF=20% lard; LF=5% lard/safflower oil; LFsupp= 5% lard/corn oil; [b]The soya protein isolate had a protease activity of less than 10 trypsin inhibitor units per mg.

food ingredients and contained equal amounts of minerals, trace elements and vitamins per unit energy (Table 2). The HF diet contained also a high level of protein. Dietary protein stimulates CCK release (19), but has no influence on pancreatic carcinogenesis (5).

Treatment. Experiment 1: Azaserine was dissolved freshly in 0.9% NaCl-solution. Each rat was given two i.p. injections of 30 mg azaserine per kg body weight at 19 and 26 days of age. The HF and the two LF groups consisted of 40 animals each while the laboratory chow group consisted of 23 animals. The laboratory chow group was an additional group, treated with azaserine according to the same protocol as the other rats, but maintained on cereal based laboratory chow for the whole experimental period.

Experiment 2: Azaserine was dissolved freshly in 0.9% NaCl solution. Each rat was given three i.p. injections of 30 mg azaserine per kg body weight at 19, 28 and 52 days of age. After the first injection of azaserine the animals were maintained on the HF diet for 4 months in order to enhance the yield of atypical acinar cell nodules (AACN). During this period the animals were gradually accustomed to eat for 4 hours per day (08.00-12.00). This dietary regimen was continued for the rest of the study. After the 4-month acclimatization period the animals were allocated to six different groups of 40 animals each by a computerized randomization procedure. Thereafter, all animals in groups 1 to 4 were maintained on the LF diet and received one of the following treatments (s.c. injection, once daily, 3 consecutive days/week for 8 months): group 1, 0.9% NaCl (saline control); group 2, CCK in gelatin (2.5 μg/kg body wt); group 3,

Table 2 Weight % composition of the diets (Experiment 2).

Ingredients	High fat	Low fat
Corn oil	20.0	5.0
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
Total	100.0	100.0

lorglumide (12 mg/kg body wt); group 4, CCK (2.5 μg/kg body wt) in combination with lorglumide (12 mg/kg body wt). The animals in group 5 and 6 were given the HF diet on 3 consecutive days per week. On these days the animals in group 6 were treated with lorglumide (12 mg/kg body wt), while the animals in group 5 were injected with saline. The other 4 days of the week these animals were maintained on the LF diet. Lorglumide was dissolved in distilled water to a concentration of 0.4% and adjusted to pH 9 with 0.1M NaOH and subsequently administered to the animals 30 min before injection of CCK and 30 min before the animals received their feed. Drinking water was available *ad libitum*. The dose of CCK used was based on plasma concentration-time curves for CCK obtained from a previously described 2-week study in rats and hamsters (27). Subcutaneous injection of 2.5 μg/kg body wt CCK, dissolved in 16% hydrolysed gelatin, resulted in plasma CCK levels that were only slightly supraphysiological and comparable with those seen after dietary administration of corn oil (19).

Monitoring. Experiment 1: Body weights and food and liquid intake were recorded weekly during the

first three months and monthly thereafter. The general condition and behaviour of the animals were checked daily. Seven animals died before terminal autopsy, and were excluded from the results. No cause of death was established for these animals.

Experiment 2: Body weights were recorded weekly during the first 2 weeks, every 2 weeks for 16 week thereafter and monthly for the rest of the experimental period. The general condition and behaviour of the animals were checked daily. A total of 31 animals, involving all groups, died before terminal autopsy. The rats that died after 350 effective days (N=4) in the study have been included in the results. Twenty-seven ra (11%), were excluded from the results because they died or were killed *in extremis* before day 350 of the study. Six of these animals died or were killed owing to a bad condition caused by the repeated injections (N=3) or the malocclusion syndrome and subsequent anorexia (N=3). Five animals died presumably of renal failure, one rat of a bone tumour and another one of a squamous cell carcinoma of the Zymbal's gland. No cause of death could be established for fourteen of the animals because no autopsy was performed due to early death (before day 85; N=11) or to cannibalism (N=3).

Analyses. Terminal autopsy was at 482, 483, 484 or 485 days (Experiment 1) and 371 days (Experimen 2) after the last injection of azaserine. The animals were anaesthetized with ether, exsanguinated by cannulating the abdominal aorta, and examined for gross pathological changes. The entire pancreas, liver an all gross lesions were excised. The pancreas and liver of each animal were weighed. All excised organs and all gross abnormalities suspected of being tumours were fixed in 10% buffered formalin. The entire pancrea were processed for microscopy by conventional methods, step-sectioned at 5 μm, stained with haematoxylin and eosin (H&E) and examined by light microscopy. All pancreatic lesions were identified and classified according to the criteria of Longnecker (28) and Rao *et al.* (29).

Atypical acinar cell nodules (AACN) were recognised by phenotypic changes comprising an increased rate of cell division, altered zymogen content of the cells, changes in nuclear size, and loss of differentiatior Two different populations of AACN have been characterised in H&E-stained tissue sections by their markedly basophilic or intense acidophilic cytoplasm. AACN have been defined as those with a diameter smaller than 3 mm. Some lesions reached diameters of 2-3 mm and a few reached diameters of 3-7 mm. Lesions of the latter group that retained a high degree of differentiation have been designated acinar cell adenomas. Carcinoma *in situ* (CIS) is a lesion showing some degree of anaplasia that suggests malignant growth potential but without evidence of local invasion. Microcarcinoma was used for a carcinoma *in situ* o an adenoma-like lesion exhibiting anaplasia and focal invasion of the fibrous capsule or the surrounding normal pancreatic tissue. Carcinomas show invasion of adjacent tissues and may metastasise in periaortic lymphnodes, liver and lungs.

Quantitative determination of the number of AACN per cm^3 of pancreas was performed by using a grid inside the ocular as described previously (8). The calculated volumetric data were evaluated by analysis of variance. To minimize the SEM some mathematical transformations were performed. The total number of observed AACN per cm^2, the mean transection area and the percentage of pancreas area occupied by focus tissue were logarithmically transformed before statistical evaluation. The calculated total number of AACN per cm^3 was prepared for statistical evaluation by taking the square root. The mean diameters of AACN wer not mathematically transformed. The number of pancreatic lesions was evaluated by a generalized linear regression model (error is Poisson, link function is log). The incidence and severity of pancreatic neoplasms were evaluated by a log-linear model followed by χ^2 tests for goodness of fit.

Results

Food intake. Experiment 1. Food intake was significantly ($P < 0.01$) less in animals fed the HF diet as compared to the LF diets. As the energy intake per animal per week was similar in all groups, the two LF groups had to convert their food less efficiently, which is reflected in significantly lower FCE-values than in the HF group. These data are presented in Table 3.

Experiment 2. Food intake was not recorded.

Table 3 Mean food and energy intake and food conversion efficiency.

Diet group[a]	No. of rats	Food[b]	Energy[c]	FCE[d]
HF	38	100.8	1788.2	0.40
LF	37	118.3**	1786.3	0.32***
LFsupp	38	120.4**	1818.0	0.33***

[a]HF, high fat; LF, low fat; [b]Food intake in g/animal per week; [c]Energy intake in KJ/animal per week; [d]FCE, Food Conversion Efficiency for the first 28 days on the respective diets: g weight gain per g food consumed. Statistics: 2-way analysis of variance: **P<0.01; ***P<0.001.

Body and organ weights. Experiment 1: Body weights and liver and pancreas weights were comparable among the various groups, controls included. Absolute liver weights were slightly increased in the laboratory chow group (15.16 g ± 0.53 vs 13.54 ± 0.41 in controls).

Experiment 2: Body weights remained similar for all groups. Both absolute and relative liver weights of all treated animals were comparable with those of controls. The pancreata of animals treated with CCK increased significantly in weight as compared with controls ($P<0.01$).

Microscopy. Experiment 1: The results of the quantitative analysis of the pancreatic H&E-stained paraffin sections are presented in Table 4. The number and size of the acidophilic foci were not significantly different among the different diet groups. The mean diameter of the acidophilic foci observed in the laboratory chow group was significantly smaller as compared to the HF group ($P < 0.05$). In the laboratory chow group the number of basophilic foci observed was comparable with those seen in the HF and LFsupp group. The LFsupp group showed an increase in number of basophilic foci of the smallest size group scored (diameter 272.5 μm), as well as an increase in total number of basophilic foci per cm^3 when compared to the LF group ($P < 0.05$).

Table 4 Effects of a HF, LF, LFsupp or laboratory chow diet on development of putative preneoplastic lesions in rat pancreas (N=15) induced by azaserine[a,b].

Post-initiation diet group	Observed transection data of foci		Calculated volumetric data of foci						
			n/cm^3 with mean diameter (μm)						
	Total n/cm^2	Transection area (mm^2)	385	545	770	1090	Total n/cm^3	Mean diameter (μm)	Area as % of pancreas
Acidophilic foci									
HF	15.80	0.34	182	143	30	50	445	575	5.31
LF	15.49	0.30	211	107	22	39	427	522	4.66
LFsupp	13.33	0.27	153	101	21	25	327	532	3.67
Lab. chow	12.43	0.25	196	105	20	21	380	505[c]	3.12
			n/cm^3 with mean diameter (μm)						
			272.5	385	545	770			
Basophilic foci									
HF	3.56	0.079	199	134	3	0	207	298	0.28
LF	2.56	0.072	99	8	0	0	119	296	0.19
LFsupp	4.48	0.073	264[d]	28	0	0	311[d]	290	0.33
Lab. chow	5.16[d]	0.073	203	29	1	0	248	294	0.38

[a]Values are means; [b]Data are based on H&E stained paraffin sections; Statistics: analysis of variance; [c]$P<0.05$ as compared to HF group; [d]$P<0.05$ as compared to LF group

Table 5 shows that the number of AACN with a diameter over 1 mm is significantly decreased in the LFsupp ($P < 0.01$) and the laboratory chow group ($P < 0.001$) and that the number of adenocarcinomas is lower in all LF groups including the group onlaboratory chow as compared to the HF group ($P < 0.001$). Overall the animals maintained on the HF diet for 15 months developed more adenomas (35 vs 25) and microcarcinomas (10 vs 6) but less CIS (4 vs 10) than the animals in the LF group. However, these differences did not reach the level of statistical significance. Unexpectedly, the number of benign and malignant tumours was consistently lower in the LFsupp group as compared to the LF group ($P < 0.05$). The incidence of adenocarcinomas (Table 6) was highest in the HF group but the difference with the LFsupp and the laboratory chow group was not significant. None of the animals of the laboratory chow group developed CIS or microcarcinoma. The number of tumour bearing animals was the highest in the HF group and the lowest in the laboratory chow group.

Table 5 Number of pancreatic lesions in azaserine-treated rats maintained on a HF, LF, LFsupp or laboratory chow diet for 15 months.

(Pre)neoplastic lesions observed	Number of lesions[a]			
	HF	LF	LFsupp	Lab.Chow
No. of animals examined	38	37	38	23
AACN (<1mm)	176	155	110^{**,b}	58^{***,c}
Adenoma	35	25	17	13
CIS	4	10	6	0
Microcarcinoma	10	6	3	1
Adenocarcinoma	17	7**	2^{***,b}	2^{***,b}
Total carcinoma	31	23	11	3

[a]HF, high fat; LF, low fat; Lab.chow, laboratory chow. Statistics: generalized linear model (one tailed) with a Poisson error distribution; link function is log, $^{**}P<0.01$; $^{***}P<0.001$; significantly different as compared to the HF group; $^{b}P<0.05$; $^{c}P<0.001$; significantly different as compared to the LF group.

Experiment 2: Animals of the LF + lorglumide group and those of the HF group irrespective of lorglumide treatment, showed no differences in number and size of acidophilic AACN from controls on a LF diet (Table 7).
In rats treated with CCK we found an increase in number ($P<0.001$), but not in mean

diameter, of acidophilic lesions resulting in an increase in area of pancreas occupied by acidophilic tissue (P<0.001). Treatment with lorglumide 30 minutes before CCK injectio caused a significant inhibition of the promoting effect of CCK on growth of acidophilic AACN (P<0.001). The total number of acidophilic AACN per cm^3 in the CCK + lorglumide group was similar to that in controls. The area of pancreas occupied by acidophilic focus tissue had also decreased significantly (P<0.001) in the group treated with CCK and lorglumide in comparison with the group treated with CCK alone. Interestingly, the effect of CCK on growth of acidophilic AACN was accompanied by a significant inhibitory effect on growth of basophilic AACN as reflected in a decrease in total number of basophilic nodules per cm^3 (P<0.001), a decrease in mean diameter (P<0.05) and in area of pancreas occupied by basophilic focus tissue (P<0.01). Pre-treatment with lorglumide reduced significantly (P<0.05) the inhibitory effect of CCK or growth of basophilic foci. The HF diet given for 3 days/wk, 4 h per day for 8 months caused a decrease (P<0.05) in number of basophilic AACN in comparison with animals maintained on the LF diet. Treatment of the animals with lorglumide, 30 minutes before they received the HF diet, resulted in a number of basophilic AACN similar to that in controls.

Table 6 Incidence of pancreatic neoplasms (%) in azaserine-treated rats maintained on a HF, LF, LFsupp or laboratory chow diet for 15 months[a]

Neoplastic lesions observed	HF	LF	LFsupp	Lab. chow
Number of rats examined	38	37	38	23
No. of tumour-bearing rats	17 (45)	12 (32)	11 (29)	5 (22)
Adenoma	4 (11)	4 (11)	4 (11)	3 (13)
CIS	1 (2)	1 (2)	1 (2)	0
Microcarcinoma	3 (8)	2 (5)	4 (11)	0
Adenocarcinoma	9 (24)	5 (14)	2 (5)	2 (9)

[a]Incidences are based on scoring each animal once in the category of the most advanced lesion; HF, 20% lard; LF, 5% lard/safflower oil; LFsupp, 5% lard/corn oil; Lab. chow, laboratory chow.

Treatment of rats with CCK increased the number of adenomas (P<0.01; Table 6) an the number of AACN with a diameter > 1.0 mm (P<0.001). Lorglumide inhibited this promoting effect of CCK on pancreatic carcinogenesis significantly. Furthermore, CCK

Table 7 Effects of CCK and a HF diet, either alone or in combination with lorglumide, on development of putative preneoplastic pancreatic foci induced in rats by azaserine[†]

ACIDOPHILIC FOCI	Observed transection data of foci			Calculated volumetric data of foci		
Treatment group[††]	No. of rats	Total no./cm^2	Transection area (mm^{2}x100)	Total no./cm^3	Mean diameter (μm)	Area as % of pancreas
LF	36	5.4	18.3	240	447	0.99
LF/CCK	37	22.2^c	23.1^b	717^c	474	5.12^d
LF/lorglumide	35	5.7	21.0	254	457	1.20
LF/CCK/lorglumide	35	9.2^{a,d}	20.2	362^d	463	1.86^{a,d}
HF	34	4.2	19.4	164	455	0.80
HF/lorglumide	36	5.6	20.7	202	464	1.16
BASOPHILIC FOCI	Observed transection data of foci			Calculated volumetric data of foci		
Treatment group[††]	No. of rats	Total no./cm^2	Transection area (mm^{2}x100)	Total no./cm^3	Mean diameter (μm)	Area as % of pancreas
LF	36	4.6	8.3	274	309	0.38
LF/CCK	37	2.4^b	7.7	131^c	292^a	0.18^b
LF/Lorglumide	35	4.4	7.3	289	293^a	0.32
LF/CCK/Lorglumide	35	2.8^a	7.7	161^a	299	0.21^a
HF	34	2.9	7.9	174^a	308	0.23
HF/Lorglumide	36	3.7	8.3	222	305	0.31

[†]Values are means; [††]LF, low fat; HF, high fat; Statistics: Analysis of variance followed by Student's t-test (two-tailed); $^aP<0.05$; $^bP<0.01$; $^cP<0.001$; as compared to LF controls; $^dP<0.001$; as compared to the LF/CCK-group.

alone enhanced and lorglumide alone inhibited the development of microcarcinomas (Table 8) and CCK also increased the total number of carcinomas as compared to contro ($P<0.05$). Lorglumide had only a slight inhibitory influence on this effect of CCK. The HF diet caused an increase ($P<0.05$) in number of pancreatic adenomas and adenocarcinomas. No difference, however, was found in total number of carcinomas (comprising adenocarcinomas, CIS and microcarcinomas) between animals maintained on a HF diet and LF controls. Pre-treatment with lorglumide did not influence the promoting effects of the HF diet on pancreatic carcinogenesis.

A shift towards malignant lesions was observed in the CCK-treated group (Table 9). this group 38% of the rats developed a carcinoma versus 25% in the controls. This increase is reflected in an increased incidence ($P<0.05$) of animals with a microcarcinom in the CCK-treated group. In the group treated with lorglumide prior to CCK, the incidence of pancreatic tumours was similar to that in controls. The group maintained on HF diet and pre-treated with lorglumide showed a higher incidence ($P<0.05$) of adenocarcinomas than controls. Since lorglumide alone did not result in a rise in incidence, the latter effect is considered to be attributable to the HF diet.

Discussion

It has been proposed that unsaturated fat but not saturated fat has promoting effects o pancreatic carcinogenesis in rats (5,6). The results of the present study, however, point to an enhancing effect of saturated fat on the development of pancreatic neoplasms in azaserine-treated rats. The effects observed with lard, which were mainly seen on the multiplicity rather than on the incidence, were less pronounced than those observed with diet rich in unsaturated fat (20% corn oil). Furthermore, it appeared from the present results that the enhancing effect of lard on pancreatic cancer is not related to a high caloric intake or a high concentration of linoleic acid. Increased growth rate that may hav accelerated the development of preneoplastic pancreatic lesions in the group maintained o a high fat diet as compared to the groups on low fat did not occur because rats in the hig fat group adjusted their caloric intake to the level of the low fat groups by eating significantly ($P < 0.01$) less. This is reflected by a similar mean caloric intake among the groups and a significantly ($P < 0.001$) lower FCE of animals of the low fat groups as compared to those of the high fat group. The amount of linoleic acid in the high fat grou was also not involved in the promoting effect of lard since neither the multiplicity nor th incidence or severity of the neoplastic pancreatic lesions were influenced by supplementation of linoleic acid to the low lard group. On the contrary, in the linoleic ac supplemented group the number of AACN larger than 1.0 mm as well as the number of adenocarcinomas were significantly lower as compared to the unsupplemented low lard

Table 8 Effects of CCK and a HF diet, either alone or in combination with lorglumide, on the number of (pre)neoplastic pancreatic lesions induced in rats by azaserine[†]

Treatment[††]	No. of rats	AACN (Ø>1.0 mm)	Adenomas (Ø>3.0 mm)	CIS	Micro-carcinomas	Adeno-carcinomas	Total carcinomas
LF	36	14	0	4	5	2	11
LF/CCK	37	$90^{***,a}$	$7^{**,a}$	4	$13^{*,b}$	4	$21^{*,c}$
LF/lorglumide	35	16	2	7	$2^{*,c}$	2	11
LF/CCK/lorglumide	35	$33^{**,a}$	1	6	$4^{*,b,c}$	5	$15^{*,c}$
HF	34	23	$4^{*,a}$	2	1	$5^{*,d}$	8
HF/lorglumide	36	25	3	4	$1^{*,c}$	$9^{*,d}$	14

[†]All animals were fed for 4 h a day; [††]LF, low fat; HF, high fat; Statistics: regression analysis (error is Poisson, link function is log); $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. [a]Significantly different from the LF group; [b]Treatment with CCK caused an increase in number of microcarcinomas as compared to animals kept on a LF diet and not treated with CCK; [c]Treatment with lorglumide caused a reduction in number of microcarcinomas as compared to animals not treated with lorglumide; [d]A HF diet caused an increase in number of carcinomas as compared to animals kept on a LF diet for 7 days a week; [e]Treatment with CCK caused an increase in total number of carcinomas as compared to animals not treated with CCK.

group. Further research is needed to find an explanation for this remarkable observation.

In previously conducted 4-month experiments unsaturated fat (corn oil) was found to enhance growth of both acidophilic and basophilic foci (30-32). Roebuck (33) reported an increase in size, number and area of pancreas occupied by acidophilic AACN in rats maintained on a diet high in unsaturated fat (20% corn oil) but not on a diet rich in saturated fat. Woutersen *et al.* (12), however, found a significant higher number of basophilic foci per cm^3 in pancreata of rats maintained on a diet high in lard as compared to a low lard diet. In the present study, the high lard diet also showed a tendency, although not statistically significant, to enhance growth of basophilic foci. This observation supports the hypothesis that the basophilic foci somehow may play a role in the development of pancreatic cancer in azaserine-treated rats. Although their growth potential is much less than that of acidophilic foci (34), modulation of their development by ethanol, cholecystokinin, lard and the synthetic trypsin inhibitor Camostate has been reported (5,8,12). These findings point to a possible role of the basophilic foci in the development of pancreatic cancer in rats. Up to now, however, the significance of these observations is still a matter of debate and needs further elucidation.

The discrepancy between our findings with lard and those reported by Roebuck *et al.* (5,6), who did not find an enhancing effect of saturated fat on pancreatic carcinogenesis might be due to either differences in the type of saturated fat (lard vs coconut oil) or to the control group used (low lard vs low corn oil). Moreover, we presently found mainly a enhancing effect on the multiplicity of the pancreatic neoplasms whereas an effect on the incidence was not clearly indicated. It has been proposed that a minimum threshold level of essential fatty acid (EFA) in the form of linoleic acid is needed in the diet to yield a maximum tumorigenic effect. Below this threshold level a maximum tumorigenic effect should be impossible to reach irrespective of the amount of fat used. Using the DMBA-induced mammary cancer model, Ip *et al.* (14) demonstrated this maximum response to b at about 4% of dietary EFA. They also found that when EFA requirements were met, increasing levels of saturated fat (hydrogenated coconut oil) enhanced both the mammary tumour incidence and yield (35). Lasekan *et al.* (36) recently found a similar threshold level (i.e. 3.4% of dietary EFA) in the same model. Roebuck *et al.* (15) varied the amoun of EFA in a high fat diet (20%) by blending corn oil with hydrogenated coconut oil and found that growth of AACN had increased with increasing EFA concentrations. This increase was particularly significant between 4.4% and 8.5% of dietary EFA, indicating that at least 4.4% EFA is required in the diet to yield a maximal tumorigenic response of a high fat diet on pancreatic carcinogenesis. The concentrations of linoleic acid used in th present study were lower than 4.4% indicating that the observed enhancing effect of a hig lard diet may have been more pronounced in case the threshold level of EFA would have been met. However, at the dietary EFA-levels used in this study, which are far above the concentration of EFA required to obtain normal growth of the rats (0.6% for male rats), a

Table 9 Effects of CCK and a HF diet, either alone or in combination with lorglumide, on the incidence of (pre)neoplastic pancreatic lesions induced in rats by azaserine[†]

Treatment group[††]	No. of rats	Tumour-bearing rats (%)	Carcinoma-bearing rats (%)	AACN (Ø>1.0 mm)	Adenomas (Ø>3.0 mm)	CIS	Micro-carcinomas	Adeno-carcinomas
LF	36	9 (25)	9 (25)	8	0	4	3	2
LF/CCK	37	18 (49)	14 (38)	12	4	4	6[a]	4
LF/CCK/lorglumide	35	9 (26)	7 (20)	6	2	4	1	2
LF/lorglumide	35	10 (29)	10 (29)	6	0	4	2	4
HF	34	10 (29)	7 (21)	8	3	1	1	5
HF/lorglumide	36	14 (39)	13 (36)	9	1	3	1	9[a]

[†]All animals were fed for 4 h a day; [††]LF, low fat; HF, high fat. Statistics: Log-linear model followed by χ^2-tests for goodness of fit; [a]$P<0.05$, as compared to LF controls

diet high in saturated fat appeared to be able to accelerate development of acidophilic AACN to pancreatic neoplasms.

The present observation that a standard laboratory chow (containing 6% fat and ca. 3% of linoleic acid: partly derived from natural ingredients and partly added as soya oil) inhibits the development of acidophilic pancreatic AACN in rats treated with azaserine is in agreement with a previous observation of Longnecker *et al.* (7). Moreover, Carroll *et a* (16) found an inhibitory effect of a natural ingredient diet on DMBA-induced mammary tumours, which remained present when the dietary fat content was varied from 5-20%, indicating that the overall decrease in tumour incidence observed in the group maintained on a natural ingredient may be ascribed to dietary factors other than fat. Purified diets contain animal protein (casein), while commercial or standard laboratory chows contain protein largely derived from plant sources. Since epidemiological studies have shown that mortality from breast cancer in humans was positively related to animal-protein intake, Carroll *et al.* (16) compared the mammary tumour yield in DMBA-treated rats maintained on a semi-synthetic diet high in animal- or plant-protein. These experiments did not provide any indication for an inhibitory effect of dietary plant-protein on mammary gland carcinogenesis induced in rats by DMBA. Recent research in our Institute showed that animals given a standard laboratory chow, as compared to animals maintained on a semi-purified diet, developed symptoms that are indicative for animals fed a diet rich in low digestibility carbohydrates, which are characterized by enlargement of the caecum and lov faecal pH. These observations led to the hypothesis that the significant inhibition of pancreatic carcinogenesis in rats fed a standard laboratory chow as compared to rats fed a semi-purified diet is due to dietary fibre (37). The results of other animal studies also point to an inhibitory effect of dietary fibre on carcinogenesis. Sinkeldam *et al.* (38) recently reported a decrease in incidence of tumours and adenomatous polyps in a high fat/medium fibre diet and a decreased multiplicity of these neoplasms in a high fat/high fibre diet as compared to a high fat/low fibre diet in N-methyl-N'-nitro-N-nitroguanidine-induced colon cancer in rats. Their overall results indicated that fibre may have protective properties, but that fibre and fat affect colon carcinogenesis in a complex, interactive manner. Fisher *et al.* (39) found in a lifespan study in rats significantly fewer spontaneously occurring mammary gland tumours in rats fed a high fibre stock diet as compared to rats fed low fibre purified diets. Experiments at our Institute confirmed these observations (40) and showed that the inhibitory effect was independent of the amount of dietary fat. Furthermore, the growth of N-nitrosomethylurea-induced mammary tumours ir rats was found to be inhibited in rats maintained on a high fibre diet as compared to a lov fibre diet (41).

The second study was conducted to investigate whether dietary fat-promoted pancreati carcinogenesis in rats is mediated via an increased duodenal release of CCK. It has been demonstrated that lorglumide, a specific CCK-receptor antagonist, remains in its active

form for about 4 to 6 hours (22). To be able to modulate the CCK release induced by the ingestion of food we chose a dietary regimen consisting of one 4-hour meal per day. To establish whether the enhancing effect of a HF diet on pancreatic carcinogenesis is indeed mediated via CCK, we injected lorglumide 30 minutes before the animals received their respective diets to occupy the CCK receptors before CCK is released from the duodenum. A disadvantage of the 4-hour meal regimen used was a significant decrease in body weight gain as compared to a parallel study with azaserine-treated rats fed *ad libitum* (average body weight in the present study after 12 months was 400 g versus 467 g in the parallel study). The decrease in body weight gain might be a confounding factor in the present study, since it is well known that an energy-restricted diet (10%) has a significant inhibitory effect on azaserine-induced pancreatic carcinogenesis in rats (5). Indeed, the tumour incidences in the present study appeared to be consistently lower than in the parallel study in which the animals were fed *ad libitum*.

The present results obtained with CCK are in agreement with those of previous studies (23-25). CCK administration enhances pancreatic growth as well as pancreatic carcinogenesis in azaserine-treated rats in spite of the restricted feeding regimen.

Also of great interest is the observation that, whereas CCK enhances growth of acidophilic AACN, it inhibits the growth of basophilic foci. This effect of CCK is slightly inhibited by lorglumide. In a previous study we have observed the same phenomenon with the synthetic trypsin inhibitor Camostate (23). These results support our conclusion that, besides acidophilic foci, also basophilic foci may be responsive to modulators of carcinogenesis and may play a role in the development of pancreatic cancer in azaserine-treated rats (8,11). It is, therefore, of paramount importance not to neglect these putative preneoplastic lesions in studies on pancreatic carcinogenesis. Moreover, the significance of these observations needs further elucidation.

Even under the circumstances of a single 4-hour meal the HF diet enhances the development of pancreatic acinar adenocarcinomas, albeit less pronounced than in rats fed a HF diet *ad libitum* (9). Lorglumide did not influence the promoting effects of the HF diet on pancreatic carcinogenesis. This observation is not in agreement with that of Smith *et al.* (42), who reported that L 364,718, a potent CCK-receptor antagonist, decreased the volume and weight of a xenografted human pancreatic tumour cell line in athymic mice. Moreover, they also found a reduction in dietary fat-promoted growth of these xenografts by L 364,718. These apparently contradictory findings are most probably due to differences in tumour types induced by azaserine in rats (almost exclusively acinar adenocarcinomas) and those occurring in man (ductular adenocarcinomas). Syrian golden hamsters treated with N-nitrosobis(2-oxopropyl)amine (BOP) develop ductular tumours which resemble those occurring in man. In the BOP-hamster model CCK has been found to have no effect or even an inhibitory effect on pancreatic carcinogenesis (43,44).

In the present study, CCK enhanced multiplicity and incidence of pancreatic tumours.

The HF diet also promoted development of pancreatic tumours, but in a less pronounced manner. Lorglumide largely inhibited the CCK effect on pancreatic tumour development, but did not influence the effect of the HF diet. In fact, the number and incidence of adenocarcinomas was highest in the HF + lorglumide group. The latter observation may indicate a possible unknown interaction between HF and lorglumide. However, the lack c statistical evidence for such an interaction and the slight inhibitory effect observed with lorglumide alone, suggest that the promoting effect of HF + lorglumide is attributable to HF. The number of rats bearing a microcarcinoma was significantly higher in animals treated with CCK than in the other groups. Pre-treatment with lorglumide completely inhibited this effect. Moreover, CCK, but not the HF diet, enhanced pancreatic weight. These results indicate that both CCK and a HF diet enhance pancreatic carcinogenesis in azaserine-treated rats. The mechanism by which these two promoters enhance pancreatic carcinogenesis, however, seems to be different. Lorglumide largely inhibited the enhanci effect of CCK, but not of dietary fat, indicating that it is unlikely that the promoting effe of dietary unsaturated fat on pancreatic carcinogenesis is mediated via CCK. The mechanism by which dietary (un)saturated fat promotes carcinogenesis is still largely unknown. Several mechanisms have been postulated by which a diet high in (un)saturate fat may be linked to a high risk for some cancers (45). A hypothetical explanation for dietary fat-promoted tumour growth involves the acceleration of linoleic acid-derived arachidonic acid metabolism resulting in enhanced production of eicosanoids such as prostaglandins, thromboxanes and leukotrienes (46). Investigations are currently in progress to find out whether prostaglandins play a role in dietary fat-promoted pancreatic carcinogenesis using both the azaserine-rat and the BOP-hamster model.

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Effects of dietary linoleic acid on pancreatic carcinogenesis in rats and hamsters[1]

Marko J. Appel, Annemarie van Garderen-Hoetmer and Ruud A. Woutersen[2]
Department of Pathology, Division of Toxicology, TNO Nutrition and Food Research Institute, P.O. Box 360, 3700 AJ Zeist (The Netherlands)

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Abstract

It has been suggested that linoleic acid (LA[3]) is responsible for the promoting effect of dietary polyunsaturated fat on pancreatic carcinogenesis via an accelerated prostaglandin (PG) synthesis, caused by metabolism of LA-derived arachidonic acid (AA) in (pre)neoplastic tissue. The purpose of the present study was to investigate whether dietary LA is the cause of pancreatic tumour promotion by a high fat diet. Five groups of 30 azaserine-treated rats and 5 groups of 30 N-nitrosobis(2-oxopropyl)amine (BOP)-treated hamsters were maintained for 6 months (rats) and 12 months (hamsters) on high fat (25 wt%) AIN[76] diets containing 2, 4, 6, 10 or 15 wt% LA. The results indicated that the strongest enhancing effect on the growth of pancreatic (pre)neoplastic lesions in rats and hamsters was obtained with 4 and 2 wt% of dietary LA, respectively. At higher LA levels the tumour response seemed to decrease rather than to increase. In both rats and hamsters the fatty acid profiles of blood plasma and pancreas showed an accurate reflection of the dietary fatty acid profiles: a proportional increase in LA levels was observed in plasma and pancreas with increasing dietary LA. In both species plasma and pancreatic AA levels remained constant, except for AA levels in rat plasma, which significantly increased with

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: LA, linoleic acid; AA, arachidonic acid; OA, oleic acid; PUFA, polyunsaturated fatty acids; BOP, N-nitrosobis(2-oxopropyl)amine; AACF, atypical acinar cell foci; PG, prostaglandin; TXB_2, thromboxane B_2.

increasing dietary LA levels. Fatty acid profiles in hamster pancreatic tumours did not differ from fatty acid profiles in non-tumorous pancreatic tissue from hamsters fed the same diet. PGE_2-, 6-keto-$PGF_{1\alpha}$-, $PGF_{2\alpha}$- and TXB_2-concentrations in non-tumorous pancreatic tissue were similar among the diet groups. Ductular adenocarcinomas from hamster pancreas showed significantly higher levels of 6-keto-$PGF_{1\alpha}$, $PGF_{2\alpha}$ and TXB_2, but not of PGE_2 in comparison with non-tumorous pancreas.
It is concluded that the strongest pancreatic tumour promotion by dietary LA is 4 wt% in rats and 2 wt% or less in hamsters, and that PGs may be involved in the development of ductular adenocarcinomas induced in hamster pancreas by BOP.

Introduction

Initiation of cancer is generally caused by a primary genetic event, followed by a series of genetic and/or epigenetic alterations that promote development from precancerous cells to malignant tumours.

It has been demonstrated that high levels of dietary polyunsaturated fatty acids (PUFA) promote tumour growth in several animal models (1-3), including pancreatic cancer models (4-6). Linoleic acid (LA) has been implicated to cause this effect (4,7,8). One hypothesized mechanism involves an accelerated prostaglandin synthesis, caused by metabolism of LA-derived arachidonic acid (AA), in (pre)neoplastic tissue (9-11). This theory is supported by a frequently seen tumour growth inhibition concurrent with biochemical inhibition of the prostaglandin-synthesizing enzyme cyclooxygenase by indomethacin (12) or by competitive inhibition by ω-3 fatty acids from fish oils (13-15).

To study the specific role of PUFA in dietary fat-promoted pancreatic carcinogenesis, experiments with azaserine-treated rats (model for acinar pancreatic cancer) and BOP-treated hamsters (model for ductular pancreatic cancer) were performed. Rats and hamsters were maintained on a high fat diet (25 wt%) with increasing LA concentrations, in order to find the dietary LA level with the strongest enhancing effect on pancreatic carcinogenesis and to evaluate the involvement of prostaglandins in this process by determination of the fatty acid composition of blood plasma and pancreas as well as the prostaglandin content of the pancreas.

Materials and methods

Animals. Fifty five one-week pregnant female Wistar rats were obtained from Harlan-CPB, Austerlitz, The Netherlands. During pregnancy the rats were kept solitary, in stainless steel cages fitted with wire-mesh floors and fronts and were fed a standard laboratory chow. Two weeks (± 1 day) after arrival the rats gave birth to a mean of eight pups. After 4 days the pups were sexed. All females, the surplus of male pups and 33 mothers were killed and a total of 175 male pups were divided among the remaining 22 mothers. At 14 and 21 days of age 150 pups were given an i.p. injection of 30 mg azaserine (Calbiochem-Behring Corp., La Jolla, CA) per kg body wt, which was dissolved freshly in 0.9% NaCl-solution. Twenty five control animals received injections with 0.9% NaCl solution only. Directly after the second injection the animals were weaned and randomly allocated to 5 groups of 35 animals each (five 0.9% NaCl-treated controls plus 30 azaserine-treated animals).

Table 1 Weight percentage composition of the AIN[76] diet and the percentage of fatty acid composition of the oils[a]

Dietary component		Fatty acid	Safflower oil	Sunflower oil
Casein	25.00	C14:0	0.1	0.1
DL-Methionine	0.38	C16:0	7.0	3.8
Wheat starch	35.72	C16:1	0.1	0.1
Cellulose	6.25	C18:0	2.6	4.0
Choline bitartrate	0.25	C18:1	13.1	82.6
AIN[76] minerals	4.38	C18:2	76.0	7.7
AIN[76] vitamins	1.25	C18:3	0.3	0.1
CaH_2PO_4	1.77	C20:0	0.3	0.3
Fat	25.00	C20:1	0.2	0.2
		C22:0	0.2	0.9
Total	100.00		99.9	99.9

[a]The diets were prepared freshly every 2 months. All diets were stored in sealed, nitrogen-flushed plastic bags at -20°C until use.

Hundred seventy five four-week old Syrian golden hamsters were obtained from Harlan-CPB, Austerlitz, The Netherlands. The hamsters were kept in macrolon cages, 5 animals per cage, on a softwood bedding and

under standard laboratory conditions. During the initiation phase the hamsters were fed a standard laboratory chow. At 5, 6 and 7 weeks of age 150 hamsters received a s.c. injection of 20 mg N-nitrosobis(2-oxopropyl)amine (BOP; Nacalai Tesque Inc., Kyoto, Japan) per kg body wt, which was dissolved freshly in 0.9% NaCl-solution. Twenty five control animals received injections with 0.9% NaCl-solution only. Directly after the third injection the animals were randomly allocated to 5 groups of 35 animals each (five 0.9% NaCl-treated controls plus 30 BOP-treated animals).

Diets. One week after the last injection the animals were maintained on an AIN[76] diet high in fat (25 wt%; Table 1). The fat was blended to contain an increasing percentage of linoleic acid by mixing high linoleic safflower oil (Unilever, Vlaardingen, The Netherlands) with high oleic sunflower oil (Trisun™; Contined, Bennekom, The Netherlands). The (calculated) dietary levels of linoleic acid over the five experimental groups were 2, 4, 6, 10 and 15 wt%. After reextraction of the oil mixtures, the prepared diets actually contained 2.0, 3.8, 5.9, 9.9, and 15.0 wt% of linoleic acid, respectively. The fatty acid compositions of the oils are summarized in Table 1. The diets were prepared freshly every two months and were stored in sealed, nitrogen flushed plastic bags at -20°C until use. Food and drinking water were available *ad libitum*.

Monitoring and autopsy. Body weights and food intake were recorded weekly during the first 3 months and monthly thereafter. The general condition and behaviour of the animals were checked daily. Two rats died during the study (at day 54 and 104, respectively). Their cause of death could not be established due to severe autolysis and cannibalism. Sixty seven hamsters (38%), involving all groups, did not reach the end of the study. Forty seven of these animals (20 found dead and 27 killed *in extremis*) were included in the results. Their effective stay in the study was at least 250 days. The 20 remaining animals were excluded from the results due to very early death (before day 171), autolysis or cannibalism.

Terminal autopsy was performed at day 188 and 189 (rats) and at day 371 and 372 (hamsters) after the last injection with carcinogen. The animals were anaesthetized with ether, exsanguinated by cannulating the abdominal aorta and examined for gross pathological changes. The blood was collected in heparin-containing tubes and centrifuged at 1700 g/4°C for 20 min. The plasma was kept at -80°C until analysis. The entire pancreas, liver, kidneys and all gross lesions were excised. The pancreas and liver (rats) or the pancreas only (hamsters) of each animal were weighed. All pancreata and all grossly observed abnormalities suspected of being tumour were fixed in 10% buffered formalin. About one-third of the pancreas of 2 animals per cage was snap-frozen in liquid nitrogen and stored at -80°C until fatty acid or prostaglandin analysis. The remaining two-thirds of these and all other pancreata were processed for microscopy by conventional methods, step-sectioned at 5 μm (about 6 sections per pancreas), stained with haematoxylin and eosin (H&E) and examined by light-microscopy.

A total area of 100-200 mm^2 of pancreatic tissue was examined per rat. Atypical acinar cell foci (AACF), acinar cell adenomas, localized carcinomas (carcinoma *in situ*, CIS) were identified and classified according to the criteria of Longnecker (16) and Rao *et al.* (17). The diameter of the nodule transections was determined by using an intra-ocular grid as described previously (18).

In hamster pancreas, borderline lesions, carcinomas *in situ* and ductular adenocarcinomas were classified according to the criteria of Meijers *et al.* (19) and Pour *et al.* (20)

Analytical procedures. Fatty acids. Pancreatic microsomes were prepared by homogenizing 100-200 mg pancreatic tissue in 0.1 M Tris-KCl buffer, pH 7.4. Subsequently, the homogenate was centrifuged at 10,000 g for 30 min and the supernatant was centrifuged at 105,000 g for 60 min. The microsomal pellet was resuspended in 300 μl buffer and stored at -30°C until fatty acid analysis. Total lipids were extracted from 50 μl aliquots of pancreatic microsomes or from 100 μl aliquots of blood plasma as described by Folch *et*

al. (21). Fatty acid composition was determined by GLC. The samples were eluted on a capillary BD23 column (J&W Scientific) after saponification with NaOH in methanol and transmethylation of the fatty acids with borontrifluoride-methanol.

Prostaglandins. Pancreatic tissue (100-200 mg) was homogenized in 0.1 M PBS (Ph 7.4) containing 15% methanol and applied to Sep-pak C-18 columns (J.T. Baker Inc, Phillipburg, NJ, USA). After washing with 6 ml 15% methanol/PBS and 6 ml petroleum ether, the samples were eluted with 6 ml methanol. After evaporation of the methanol under N_2, the samples were dissolved in 1.0 ml buffer and subsequently analyzed by using enzyme immunoassay kits for PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 (Cascade Biochem Ltd, Reading, UK).

Statistics. Food and energy intake and body and pancreatic weights were statistically evaluated by two-way analysis of variance followed by Dunnetts test, prostaglandin levels were evaluated by analysis of variance followed by Student's *t* test, the number of pancreatic lesions was evaluated by two sample *t* test or by one-way analysis of variance followed by linear trend tests with orthogonal contrasts. The number of tumour bearing animals (incidence) was analyzed by χ^2-test. Fatty acid compositions were evaluated by two-way analysis of variance using percentage of dietary linoleic acid and carcinogen-treatment as factors, and by one-way analysis of variance followed by linear trend tests with orthogonal contrasts.

Results

Food consumption and body and pancreatic weights

Mean food consumption was similar among the experimental groups (rats: 12.7-13.0 g/animal/day; hamsters: 5.1-6.0 g/animal/day). Mean body weight gain showed no significant differences among groups (Fig 1). Body and pancreatic weights at autopsy were similar among the diet groups and are summarized in Table 2.

Microscopy

Rats. Both acidophilic and basophilic AACF were identified in pancreatic tissue of azaserine-treated rats. Basophilic AACF were not scored because they were too scarce to justify evaluation. The microscopic data on acidophilic AACF, which are summarized in Table 3, show that the area and volume as % of pancreas occupied by AACF tissue is highest in the group fed 4% dietary LA. At higher levels of LA in the diet the area as % pancreas occupied by acidophilic nodule tissue had decreased rather than increased in comparison with the 4% LA diet group. Furthermore, rats maintained on the 4% LA-diet developed significantly ($P<0.05$) more acidophilic AACF with a diameter between 1 mm and 3 mm than rats in the diet groups containing more than 4% LA (Table 4). The number of carcinomas was also higher in the 4% LA-group in comparison with the other groups, although the difference did not reach the level of statistical significance. No significant

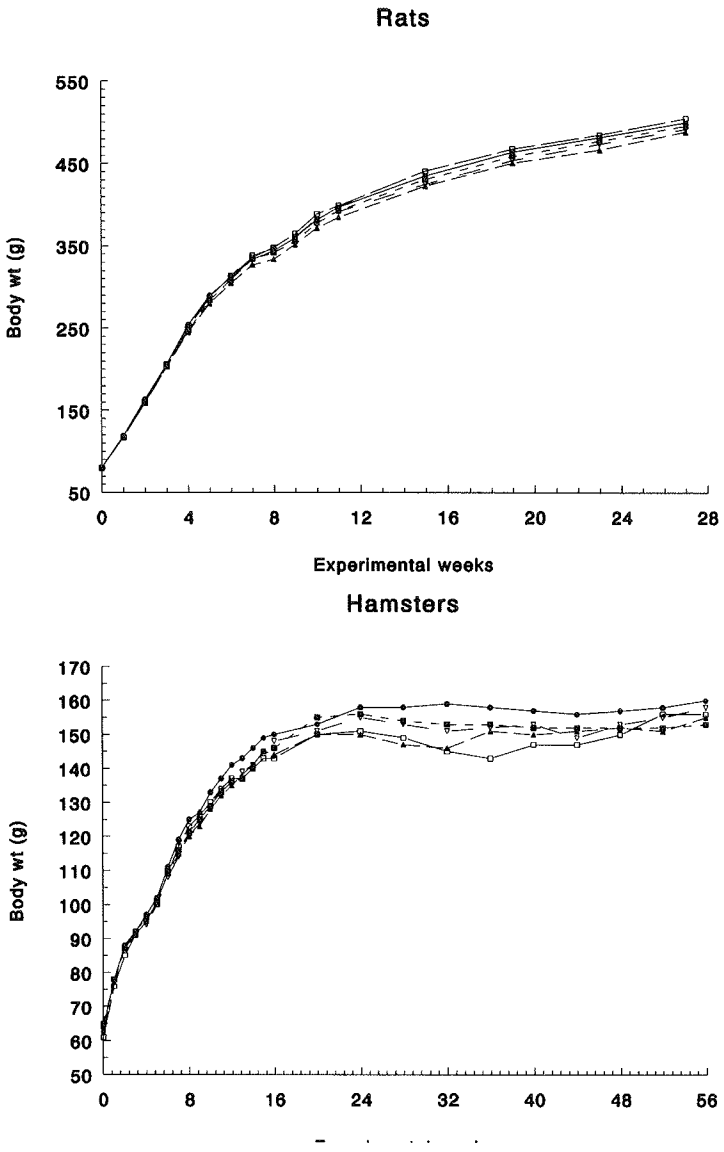

Figure 1. Body weight gain of azaserine-treated rats and BOP-treated hamsters maintained on a high fat die containing increasing levels of linoleic acid for 6 and 12 months, respectively. ●, 2% LA; △, 4% LA; ■, 6% LA; ▽, 10% LA; □, 15% LA.

Table 2 Body and pancreatic weights at autopsy[a]

	Rats				Hamsters			
	Body wt (g)		Pancreatic wt (g)		Body wt (g)		Pancreatic wt (g)	
Diet group	Controls	Aza[b]-treated	Controls	Aza-treated	Controls	BOP-treated	Controls	BOP-treated
2% LA	494±7 (5)	500±7 (30)	1.14±0.06 (5)	1.23±0.04 (30)	146±6 (4)	160±5 (20)	0.39±0.03 (4)	0.35±0.03 (17)
4% LA	483±6 (5)	489±8 (29)	1.01±0.08 (5)	1.26±0.04 (28)	153±5 (3)	155±3 (18)	0.32±0.08 (3)	0.45±0.02 (17)
6% LA	480±8 (5)	496±8 (30)	1.21±0.16 (5)	1.21±0.05 (30)	158±5 (5)	153±3 (22)	0.44±0.05 (5)	0.44±0.02 (22)
10% LA	482±9 (5)	492±6 (29)	1.07±0.05 (5)	1.20±0.04 (29)	168±4 (3)	158±4 (13)	0.40±0.06 (3)	0.37±0.02 (11)
15% LA	503±9 (5)	505±7 (30)	0.96±0.08 (5)	1.24±0.05 (30)	143±7 (5)	156±5 (15)	0.33±0.05 (5)	0.51±0.04 (14)

[a]Values are means ± SEM (N). Statistics: analysis of variance plus Dunnett's tests.
[b]Aza, azaserine.

linear trend could be observed in number of lesions in rat pancreas with increasing dietary LA.

Hamsters. The total number of ductular carcinomas in hamster pancreas was highest in the group fed 2% dietary LA and was significantly ($P<0.05$) higher as compared to the group maintained on 6% LA (Table 4). No significant linear trend could be observed in number of pancreatic lesions with increasing dietary LA. The incidence of pancreatic tumours (carcinoma *in situ* + adenocarcinomas) was also highest in the 2% LA group in comparison with the other groups, although no significant differences were observed when analyzed by χ^2-test.

Fatty acid composition of blood plasma and pancreatic microsomes

Untreated *versus* carcinogen-treated animals. Two-way analysis of variance revealed significant differences between control animals and carcinogen-treated animals for most fatty acids in both plasma and pancreas (Tables 5 to 9). Azaserine-treated rats exhibited a higher level of C18:2 along with lower levels of C18:3 (ω-6) and C20:4 in plasma in comparison with controls. Remarkably, in hamsters the opposite was observed: BOP-treated hamsters demonstrated a lower level of C18:2 along with higher levels of C18:3 (ω-6) and C20:4 in both plasma and pancreas, in comparison with controls. Azaserine-treatment caused almost no differences in fatty acid profiles of rat pancreas.

Effects of dietary LA on fatty acid composition of plasma and pancreas. The complete fatty acid profiles of plasma and pancreas of rats and hamsters are given in Tables 5, 6, 7 and 8, respectively.

In plasma as well as pancreatic microsomes of both species LA significantly increased ($P<0.001$) with increasing LA concentration in the feed. This dose related increase of LA was accompanied by a significant decrease ($P<0.001$) in oleic acid (OA). AA in rat plasma increased significantly ($P<0.001$) with increasing dietary LA, whereas AA levels in pancreatic tissue of rats as well as in plasma and pancreatic tissue of hamsters remained constant. C16:1trans decreased significantly ($p<0.01$) in plasma and pancreas of both species. C18:3 (ω-6) increased significantly ($P<0.001$) in plasma, but remained unchanged in pancreas of both rats and hamsters.

The effects of dietary LA on five of the most relevant fatty acid profiles are depicted in Figures 2 and 3. Parts of all grossly visible hamster tumours were analyzed for fatty acid profiles and prostaglandins. Microscopic examination of the grossly visible pancreatic lesions indicated that the number of adenocarcinomas in the 2% LA group was high enough to justify statistical analysis. It appeared that the fatty acid profile in tumour tissue was similar to that in normal tissue. Marginally significant increases were found in

Table 3 Effects of increasing levels of linoleic acid on development of acidophilic atypical acinar cell foci induced in rat pancreas by azaserine[a].

Diet group	Observed transection data of foci		Calculated volumetric data of foci								
			no./cm^3 with mean diameter (μm)								
	Total no./cm^2	Transection area (mm^2)	272.5	385	545	770	1090	Total no./cm^3	Mean diameter (μm)	Area as % of pancreas	Vol. as % of pancreas
2% LA	34.7	0.148	507	381	222[b]	46	11	1167	386	5.61	1.72
4% LA	34.0	0.163	454	410	163[b,c]	57	22	1106	389	6.28	1.91
6% LA	28.2	0.132	550	372	118[c]	23	10	1073	359	4.04	1.25
10% LA	30.1	0.152	458	361	137[c]	52	16	1024	379	5.28	1.61
15% LA	28.5	0.147	455	379	122[c]	35	15	1006	371	4.64	1.43

[a]Values are means; standard errors are taken into account in the statistical calculations, but are omitted from the table for reasons of clarity. Data are based on haematoxylin- and eosin-stained paraffin sections. The untreated controls showed no histological abnormalities in the pancreas based on H&E-stained paraffin sections. Statistics: analysis of variance followed by Student's *t* tests (2-tailed); [b,c]Values with different superscripts are significantly different ($P<0.05$).

Table 4 Number of pancreatic lesions in azaserine-treated rats and BOP-treated hamsters maintained on a high fat diet containing increasing levels of linoleic acid for 6 or 12 months, respectively.

	No. of lesions					
(Pre)neoplastic lesions observed	2% LA	4% LA	6% LA	10% LA	15% LA	*P*
Rats						
No. of animals	30	29	30	29	30	
No. of tumour-bearing animals (%)[a]	4 (13)	6 (21)	1 (3)	4 (14)	3 (10)	
AACF (1<Ø<3 mm)	19[b,c]	30[c]	9[b]	12[b]	12[b]	0.112
Adenoma (Ø>3 mm)	2	1	3	1	0	0.347
Carcinoma *in situ*	3	7	0	6	3	0.678
Acinar adenocarcinoma	1	0	1	0	0	0.615
Hamsters						
No. of animals	28	24	27	22	26	
No. of tumour-bearing animals (%)	12 (43)	5 (20)	5 (19)	8 (36)	6 (23)	
Borderline lesion	17	7	21	12	19	0.361
Carcinoma *in situ*	3	2	2	5	2	0.692
Ductular adenocarcinoma	11	4	3	4	6	0.321
Total no. of carcinomas (av.)	14 (0.50)[b]	6 (0.25)[b,c]	5 (0.19)[c]	9 (0.41)[b,c]	8 (0.31)[b,c]	0.528

[a]Tumour-bearing animal, animal that bears one or more carcinoma (*in situ*) in the pancreas. Statistics: 1-way analysis of variance plus linear trend tests (orthogonal contrasts); χ^2-test (no. of tumour-bearing animals) or 2-sample *t* test (no. of lesions); [b,c]groups with different superscripts are significantly different ($P<0.05$).

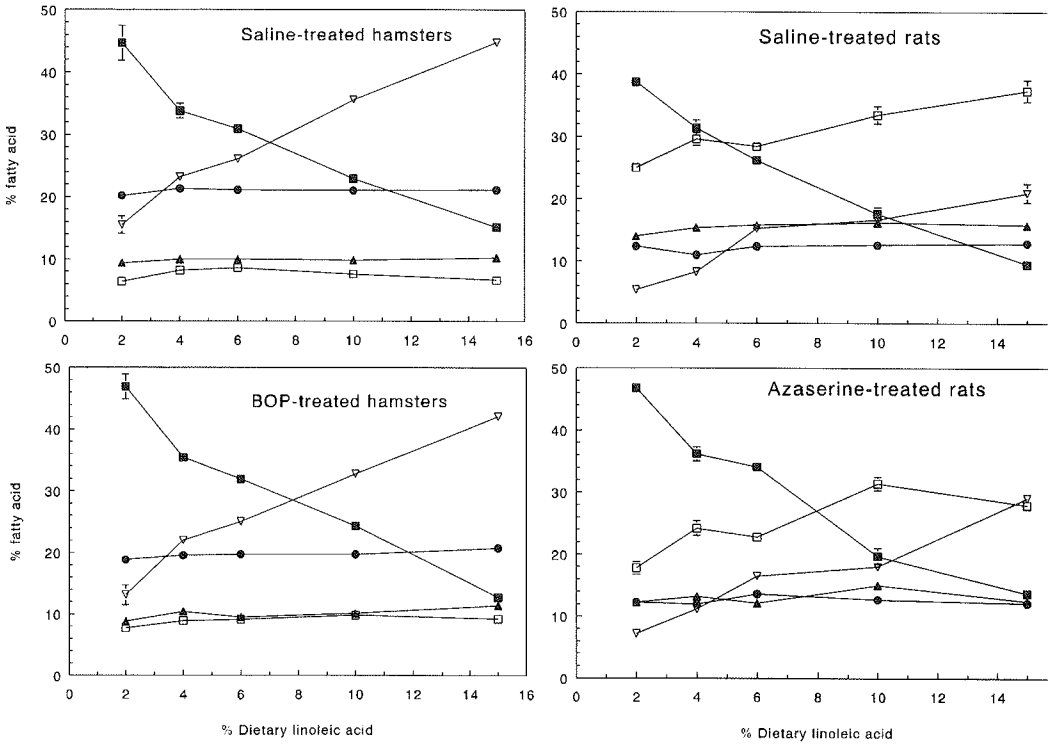

Figure 2. Fatty acid profiles in plasma of rats and hamsters as a function of dietary linoleic acid. ●, C16:0; ○, C18:0; ■, C18:1; ▽, C18:2; □, C20:4. Values are means ± SEM (N=3).

C16:1trans and C18:0 levels ($P<0.05$; data not shown).

Prostaglandins. In rats and hamsters, PGE_2-, 6-keto-$PGF_{1\alpha}$-, $PGF_{2\alpha}$- and TXB_2-levels in pancreatic tissue of carcinogen-treated animals were not different among the diet groups (Figures 4 and 5). However, in BOP-treated hamsters, 6-keto-$PGF_{1\alpha}$-, $PGF_{2\alpha}$- and TXB_2-levels, but not PGE_2-levels in pancreatic tumours were significantly elevated ($P<0.001$, $P<0.05$ and $P<0.05$, respectively) in comparison with non-tumorous pancreas of BOP-treated hamsters (Fig 5).

Discussion

The main purpose of the present study was to find out whether increasing levels of

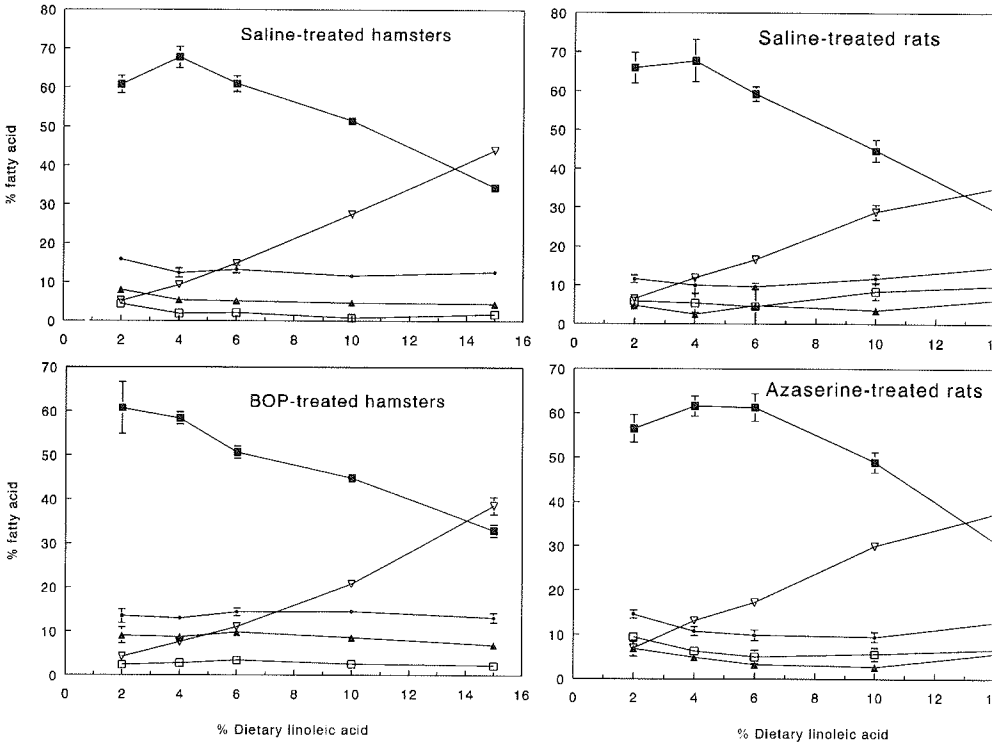

Figure 3. Fatty acid profiles in pancreas of rats and hamsters as a function of dietary linoleic acid. ●, C16:0; ○, C18:0; ■, C18:1; ▽, C18:2; □, C20:4. Values are means ± SEM (N=3).

essential polyunsaturated fatty acid (in fact LA) proportionally enhance the development of putative (pre)neoplastic acinar lesions in the pancreas of azaserine-treated rats and ductular (pre)neoplastic lesions in the pancreas of BOP-treated hamsters. Since dietary fat has a strong promoting effect on pancreatic carcinogenesis, the amount of fat in the diets of the various groups was kept at a constant high (25 wt%) level. To obtain an increasing concentration of LA in the high fat diet without influencing the chain length of the fatty acids, high linoleic safflower oil (ca. 75% LA) was blended with high oleic Trisun sunflower oil (ca. 80% OA). The results of the present studies demonstrate that 2 and 4 wt% LA in a high fat diet of hamsters and rats, respectively, give the strongest tumour response. Higher concentrations of LA resulted in a decrease rather than an increase in tumour response, indicating that above a certain threshold a dose-effect relationship between dietary LA and tumour response does not exist as far as the growth of pancreatic

Table 5 Fatty acid composition of plasma of untreated and azaserine-treated rats maintained on a high fat diet containing increasing levels of linoleic acid for 6 months[a]

	Dietary linoleic acid										
	2%		4%		6%		10%		15%		
Fatty acid	Saline treated	Azaserine treated	Saline treated	Azaserine treated	Saline treated	Azaserine treated	Saline treated	Azaserinet reated	Saline treated	Azaserine treated	*P*
C14:0	0.2±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	NS[b]
C16:0	12.4±0.4	12.3±0.6	11.9±0.0	12.0±0.2	12.3±0.3	11.6±0.1	12.6±0.3	12.7±0.4	12.9±0.1	13.6±0.3	NS
C16:1*trans*	1.0±0.1	0.9±0.1	0.8±0.0	0.7±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.1	0.2±0.0	0.4±0.1	<0.001
C16:1*cis*	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	NS
C18:0	14.0±0.5	12.3±0.6	15.4±0.5	13.2±0.3	15.8±0.4	12.1±0.0	16.2±0.5	15.0±0.7	15.8±0.3	12.4±0.1	NS
C18:1	38.8±0.9	46.8±0.4	31.4±1.2	36.2±1.1	26.2±0.6	34.1±0.3	17.6±1.0	19.7±1.3	9.5±0.6	13.7±0.3	<0.001
C18:2	5.4±0.3	7.2±0.3	8.3±0.2	11.2±0.5	15.3±0.3	16.5±0.3	16.7±0.5	18.0±0.4	21.0±1.5	29.1±0.9	<0.001
C18:3*cis*6,9,12	0.3±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.7±0.0	0.6±0.0	0.7±0.0	0.7±0.0	<0.001
C20:0	0.1±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.2±0.0	NS
C20:1	0.4±0.0	0.4±0.0	0.3±0.0	0.4±0.0	0.4±0.1	0.3±0.1	0.5±0.3	0.3±0.3	0.2±0.0	0.2±0.0	NS
C20:2	1.0±0.0	0.7±0.1	0.6±0.0	0.5±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.5±0.0	0.5±0.0	<0.01
C20:3	0.4±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.6±0.0	0.5±0.1	0.5±0.0	0.5±0.1	<0.01
C20:4	25.0±0.7	17.8±1.0	29.6±1.0	24.3±1.1	28.4±0.7	22.8±0.2	33.5±1.4	31.4±1.4	37.4±1.7	27.9±0.2	<0.001
C22:6	0.6±0.0	0.6±0.2	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.0	NS

[a]Values are mean ± SEM (N=3); Statistics: 1-way analysis of variance plus linear trend tests (orthogonal contrasts); [b]NS, not significant.

Table 6 Fatty acid composition of pancreatic microsomes of untreated and azaserine-treated rats maintained on a high fat diet containing increasing levels of linoleic acid for 6 months[a]

	Dietary linoleic acid										
	2%		4%		6%		10%		15%		
Fatty acid	Saline treated	Azaserine treated	Saline treated	Azaserine treated	Saline treated	Azaserine treated	Saline treated	Azaserine treated	Saline treated	Azaserine treated	P
C12:0	1.7±0.4	1.7±0.7	0.5±0.0	1.0±0.1	2.3±0.4	0.6±0.1	1.0±0.0	0.7±0.0	3.4±0.9	1.7±0.8	NS[b]
C14:0	0.5±0.0	0.8±0.1	0.6±0.1	0.0±0.0	0.0±0.0	0.6±0.1	1.0±0.1	0.8±0.0	0.8±0.0	0.5±0.2	NS
C16:0	11.6±1.0	14.6±0.9	10.0±2.0	10.8±1.0	9.7±0.8	9.9±1.2	11.8±1.1	9.5±1.1	15.4±0.5	13.6±2.3	NS
C16:1*trans*	1.4±0.1	0.9±0.1	0.9±0.1	0.9±0.1	0.9±0.1	0.8±0.0	0.7±0.0	0.6±0.0	0.6±0.1	0.7±0.1	<0.01
C16:1*cis*	0.3±0.1	0.3±0.0	0.4±0.1	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0	NS
C18:0	4.8±1.2	6.8±1.7	2.5±0.6	4.9±0.7	4.8±1.1	3.2±0.6	3.5±0.4	2.7±0.4	6.9±0.9	6.4±1.7	NS
C18:1	65.9±3.9	56.1±3.1	67.7±5.7	61.6±2.3	59.2±1.9	61.3±3.1	44.7±2.8	49.0±2.3	25.5±0.7	26.7±2.1	<0.001
C18:2	6.6±0.3	7.0±0.5	11.9±0.3	13.2±0.9	16.6±0.5	17.3±0.3	28.9±1.9	30.0±0.8	36.6±1.0	39.1±4.5	<0.001
C18:3*cis*6,9,12	1.1±0.2	1.2±0.5	0.2±0.0	0.6±0.1	1.1±0.4	0.3±0.1	0.4±0.0	0.3±0.0	1.6±0.5	1.2±0.5	NS
C18:3*cis*9,12,15	0.2±0.0	0.4±0.0	0.1±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.3±0.0	0.2±0.0	NS
C20:0	0.4±0.0	0.5±0.1	0.1± 0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.4±0.1	0.3±0.1	NS
C20:1	0.3±0.0	0.6±0.2	0.3±0.0	0.4±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	<0.05
C20:2	0.3±0.1	0.7±0.0	0.2±0.1	0.3±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.5±0.1	0.5±0.1	NS
C20:3	0.2±0.0	0.3±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.3±0.0	0.2±0.0	NS
C20:4	5.9±1.5	9.5±1.1	5.4±2.4	6.3±0.9	4.6±1.0	5.0±1.5	8.4±2.1	5.6±1.5	10.1±0.5	6.8±1.8	NS
C22:6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	

[a]Values are mean ± SEM (N=3); Statistics: 1-way analysis of variance plus linear trend tests (orthogonal contrasts); [b]NS, not significant; ND, not detected.

Table 7 Fatty acid composition of plasma of untreated and BOP-treated hamsters maintained on a high fat diet containing increasing levels of linoleic acid for 12 months[a]

	Dietary linoleic acid										
	2%		4%		6%		10%		15%		
Fatty acid	Saline treated	BOP treated	Saline treated	BOP treated	Saline treated	BOP treated	Saline treated	BOP treated	Saline treated	BOP treated	P
C12:0	0.2±0.0	0.2±0.0	0.2±0.0	0.0±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.0±0.0	0.2±0.0	0.2±0.0	NS[b]
C14:0	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.3±0.0	0.2±0.0	NS
C16:0	20.2±0.6	18.8±0.3	21.3±0.4	19.5±0.6	21.1±0.4	19.7±0.1	21.0±0.4	19.7±0.4	20.0±0.9	20.7±0.3	NS
C16:1*trans*	0.4±0.0	0.7±0.0	0.4±0.0	0.6±0.0	0.5±0.0	0.7±0.0	0.3±0.1	0.6±0.0	0.2±0.0	0.3±0.0	<0.001
C16:1*cis*	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.2±0.0	NS
C18:0	9.4±0.3	8.8±0.2	10.0±0.3	10.4±0.3	10.0±0.2	9.5±0.3	9.8±0.3	10.1±0.3	10.2±0.4	11.4±0.3	NS
C18:1	44.7±2.8	46.9±2.0	33.8±1.2	35.4±0.6	30.9±0.8	31.9±0.8	22.9±0.6	24.3±0.8	15.1±0.1	12.7±0.2	<0.001
C18:2	15.5±1.4	13.1±1.6	23.2±0.7	22.0±0.1	26.1±0.5	25.8±0.7	35.6±0.9	32.8±0.5	44.8±0.7	41.1±0.2	<0.001
C18:3*cis*6,9,12	0.4±0.0	0.6±0.0	0.6±0.0	0.7±0.1	0.6±0.1	1.1±0.0	0.9±0.1	1.1±0.1	0.9±0.0	1.4±0.1	<0.001
C20:0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0	NS
C20:1	0.3±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	NS
C20:2	0.6±0.1	1.3±0.1	0.6±0.0	0.5±0.0	0.5±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.4±0.0	0.3±0.0	<0.001
C20:3	0.8±0.0	0.6±0.1	0.4±0.0	0.6±0.0	0.3±0.0	0.6±0.1	0.2±0.0	0.4±0.1	0.3±0.0	0.4±0.0	<0.01
C20:4	6.4±0.5	7.7±0.4	8.2±0.4	8.9±0.2	8.6±0.2	9.1±0.6	7.6±0.5	9.8±0.5	6.6±0.2	9.2±0.2	NS
C22:6	0.4±0.0	0.5±0.1	0.4±0.0	0.5±0.0	0.4±0.0	0.4±0.0	0.3±0.0	0.4±0.1	0.3±0.0	0.4±0.0	NS

[a]Values are mean ± SEM (N=3); Statistics: 1-way analysis of variance plus linear trend tests (orthogonal contrasts); [b]NS, not significant

Table 8 Fatty acid composition of pancreatic microsomes of untreated and BOP-treated hamsters maintained on a high fat diet containing increasing levels of linoleic acid for 12 months[a]

	Dietary linoleic acid										
	2%		4%		6%		10%		15%		
Fatty acid	Saline treated	BOP treated	Saline treated	BOP treated	Saline treated	BOP treated	Saline treated	BOP treated	Saline treated	BOP treated	P
C12:0	0.0±0.0	4.3±1.2	0.5±0.1	3.8±0.5	0.5±0.1	3.8±0.6	0.8±0.3	3.5±0.9	0.4±0.1	2.2±0.4	NS[b]
C14:0	1.8±0.3	2.3±0.6	0.6±0.1	2.3±0.5	0.7±0.1	2.4±0.3	1.0±0.1	2.0±0.2	0.5±0.1	1.2±0.1	NS
C16:0	15.9±0.6	13.5±1.6	12.4±1.2	13.0±0.6	13.3±1.0	14.4±0.9	11.6±0.5	14.5±0.5	12.6±0.7	13.1±0.1	NS
C16:1*trans*	0.6±0.1	0.8±0.0	0.5±0.0	0.8±0.0	0.5±0.0	0.7±0.1	0.5±0.0	0.6±0.1	0.3±0.0	0.5±0.1	<0.01
C16:1*cis*	0.4±0.0	0.6±0.0	0.3±0.0	0.5±0.0	0.4±0.0	0.5±0.1	0.4±0.0	0.4±0.0	0.3±0.0	0.5±0.1	NS
C18:0	8.0±0.7	9.1±1.8	5.5±0.6	8.7±0.5	5.2±0.5	9.8±0.5	4.7±0.3	8.6±0.3	4.4±0.4	6.9±0.8	NS
C18:1	60.8±2.3	60.7±5.9	67.8±2.7	58.4±1.6	61.0±2.1	50.7±1.4	51.4±0.6	44.9±0.6	34.4±0.7	32.9±1.4	<0.001
C18:2	5.2±0.2	4.2±0.1	9.3±0.1	7.6±0.3	14.8±0.2	11.0±0.4	27.5±0.6	20.8±0.5	44.0±0.9	38.6±1.9	<0.001
C18:3*cis*6,9,12	0.8±0.1	1.2±0.3	0.3±0.0	1.1±0.1	0.7±0.2	1.3±0.2	0.7±0.2	1.2±0.1	0.5±0.1	0.9±0.2	NS
C18:3*cis*9,12,15	ND	0.4±0.1	0.5±0.4	0.3±0.0	0.2±0.1	0.9±0.5	0.2±0.1	0.3±0.0	0.1±0.0	0.3±0.1	NS
C20:0	0.8±0.1	0.7±0.2	0.3±0.0	0.6±0.1	0.4±0.0	0.7±0.1	0.4±0.1	0.7±0.1	0.3±0.0	0.5±0.1	NS
C20:1	0.3±0.0	0.4±0.1	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0	<0.05
C20:2	0.2±0.1	0.2±0.0	0.2±0.1	ND	0.2±0.0	0.2±0.0	0.1±0.0	ND	0.2±0.0	0.3±0.0	NS
C20:4	4.4±0.5	2.4±0.6	1.9±0.8	2.8±0.3	2.1±0.8	3.4±0.5	0.8±0.1	2.6±0.2	1.8±0.5	2.2±0.7	NS
C22:6	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	

[a]Values are mean ± SEM (N=3); Statistics: 1-way analysis of variance plus linear trend tests (orthogonal contrasts); [b]NS, not significant; ND, not detected.

Table 9 Significance (P) of the differences in fatty acid composition in plasma and pancreas of rats (Tables 5 and 6) and hamsters (Tables 7 and 8) evaluated by 2-way analysis of variance comparing controls with carcinogen-treated animals

	Rats		Hamsters	
Fatty acid	Plasma	Pancreas	Plasma	Pancreas
C14:0	<0.001	<0.05	<0.01	<0.001
C16:0	NS[a]	NS	<0.05	NS
C16:1*trans*	NS	NS	<0.05	NS
C16:1*cis*	NS	NS	NS	<0.001
C18:0	<0.001	NS	NS	<0.01
C18:1	<0.001	NS	NS	NS
C18:2	<0.001	NS	<0.05	<0.001
C18:3*cis*6,9,12	<0.01	NS	<0.001	<0.001
C20:0	<0.05	NS	NS	<0.01
C20:1	NS	<0.05	NS	<0.05
C20:2	<0.05	NS	NS	NS
C20:3	NS	NS	NS	NS
C20:4	<0.001	NS	<0.01	NS
C22:6	NS		<0.05	

[a]NS, not significant.

AACF (rat) and pancreatic ductular lesions (hamster) is concerned. The present results are in contrast to those of Roebuck *et al.* (22), who found that in azaserine-treated rats the acidophilic AACF increased in number and size as the LA content in the diet increased. This promoting effect of LA was particularly apparent from 4.4% to 8.5% dietary LA. At higher or lower LA concentrations the tumour response did not show a clear dose-effect relationship, indicating that 4 up to 8 wt% LA is required for optimal enhancement of azaserine-induced pancreatic carcinogenesis by a high fat diet.

The difference between the present results and those obtained by Roebuck *et al.* (22) are most probably caused by differences in the composition of the diets used. Roebuck *et al.* (22) used diets compounded by blending corn oil with a high percentage of LA with coconut oil containing mainly short chain saturated fatty acids (C10:0, C12:0, C14:0 and C16:0) and only 10% C18:0, C18:1 and C18:2. In the present study LA (C18:2) was exchanged for OA (C18:1). OA forms a large part of many natural dietary lipid sources and in contrast to LA and AA cannot be converted by mammals to ω-6 fatty acids which are known to stimulate tumour growth. Short chain saturated fatty acids are differently absorbed and metabolized than long chain unsaturated fatty acids, leading to a difference in the fatty acid composition of the membranes of the pancreatic cell and hence a

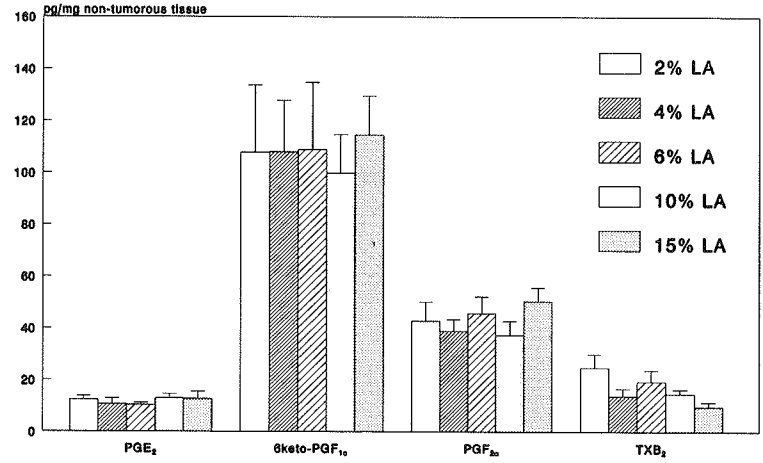

Figure 4. Prostaglandin levels in azaserine-treated rats maintained on a high fat diet containing increasing levels of linoleic acid for 6 months.

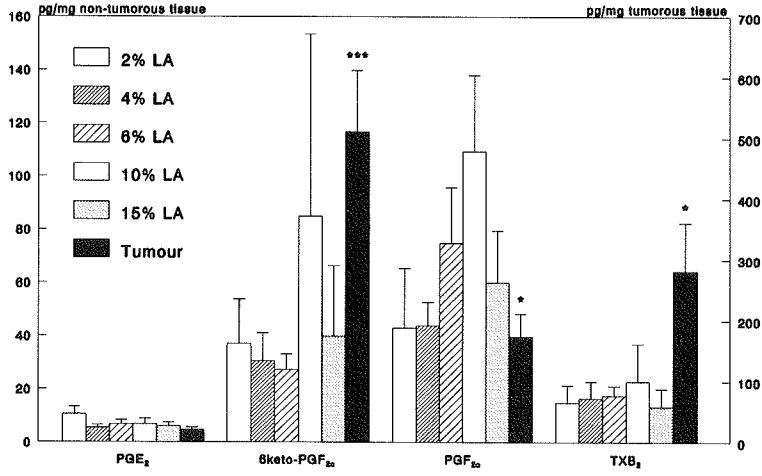

Figure 5. Prostaglandin levels in pancreatic tumours and non-tumorous pancreas of BOP-treated hamsters maintained on a high fat diet containing increasing levels of linoleic acid for 12 months.

difference in the modulating effects on carcinogenesis. It is not illogical to assume that the difference observed between our results and those of Roebuck *et al.* (22) are caused by this phenomenon, since our data clearly demonstrate that feeding increasing levels of LA to rats or hamsters altered the composition of fatty acids in the plasma and the incorporation of fatty acids in the microsomes of normal pancreatic cells as well as of pancreatic tumour cells. Apart from changes in membrane structure and function, metabolic processes that take place in the cell membranes and in the cytoplasm of the cell, utilizing liberated membrane constituents may very well be influenced by alteration of the fatty acid profile of the cell membrane. It is hypothesized that the promoting effect of a high fat diet on carcinogenesis is the result of an accelerated formation of AA and subsequently of PGs (10). Our results, however, indicate that AA is not formed in direct proportion to tissue LA which may point to elongation and desaturation of fatty acids as a strictly regulated mechanism. Furthermore, the present results are indicative of an impaired LA metabolism in plasma due to carcinogen treatment: an inhibited LA conversion to C18:3 (ω-6) and C20:4 in rats and an accelerated LA metabolism in hamsters. This alteration may be caused by the presence of (pre)neoplastic lesions in the tissue of carcinogen-treated animals, but no direct evidence is available for this assumption. However, the observation points to a contrasting species difference.

The overall high relative concentration of AA in rat plasma may be the result of a basically higher elongation and desaturation potency of rat liver in comparison with hamster liver. The significant dose-response relationship observed between the amount of LA in the diet and the AA concentration in the plasma of rats but not of hamsters may also be ascribed to a difference in hepatic metabolic activity between the two species.

Interestingly, the diet groups showing a relatively high incidence of (pre)neoplastic lesions in pancreas of both rats and hamsters contained a high percentage of OA. Recently, Khoo *et al.* (23) studied the effect of stearic and oleic acid on pancreatic fatty acids and the development of AACF in azaserine-treated rats and demonstrated an enhancing effect of oleic acid on pancreatic carcinogenesis. However, foci were absent in the group treated with azaserine alone and in the other groups the number of foci per pancreas observed was unusually low in comparison with the numbers of foci observed by other workers in this field (24,25). Since OA cannot be metabolized to LA or other long chain PUFA and PGs, the findings by Khoo *et al.* (23) are hard to explain mechanistically. However, the presently observed absence of a concentration-related effect of LA on pancreatic carcinogenesis might be due to the presence of a high concentration of OA in the diets containing low concentrations of LA. Moreover, saturated fats such as lard and beef tallow contain 40 - 50% OA which may be responsible for the promoting effects of lard on pancreatic carcinogenesis in rats (26) and of beef tallow on pancreatic

carcinogenesis in hamsters (27). The findings of Khoo *et al.* (23) and those presented in this paper warrant further elucidation of the role of OA in the process of carcinogenesis.

Notwithstanding the possible promoting effects of OA on pancreatic carcinogenesis it is beyond doubt that a diet high in unsaturated fat and containing at least 2-4 wt% of LA has stronger promoting effects on pancreatic carcinogenesis than a diet high in saturated fat containing a high concentration of OA. Since LA and not OA can give rise to AA and the biologically active PGs, the role of these eicosanoids in carcinogenesis needs further study.

The ω-6 PUFA, particularly LA may give rise to PGs of the 2-series via AA. It has been demonstrated that some of these PGs may stimulate cell proliferation ($PGF_{2\alpha}$; ref. 2 or have immunosuppressive properties (PGE_2; ref. 29) and hence may either promote tumour growth or disturb inhibition of tumour development. Quantitative determination o PG-levels in pancreatic tissue of rats and hamsters did not show any difference among th various diet groups, whereas PG levels in the ductular hamster tumours observed in the present study were significantly elevated in comparison with non-tumorous tissue, despite unchanged tissue LA and AA levels.

This observation suggests that PGs play a role in development of pancreatic tumours but more research is needed to establish whether PGs are needed for the development of putative preneoplastic lesions to tumours and, moreover, to elucidate whether ω-3 fatty acids have the potency to influence this process. Studies with combinations of LA and MaxEPA are currently running in our Institute in order to find answers to these questions

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Modulation of growth and cell turnover of preneoplastic lesions and of prostaglandin levels in rat pancreas by dietary fish oil[1]

M.J. Appel and R.A. Woutersen[2]
Department of Pathology, Division of Toxicology, TNO Nutrition and Food Research Institute, P.O. Box 360, 3700 AJ, Zeist (The Netherlands)

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Abstract

In the present study the modulating effects of dietary fish oil (MaxEPA) on unsaturated fat-promoted pancreatic carcinogenesis in azaserine-treated rats were investigated. Three groups of 20 rats (each group comprised 5 saline-treated and 15 azaserine-treated animals) were fed an AIN[76]- based purified diet containing (i) 5 wt% fat, (ii) 25 wt% fat including 5 wt% linoleic acid or (iii) 25 wt% fat including 5 wt% linoleic acid and 9.4 wt% (20 cal%) MaxEPA for 6 months.

The number and size of pancreatic atypical acinar cell foci was significantly higher ($P<0.01$) in azaserine-treated animals maintained on a high fat diet than in those fed a low fat diet. MaxEPA did not influence the promoting effect of the high fat diet. The Labeling Index of atypical acinar cell foci in animals maintained on both a low fat or a high fat/MaxEPA diet was significantly ($P<0.01$) lower than that in rats fed a high fat diet without MaxEPA. The linoleic acid concentration was higher whereas the arachidonic acid concentration was lower in blood plasma and to a lesser extent also in pancreas of animals given MaxEPA in comparison with the other groups. Furthermore, animals fed MaxEPA showed lower 6-keto-$PGF_{1\alpha}$-, $PGF_{2\alpha}$-, and TXB_2-levels , but not PGE_2-levels in pancreatic tissue in comparison with the other groups.

It is concluded that a high fat diet containing 5 wt% linoleic acid has a strong promoting effect on pancreatic carcinogenesis in azaserine-treated rats. Dietary MaxEPA

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[2] To whom requests for reprints should be addressed.

did not influence the promoting effect of unsaturated fat on pancreatic carcinogenesis, although it caused a decrease in both cell proliferation in atypical acinar cell foci and prostaglandin levels in the pancreas.

Introduction

Epidemiological indications of the involvement of dietary fat in the etiology of variou human cancers (1-3) have been further substantiated with data from animal studies. In experimental mammary, colon and pancreatic cancer models dietary fat, in particular ω-6 polyunsaturated fat (PUFA[1]), has been shown to promote carcinogenesis in the post-initiation phase (4-7). ω-6 PUFA (mainly linoleic acid; LA; C18:2) may give rise to prostaglandins (PGs) of the 2-series via arachidonic acid (AA). Some of these PGs stimulate cell proliferation *in vitro* ($PGF_{2\alpha}$) or have immunosuppressive properties (PGE_2) hence may promote tumour growth or prevent inhibition of tumour growth, respectively (8,9).

Inhibitors of PG-producing cyclooxygenase, like indomethacin or carprofen have the ability to inhibit carcinogenesis. Carter *et al.* (10) found that 0.004% indomethacin in the diet blocked the promoting effect of a high fat diet on the development of mammary tumours induced in rats by DMBA. Furthermore, they reported inhibition of mammary carcinogenesis by dietary indomethacin in rats fed high fat diets containing 4% or 12% LA, but not in rats fed a diet containing 0.5% LA (11). In experimental pancreatic carcinogenesis, Takahashi *et al.* (12) found that the cyclooxygenase inhibitor phenylbutazone caused a decrease in incidence and multiplicity, while indomethacin caused a decrease in multiplicity of pancreas tumours induced in hamsters by N-nitrosobis(2-oxopropyl)amine. It is suggested that this anti-carcinogenic effect of inhibitor of cyclooxygenase may be ascribed to inhibition of PG formation. This mechanism may also play a role in the inhibitory effects of ω-3 PUFAs from fish oil on carcinogenesis. Both ω-3 and ω-6 PUFAs are substrates for cyclooxygenase, leading to 3-series and 2-series PGs, respectively. ω-3 PUFAs from fish oil (eicosapentaenoic acid: EPA; docosahexaenoic acid: DHA) have been shown to inhibit tumour growth in several experimental animal models (13). Competitive inhibition of formation of the 2-series PGs by ω-3 PUFAs may be responsible for the anti-tumour effects observed with dietary fish

[1] Abbreviations: PUFA, polyunsaturated fatty acid; LA, linoleic acid; PG, prostaglandin; AA, arachidonic acid; DMBA, 7,12-dimethylbenz(a)anthracene; EPA, eicosapentaenoic acid; DHA, docosahexaenoic aci HF, high fat; LF, low fat; AACF, atypical acinar cell focus; AACN, atypical acinar cell nodule; H&E, haematoxylin and eosin; CIS, carcinoma *in situ*; LI, labeling index.

oil.

The present study with azaserine-treated rats was performed to investigate whether pancreatic cancer promotion by a high fat (HF) diet containing a fixed level of LA (14) can be inhibited by fish oil (MaxEPA), using number and size of atypical acinar cell foci (AACF) as parameter. Furthermore, the effects of dietary fish oil on cell-turnover in the exocrine pancreas and on the fatty acid profile and PG-levels of pancreatic tissue were studied.

Materials and methods

Animals and diets

Sixty male SPF Wistar rats were obtained from Harlan-CPB, Austerlitz, The Netherlands. Forty five animals were given an i.p. injection of 30 mg azaserine (Calbiochem-Behring Corp., La Jolla, CA) per kg body wt, which was dissolved freshly in 0.9% NaCl-solution, at 14 and 21 days of age. Fifteen animals received injections with 0.9% NaCl-solution alone. Directly after the second injection the animals were weaned and randomly allocated to 3 groups of 20 animals each (5 saline-treated and 15 azaserine-treated animals). The animals were kept in stainless steel cages, with wire-mesh floors and fronts, 5 animals per cage and under standard laboratory conditions. One week after carcinogen treatment the rats were fed an AIN^{76}- based purified diet containing either 5 wt% (low fat, LF) or 25 wt% (high fat, HF) fat.

Group 1 received 5 wt% lard (Best Food, The Netherlands), containing a marginal (0.61 wt%) but sufficient level of LA (15). Group 2 received HF containing 5 wt% LA and group 3 received HF/5 wt% LA including 9.4 wt% (20 cal%) MaxEPA. The diets were compounded by mixing high linoleic safflower oil (Unilever, Vlaardingen, The Netherlands) with high oleic sunflower oil (Contined, Bennekom, The Netherlands) and MaxEPA (fish oil; Seven Seas, Hull, United Kingdom). The composition of the AIN^{76}-based diets and the fatty acid composition of the oils are summarized in Table 1. The diets were prepared monthly and stored at -20°C until use. The animals received fresh feed daily to minimize oxidation of the polyunsaturated fatty acids. Peroxide values (as measured by means of the A.O.C.S. official method in terms of milliequivalents peroxide per kg) of the HF/MaxEPA containing diet, stored at -20°C for 3 months, were below 1.0 and remained below 1.0 when exposed to air at room temperature for 24 hours. Longer periods of exposure to air at room temperature caused a rapid increase of the peroxide value. Food consumption was measured daily during the first 3 months and on 7 consecutive days per month during the remainder of the study. The animals were weighed weekly during the first 3 months of the study and monthly, thereafter.

Three days before autopsy 5 saline-treated control rats and 5 azaserine-treated rats from each diet-group had an Alzet osmotic pump (Alza Corp., Palo Alto, USA, model 2001) implanted subcutaneously, containing 200 μl of a BrdU solution (Sigma Chemie, Brussels, Belgium; conc. 25 mg/ml). The release rate of this pump was 1 μl/h. Autopsy was performed 170 days after the last injection of azaserine. The animals were anaesthetized with ether and exsanguinated by cannulating the abdominal aorta. Blood was collected in heparin-containing tubes, centrifuged at 1700 g for 20 min. The blood plasma was stored at -80°C until analysis. The pancreas and liver were excised and weighed. About one-third of the pancreas of 2 animals per cage was snap-frozen in liquid nitrogen and stored at -80°C until fatty acid or prostaglandin analysis. The

Table 1 Weight percentage composition of the AIN[76]-based diets and percentage fatty acid composition of the dietary lipids

Dietary component	LF	HF	HF/MaxEPA	Fatty acid	Lard	Safflower oil	Sunflower oil	MaxEPA
Casein	20.00	25.00	25.00	C14:0	1.8	0.1	0.1	7.1
DL-Methionine	0.30	0.37	0.37	C16:0	25.6	7.0	3.8	17.5
Wheat starch	63.50	35.79	35.79	C16:1	2.9	0.1	0.1	9.9
Cellulose	5.00	6.18	6.18	C18:0	14.2	2.6	4.0	4.2
Choline bitartrate	0.20	0.25	0.25	C18:1	43.1	13.1	82.6	12.9
AIN[76]-minerals	3.50	4.32	4.32	C18:2 (ω-6)	8.7	76.0	7.7	4.2
AIN[76]-vitamins	1.00	1.24	1.24	C18:3 (ω-3)	0.6	0.4	0.1	0.0
CaH_2PO_4	1.50	1.85	1.85	C20:0	0.2	0.3	0.3	2.5
Lard	4.75	0.00	0.00	C20:1	0.8	0.2	0.3	4.4
Safflower oil	0.25	4.53	5.04	C20:4 (ω-6)	0.0	0.0	0.0	1.6
Sunflower oil	0.00	20.47	10.53	C20:5 (ω-3)	0.0	0.0	0.0	18.2
MaxEPA	0.00	0.00	9.43	C22:0	0.1	0.2	0.9	0.0
				C22:1	0.0	0.0	0.0	1.1
				C22:4 (ω-6)	0.0	0.0	0.0	1.2
				C22:6 (ω-3)	0.0	0.0	0.0	14.9
Total	100.00	100.00	100.00		98.0	99.9	100.0	99.7
ω-3/ω-6 ratio	0.05	0.01	0.63	ω-3/ω-6 ratio	0.07	0.01	0.01	4.7
Energy (MJ/kg)	15.50	19.70	19.70					

remaining two-thirds of these pancreata plus all other pancreata and all livers were fixed in 10% buffered formalin. Livers and pancreata of BrdU-treated animals were fixed for 24 hours in 10% buffered formalin followed by 72 hours in 70% ethanol. The organs were processed for microscopy by conventional methods, step-sectioned at 5 μm and collected on organosilane-coated slides. Parallel sections were stained with haematoxylin and eosin (H&E) or with a monoclonal antibody against BrdU (Beckton Dickinson, CA, USA) and examined by light microscopy. In the H&E stained slides all pancreatic lesions were identified as acidophilic or basophilic atypical acinar cell foci (AACF) or localized carcinoma (carcinoma *in situ*; CIS) according to the criteria of Longnecker (16) and Rao *et al.* (17). Basophilic AACF were not scored because of insufficient yield. The area of the acidophilic AACF was determined by using an intra-ocular grid as described before (18). In slides stained for BrdU the Labeling Index (LI) was expressed as the ratio of brown stained BrdU-positive cells to blue stained normal cells. To select a random sample of acinar cells, only nuclei that were located beneath the crossings of the horizontal and vertical lines in a 20x20 intra-ocular grid at high power magnification (400x) were counted. In normal pancreatic acinar tissue at least 1000 nuclei per animal were counted. The LI in AACF (mean = 9 AACF per animal) was determined by counting a mean of 118 nuclei per AACF.

Analytical procedures

Fatty acids. Pancreatic microsomes were prepared by homogenising 100-200 mg pancreatic tissue in 0.1 M Tris-KCl buffer, pH 7.4. Subsequently, the homogenate was centrifuged at 10,000 g for 30 min and the supernatant was centrifuged at 105,000 g for 60 min. The microsomal pellet was resuspended in 300 μl buffer and stored at -30°C until fatty acid analysis. Total lipids were extracted from 50 μl aliquots of pancreatic microsomes or from 100 μl aliquots of blood plasma as described by Folch *et al.* (19). Fatty acid composition was determined by GLC. The samples were eluted on a capillary BD23 column (J&W Scientific) after saponification with NaOH in methanol and transmethylation of the fatty acids with borontrifluoride-methanol.

Prostaglandins. Pancreatic tissue (100-200 mg) was homogenized in 0.1 M PBS (pH 7.4) containing 15% methanol and applied to Sep-pak C-18 columns (J.T. Baker Inc, Phillipburg, NJ, USA). After washing with 6 ml 15% methanol/PBS and 6 ml petroleum ether, the samples were eluted with 6 ml methanol. After evaporation of the methanol under N_2, the samples were dissolved in 1.0 ml potassium phosphate buffer (1.0 M; pH 7.4) and subsequently analyzed by using enzyme immunoassay kits for PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 (Cascade Biochem Ltd, Reading, UK).

Results

Food consumption and body and organ weights. Food consumption of animals maintained on the HF diets was significantly lower in comparison with animals on the low fat diet. However, due to a higher energy content of the HF diet, mean caloric intake was similar among LF and HF groups (Table 2). Body weights (Table 3) at autopsy were similar among all groups, except those of the azaserine-treated rats in the HF/MaxEPA group, which were significantly higher ($P<0.01$). Relative pancreatic weights were

significantly lower ($P<0.05$) in the HF groups as compared to the LF group (Table 3).

Microscopy. Transectional and volumetric data of AACF are presented in Table 4. Acidophilic AACF were found in all azaserine-treated rats whereas only two saline-treated animals showed one acidophilic AACF in the pancreas. Animals maintained on a HF diet, with or without MaxEPA, demonstrated more foci per cm^2 ($P<0.001$), more foci per cm^3 ($P<0.001$) with a larger mean diameter ($P<0.01$), leading to a larger area as % of pancreas occupied by focus tissue ($P<0.001$) than in LF controls. The HF group exhibited 8 AACN with a diameter over 1 mm and 4 carcinomas *in situ*, whereas in the HF/MaxEPA group these numbers were 12 and 6, respectively (Table 5). Neither AACN with a diameter over 1 mm nor carcinomas *in situ* were observed in the LF control group. The numbers of (pre)neoplastic lesions were not different in both HF groups.

Table 2 Mean food and energy intake (± SEM)[a].

Diet group	N	Food (g/rat/day)	Energy (KJ/kg)
LF (saline-treated)	5	15.6±0.2	242.4
LF (azaserine-treated)	15	15.7±0.2	244.0
HF (azaserine-treated)	14	12.6±0.1***	247.7
HF/MaxEPA (azaserine-treated)	15	12.8±0.2***	251.6

[a]LF, low fat; HF, high fat; HF/MaxEPA, high fat+9.4 wt% MaxEPA;
Statistics: 2-way analysis of variance: ***$P<0.001$

Labeling Index. Labeling Indices (Fig. 1) in normal pancreatic acinar cells were similar in all three groups (values < 1.0%). Cell turnover in AACF was significantly increased in comparison with normal acinar tissue ($P<0.001$). AACF in pancreata of rats given a HF diet demonstrated a significantly ($P<0.001$) higher LI than those of the LF control group (23.9±1.2% vs 15.1±1.4%). The LI in AACF of the HF/MaxEPA group (19.2±1.1%) was significantly ($P<0.01$) lower than that of the HF group, but not different from that of controls.

Fatty acids. Fatty acid composition of blood plasma and pancreatic microsomes are summarized in Table 6. No significant differences were observed between carcinogen- and

saline-treated animals. The fatty acid patterns in the various groups showed variations that were directly related to the fatty acid composition of the diets, resulting in high values of C16:0 in the LF group, high levels of C18:1 in the HF group and high levels of EPA and DHA in the HF/MaxEPA group. Although dietary supply of LA was the same in both HF groups, blood plasma and pancreatic tissue levels of LA were significantly higher in the MaxEPA supplemented HF group. In blood plasma and, to a lesser extent, also in pancreas the level of AA decreased with increasing LA levels.

Table 3 Body and organ weights[a]

			Absolute weight (g)			Relative weight (g/kg)	
Diet group[b]		N	Body wt	Pancreas wt	Liver wt	Pancreas wt	Liver wt
LF	saline	5	452± 4	1.11±0.10	11.5±0.4	2.46±0.23	25.4±0.8
	azaserine	15	459±13	1.37±0.08	12.8±0.5	2.97±0.12	27.9±0.6
HF	saline	5	507±47	1.12±0.07	14.1±1.6	2.25±0.11*	27.7±0.6
	azaserine	14	488±12	1.15±0.05	12.8±0.4	2.36±0.09**	26.3±0.6
HF/MaxEPA	saline	5	480±17	0.96±0.10*	14.1±0.4	2.04±0.27**	29.3±0.6
	azaserine	15	521±11**	1.16±0.08	14.5±0.5	2.23±0.14**	27.8±1.1

[a]Values are means ± SEM

[b]LF, low fat; HF, high fat; HF/MaxEPA, high fat+9.4 wt% MaxEPA

Statistics: Analysis of variance + Dunnetts tests; *P<0.05; **P<0.01

Prostaglandins. Saline-treated controls did not show different PG levels in comparison with azaserine-treated rats fed a LF diet. In carcinogen-treated rats, pancreatic levels of 6-keto-$PGF_{1\alpha}$ ($P<0.01$), $PGF_{2\alpha}$ ($P<0.01$) and TXB_2 ($P<0.05$), but not of PGE_2 were significantly lower in the HF/MaxEPA group as compared to the HF group (Fig 2). Pancreatic $PGF_{2\alpha}$-levels were significantly higher in the HF group as compared to LF controls ($P<0.01$).

Discussion

The results of the present study in which the effects of dietary fish oil on pancreatic

Table 4 Effects of MaxEPA on dietary fat promoted growth of putative preneoplastic foci in rat pancreas induced by azaserine[a,b,c]

Diet group[d]	N	Observed transection data of foci		Calculated volumetric data of foci								
		Total n/cm^2	Transection area (mm^2)	n/cm^3 with mean diameter (μm)						Total n/cm^3	Mean diameter (μm)	Area as % of pancreas
				192.5	272.5	385	545	770	1090			
LF	15	8.7	0.063	172	188	55	7	1	0	493	267	0.55
HF	15	54.5	0.130	511	605[1]	608[2]	187[1]	24[1]	30[1]	2153[2]	329[1]	7.03[2]
HF/MaxEPA	15	63.1	0.129	331	640[1]	699[2]	190[1]	54[1]	24[1]	2200[2]	358[1]	8.17[2]

[a]Values are means, [b]Data are based on H&E stained paraffin sections, [c]The untreated controls showed no histological abnormalities in the pancreas, [d]LF, low fat; HF, high fat; HF/MaxEPA, high fat+9.4 wt% MaxEPA, Statistics: analysis of variance followed by Student's *t* tests (2-tailed); [1]P<0.01; [2]P<0.001; HF or HF/MaxEPA vs LF. HF vs HF/MaxEPA revealed no differences.

carcinogenesis in azaserine-treated rats were investigated confirm previous findings that polyunsaturated fat has a strong promoting effect on pancreatic carcinogenesis as demonstrated by an increase in number and size of putative preneoplastic acidophilic AACF as well as by an increase in cell turnover in comparison with LF controls (7,18). Moreover, the present results indicate that dietary fish oil did not modulate the strong enhancing effect of a HF diet on growth of acidophilic AACF. The latter observation is in contrast to that of O'Connor *et al.* (20), who found a significant inhibition of growth of pancreatic AACF with a ω-3/ω-6 ratio of 0.63 in a diet containing 20 wt% fat. In the present study no inhibitory effect of dietary fish oil was found although the ω-3/ω-6 ratio varied from 0.01 in the HF group to 0.63 in the HF/MaxEPA group. The difference between our results and those observed by O'Connor *et al.* (20) most probably can be ascribed to an essential difference in the composition of the experimental diets used. O'Connor varied the ω-3/ω-6 ratio by mixing corn oil with menhaden oil, resulting in substantial differences in the LA content of the various diets. An increase of dietary ω-3 fatty acids was directly related to a decrease in LA content of the diets. In the present study the ω-3/ω-6 ratio was increased by raising the amount of ω-3 fatty acid while keeping the ω-6 fatty acid content at a constant level, hence avoiding confounding effects of differences in LA content.

The increased growth potential of acidophilic AACF induced by azaserine in the pancreas of rats maintained on either a LF or a HF diet is illustrated by a higher cell turnover in comparison with normal pancreatic tissue. Moreover, the LI in AACF observed in the HF group was significantly higher in comparison with the LF group, indicating an enhancing effect of dietary fat on the growth potential of AACF.

Remarkably, in the HF/MaxEPA group the LI in the acidophilic AACF was significantly lower than in acidophilic AACF in the HF group, notwithstanding the similar number and size of the AACF in the two groups. However, since the LI has only been determined at autopsy, only information has been obtained about the growth potential of AACF 6 months after azaserine-treatment. It seems not illogical to assume that growth curves of AACF induced in rats maintained on a diet without MaxEPA are different from those in rats maintained on a diet with MaxEPA. Moreover, docosahexaenoic acid (DHA) from MaxEPA may induce hypertrophy (swelling) of tumour cells, as recently has been demonstrated by Stillwell *et al.* (21) *in vitro*. This phenomenon may, at least in part, explain the size of AACF in the MaxEPA group. Another plausible explanation for the apparently contradictory observations that the LI in the AACF in the HF/MaxEPA group was significantly lower than in the HF group, whereas at the same time the number and size of the AACF were similar in both groups, may be that fish oil only has an inhibitory effect on the carcinogenic process at a late stage of the (pre)neoplastic development. A

study to investigate the effects of MaxEPA on azaserine-induced pancreatic cancer after a period of 12 months is currently running at our Institute. The results of this study will be the subject of a separate paper.

The LI in the occasionally observed basophilic AACF was similar to that found in normal tissue (data not shown), indicating a low growth potential of this type of lesion. This observation supports the conclusion of Rao *et al.* (17) and Scherer *et al.* (22), that basophilic AACF play only a minor role, if any, in the carcinogenic process.

Differences observed in the fatty acid profiles other than LA and AA are considered to be mainly caused by differences in the fatty acid composition of the diets and are, therefore, not discussed.

Table 5 Number of (pre)neoplastic lesions in azaserine-treated rats maintained on a low fat diet, a high fat diet or a high fat diet including 9.4 wt% MaxEPA for 6 months[a]

(Pre)neoplastic lesions observed[a]	No. of lesions		
	LF	HF	HF/MaxEPA
No. of animals	15	15	15
No. of tumour bearing animals (%)	0 (0)	4 (27)*	6 (40)*
AACN (1<Ø<3mm)	0	8*	12*
AACN (Ø>3mm)	0	0	1
Carcinoma *in situ*	0	4*	6*

[a]LF, low fat; HF, high fat; HF/MaxEPA, high fat+9.4 wt% MaxEPA; AACN, Atypical Acinar Cell Nodule; Statistics: Pearson χ^2-tests; *P<0.05.

The observation that animals maintained on a LF or a HF diet without MaxEPA exhibited similar AA concentrations in blood plasma and pancreas, indicates that an increase in amount of LA and unsaturated fat in the diet does not influence the conversion of LA to AA. However, when LA was provided to rats in the same amount (5 wt%) in combination with MaxEPA (containing EPA and DHA), an increase in LA concentrations accompanied by a decrease in AA levels in blood plasma and also, but to a lesser extent in pancreas was observed. This apparently reduced conversion of LA to AA is most probably due to competitive inhibition of the enzyme elongase by EPA, or to inhibition of

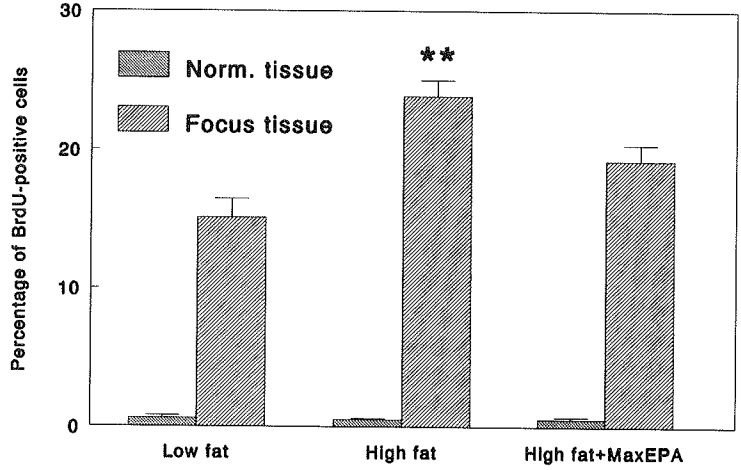

Figure 1. Labeling Index in pancreatic AACF and normal pancreatic acinar tissue of rats maintained on a low fat diet, a high fat diet or a high fat diet including 9.4 wt% MaxEPA for 6 months. Values are mean percentages ± SEM. Statistics: analysis of variance followed by Student's *t*-tests. Normal *versus* AACF: P<0.001. LI in AACF: **P<0.01 (HF *vs* LF and HF/MaxEPA).

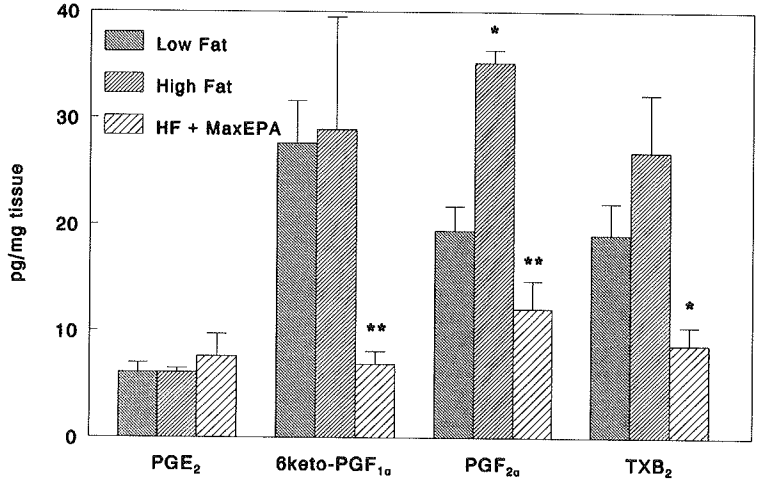

Figure 2. Prostaglandin levels in pancreas of rats maintained on a low fat diet, a high fat diet or a high fat diet including 9.4 wt% MaxEPA for 6 months. Values are means ± SEM. Statistics: analysis of variance followed by Student's *t*-tests (LF *vs* HF: P<0.05; HF *vs* HF/MaxEPA: *P<0.05, **P<0.01).

Table 6 Fatty acid composition of blood plasma and pancreatic microsomes of untreated and azaserine-treated rats maintained on a low fat diet, a high fat diet or a high fat diet including 9.4 wt% MaxEPA for 6 months (N=6)[a]

	Blood plasma				Pancreas			
Fatty acid	LF	HF	HF/MaxEPA	P<	LF	HF	HF/MaxEPA	P<
C12:0	0.3±0.0	0.3±0.0	0.4±0.1	n.s.	n.d.	n.d.	n.d.	--
C14:0	0.6±0.1^{2}	0.3±0.0	0.8±0.1^{2}	0.001	1.4±0.1^{2}	0.4±0.0^{1}	1.2±0.1^{2}	0.001
C16:0	22.0±0.6^{3}	12.0±0.6^{1}	16.8±0.1^{2}	0.001	27.4±0.3^{3}	12.0±0.6^{1}	18.3±0.7^{2}	0.001
C16:1*trans*	0.5±0.1	0.6±0.0	0.4±0.0	n.s.	0.7±0.0^{2}	0.8±0.0^{2}	0.6±0.0^{1}	0.05
C16:1*cis*	2.7±0.4^{3}	0.2±0.0^{1}	1.2±0.1^{2}	0.05	5.5±0.4^{3}	0.4±0.0^{1}	1.5±0.1^{2}	0.01
C18:0	13.3±0.4	14.0±0.4	13.1±0.4	n.s.	7.5±0.8^{1}	7.2±0.5^{1}	10.1±0.6^{2}	0.05
C18:1	20.1±0.8^{1}	30.7±1.2^{2}	19.0±0.8^{1}	0.001	43.4±1.9^{1}	59.7±1.6^{2}	40.0±1.8^{1}	0.001
C18:2 (ω-6)	9.8±0.6^{1}	12.7±0.7^{2}	17.6±0.4^{3}	0.01	8.4±0.3^{1}	13.1±0.1^{2}	17.1±0.4^{3}	0.001
C18:3 (ω-3)	0.4±0.0^{2}	0.3±0.0^{1}	0.3±0.0^{1}	0.05	0.7±0.1	0.7±0.1	1.0±0.2	n.s.
C20:0	0.1±0.0	0.2±0.0	0.2±0.0	n.s.	0.2±0.0	0.3±0.0	0.3±0.0	n.s.
C20:1	0.2±0.0	0.3±0.0	0.2±0.0	n.s.	0.3±0.0	0.3±0.0	0.4±0.1	n.s.
C20:2	0.7±0.1^{3}	0.4±0.0^{2}	0.2±0.1^{1}	0.01	n.d.	n.d.	n.d.	--
C20:3 (ω-3)	0.5±0.0^{2}	0.3±0.0^{1}	0.9±0.0^{3}	0.001	0.2±0.0	0.2±0.0	0.3±0.0	n.s.
C20:4 (ω-6)	27.4±1.9^{2}	27.4±1.3^{2}	16.0±0.9^{1}	0.001	4.1±1.2	5.0±0.6	3.2±0.3	n.s.
C20:5 (ω-3)	n.d.	n.d.	7.5±0.4	--	n.d	n.d.	3.3±0.3	--
C22:6 (ω-3)	1.5±0.1^{2}	0.5±0.0^{1}	5.4±0.2^{3}	0.001	n.d	n.d.	2.3±0.2	--
ω-3/ω-6 ratio	0.06	0.03	0.42		0.07	0.05	0.34	

[a]Values are mean percentages of total fatty acids ± SEM; Values of azaserine-treated rats were not different from untreated controls, therefore the data were pooled; Statistics: analysis of variance followed by Student's *t* tests; [1,2,3]Values with different superscripts are significantly different; n.s., not significant; n.d., not detected; LF, low fat; HF, high fat; HF/MaxEPA, high fat+9.4 wt% MaxEPA

δ^6-desaturase, the enzyme that desaturates LA to γ-linolenic acid, by long chain ω-3 fatty acids (23). Furthermore, it has been demonstrated that ω-3 fatty acids compete with ω-6 fatty acids for conversion to prostaglandins via cyclooxygenase. *In vitro*, $PGF_{2\alpha}$ has been shown to have cell proliferative properties (8), suggesting that this prostaglandin may be involved in tumour promotion. This hypothesis is supported by the present observation that animals maintained on a HF diet without MaxEPA demonstrated both a high level of $PGF_{2\alpha}$ in the pancreas as well as high LI in the AACF, whereas low pancreatic levels of $PGF_{2\alpha}$, as seen in the LF and the HF/MaxEPA groups, correlate with low LI in AACF.

It is concluded that a HF diet containing 5 wt% LA has a strong promoting effect on azaserine-induced pancreatic carcinogenesis in rats, which is not inhibited by a diet rich in fish oil, although such a diet causes a decrease in cell turnover in AACF as well as a reduction of the levels of several PGs in the pancreas.

The results of the present study warrant further investigations into the long term chemopreventive effects of fish oil on pancreatic carcinogenesis in rats.

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Dietary fish oil (MaxEPA) enhances pancreatic carcinogenesis in azaserine-treated rats[1]

M.J. Appel and R.A. Woutersen[2], TNO Nutrition and Food Research Institute, Division of Toxicology, Department of Pathology, P.O. Box 360, 3700 AJ, Zeist (The Netherlands)

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Abstract

In the present study the putative chemopreventive effect of dietary fish oil (MaxEPA) on azaserine-induced pancreatic carcinogenesis in rats was investigated. Groups of male rats were maintained on a semi-purified low fat (LF; 5 wt%) diet or on semi-purified high fat (HF; 25 wt%) diets containing 5 wt% linoleic acid (LA[3]) and including 0.0, 1.2, 2.4, 4.7, 7.1 or 9.4 wt% MaxEPA for 12 months. Animals fed a HF diet developed significantly higher mean numbers of atypical acinar cell nodules (AACN), adenomas and carcinomas than animals fed a LF diet. Dietary MaxEPA caused a significant ($P<0.01$) dose-related increase in mean number of AACN ($0.5<Ø<3.0$ mm). The mean number of adenomas and carcinomas remained similar among the groups. Cell proliferation was significantly lower in AACN from animals fed HF containing 9.4% MaxEPA in comparison with HF without MaxEPA and with LF. Fatty acid analysis revealed a significant linear increase in LA levels and a decrease in arachidonic acid (AA) levels in blood plasma and pancreas with increasing dietary MaxEPA. Feeding MaxEPA resulted in significant decreases in 6-keto-prostaglandin (PG) $F_{1\alpha}$ ($P<0.05$) and $PGF_{2\alpha}$ ($P<0.01$) in non-tumorous pancreas, whereas PGE_2, $PGF_{2\alpha}$ and thromboxane B_2 levels were significantly ($P<0.001$) higher in pancreatic tumour tissue than in non-tumorous pancreatic tissue.

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[2] To whom requests for reprints should be addressed.

[3] Abbreviations: LA, linoleic acid; AA, arachidonic acid; OA, oleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AACN, atypical acinar cell nodule; AACF, atypical acinar cell focus; PG, prostaglandin; TXB_2, thromboxane B_2; PUFA, polyunsaturated fatty acid; DMBA, 7,12-dimethylbenz(a)anthracene; MNU, methylnitrosurea; DMH, dimethylhydrazine; AOM, azoxymethane; CO, corn oil; MO, menhaden oil; LI, labeling index.

From the results of this study it is concluded that (i) dietary MaxEPA has no chemopreventive effect, but on the contrary enhances dose-relatedly growth of putative preneoplastic AACN in the pancreas of azaserine-treated rats and (ii) dietary MaxEPA inhibits the conversion of LA to AA, as well as the conversion of AA to TXB_2 or $PGF_{2\alpha}$ in non-tumorous pancreatic tissue and (iii) the high levels of PGE_2, $PGF_{2\alpha}$ and thromboxane B_2 in pancreatic adenocarcinomas indicate a possible role for these eicosanoids in modulation of tumour growth.

Introduction

It has been estimated that 30-35% of total cancer mortality in the Western world is caused by diet related factors. It is not surprising that most of the diet related cancers are located in the gastrointestinal tract, including the pancreas (1). In several epidemiological studies dietary fat has been associated with increased cancer risk (2,3). Positive associations, after controlling for total energy intake, have been found with the incidence of breast cancer and total fat, and with colon cancer and animal fat (4-7). Epidemiology has also revealed associations between total fat consumption and mortality of pancreatic cancer (8-10). The relationship between dietary fat and cancer development has become more clear in studies with experimental animals. In animal models for colon, breast, prostate and pancreatic cancer a high fat diet has been reported to promote tumour growth (11-15). Polyunsaturated fatty acids (PUFA) from the ω-6 family (mainly linoleic acid; LA; C18:2) have been implicated to cause this effect. In the DMBA-induced mammary tumour model, Ip *et al.* (16) varied the levels of dietary LA and found a threshold level of 4.4% for maximal tumour response. Similarly, Roebuck *et al.* (17) reported this level to be in the range of 4.4-8.5% of dietary LA in the azaserine-rat model for pancreatic carcinogenesis. However, we found rather an inverse relationship between tumour yield and dietary LA in the range of 4-15% in the same model (18). PUFAs from the ω-3 family (abundant in fish oil) have been shown to inhibit tumour development in the mammary, colon and pancreatic animal models mentioned above. Menhaden oil (MO) supplemented to a HF diet caused a lengthening of the tumour latency period, as well as a reduction in tumour incidence in MNU-induced (19,20) and DMBA-induced (21,22) mammary carcinogenesis in comparison to high corn oil (CO) diets.

Colon tumours are also sensitive to qualitative differences in fatty acid composition of the dietary fat. Azoxymethane (AOM) induced colonic tumour incidence was significantly lower in animals fed high fat diets containing mixtures of CO and MO in comparison to CO alone (23,24). Furthermore, feeding ω-3 PUFA at a 5% level (4.7% eicosapentaenoic

acid; EPA + 0.3 % LA, as ethyl esters) as sole lipid source, resulted in lower colonic tumour incidence and multiplicity than in ω-6 PUFA (5.0% LA as ethyl ester) fed animals (25). Growth of dimethylhydrazine (DMH)-induced colon tumours could also be inhibited by dietary fish oil (26).

Information on effects of fish oil on pancreatic carcinogenesis is scarce and comes mainly from the studies of O'Connor *et al.* (27), who observed that in azaserine-treated rats maintained on a 20% MO diet for 4 months, the number and size of pancreatic preneoplastic Atypical Acinar Cell Nodules (AACN) were significantly reduced as compared to rats fed a 20% CO diet. In a subsequent 4-month study they found a decrease of the number of AACN with increasing levels of MO in a 20% fat diet (28). However, using the same model, we did not find any difference in AACN yield in rats fed 25% fat diets with a constant 5% LA level, either or not containing 9.4% fish oil (MaxEPA), for 6 months (29). Because of the latter unexpected and contrasting result and the paucity of data on effects of fish oil on development of pancreatic tumours, we performed a 12-month study to investigate the effects of increasing levels of MaxEPA in a 25% fat/5% LA diet on pancreatic tumour development in azaserine-treated rats. Furthermore, the effects of dietary MaxEPA on cell proliferation in AACN and normal acinar pancreatic tissue, as well as fatty acid profiles and prostaglandin levels in pancreatic tissue were examined.

Materials and methods

Animals and diets. Fifty five one-week pregnant female Wistar rats were obtained from Harlan-CPB, Austerlitz, The Netherlands. During pregnancy the rats were kept solitary, in stainless steel cages fitted with wire-mesh floors and fronts and were fed a standard laboratory chow. Two weeks (± 1 day) after arrival the rats gave birth to a mean of eight pups. After 4 days the pups were sexed. All females, the surplus of male pups and the surplus of mothers were killed and a total of 210 male pups were divided among the remaining 26 mothers. One hundred seventy five pups were given an i.p. injection of 30 mg azaserine (Calbiochem-Behring Corp., La Jolla, CA) per kg body wt, which was dissolved freshly in 0.9% NaCl-solution, at 14 and 21 days of age. Thirty five control pups received injections with NaCl-solution alone. Directly after the second injection the animals were weaned and randomly allocated to 7 groups of 30 animals each (5 control animals and 25 azaserine-treated animals). The animals were kept in stainless steel cages, with wire-mesh floors and fronts, 5 animals per cage and under standard laboratory conditions. One week after carcinogen treatment the rats were fed an AIN^{76}- based purified diet containing either 5 or 25 wt% fat. The control group received a 5 wt% lard (Best Food, The Netherlands; LF) diet, containing a marginal (0.61 wt%) but sufficient level of linoleic acid (LA; ref. 30). The experimental groups received a high-fat (25 wt%; HF) diet containing 5 wt% LA and including 0.0, 1.2, 2.4, 4.7, 7.1 or 9.4 wt% (0, 2.5, 5, 10, 15 and 20 en%) MaxEPA. The diets were compounded by mixing high linoleic safflower oil (Unilever, Vlaardingen, The Netherlands) with high oleic sunflower oil (Trisun™, Contined, Bennekom, The Netherlands) and fish oil

(MaxEPA; Seven Seas, Hull, United Kingdom). The safflower oil, the sunflower oil and the MaxEPA contained 0.55 g/kg, 0.44 g/kg and 1.80 g/kg vitamin E, respectively. α-Tocopherol was added to all diets a extra antioxidant to a level of 0.450 g/kg. The composition of the AIN[76]- based diets and the fatty acid composition of the oils are summarized in Tables 1 and 2. The diets were prepared monthly and stored at -20°C until use. The animals were fed daily to minimize oxidation of the polyunsaturated fatty acids. Peroxide values (as measured by means of the A.O.C.S. official method in terms of milliequivalents peroxid per kg) of the HF/9.4% MaxEPA containing diet, stored at -20°C for 3 months, were below 1.0 and remained below 1.0 when exposed to air at room temperature for 24 hours. Longer periods of exposure to a at room temperature caused a rapid increase of the peroxide value. The profiles of the dietary fatty acids of interest are depicted in Fig 1. Food consumption was measured daily during the first 3 months and on 7 consecutive days per month during the remainder of the study. The animals were weighed weekly during th first 3 months of the study and monthly, thereafter.

Table 1 Percentage fatty acid composition of the dietary lipids[a].

Fatty acid	Lard	SA	SO	MaxEPA
C14:0	1.8	0.1	0.1	7.1
C16:0	25.6	7.0	3.8	17.5
C16:1	2.9	0.1	0.1	9.9
C18:0	14.2	2.6	4.0	4.2
C18:1	43.1	13.1	82.6	12.9
C18:2 (ω-6)	8.7	76.0	7.7	4.2
C18:3 (ω-3)	0.6	0.4	0.1	0.0
C20:0	0.2	0.3	0.3	2.5
C20:1	0.8	0.2	0.3	4.4
C20:4 (ω-6)	0.0	0.0	0.0	1.6
C20:5 (ω-3)	0.0	0.0	0.0	18.2
C22:0	0.1	0.2	0.9	0.0
C22:1	0.0	0.0	0.0	1.1
C22:4 (ω-6)	0.0	0.0	0.0	1.2
C22:6 (ω-3)	0.0	0.0	0.0	14.9
Total	98.0	99.9	100.0	99.7
ω-3/ω-6 (ratio)	0.07	0.01	0.01	4.7

[a]SA, Safflower oil; SO, Sunflower oil (Trisun)

Monitoring and autopsy. Three days before autopsy 5 saline-treated control rats and 5 azaserine-treate rats from the LF, HF/0.0% MaxEPA and the HF/9.4% MaxEPA groups had an Alzet osmotic pump (Alza Corp., Palo Alto, USA, model 2001) implanted subcutaneously, containing 200 μl of a BrdU solution (Sigm

Table 2 Weight percentage composition of the AIN[76]-based diets.

Premix				LF	HF/ wt% MaxEPA					
Dietary components	LF	HF			0.0%	1.2%	2.4%	4.7%	7.1%	9.4%
Casein	20.00	25.00	Premix	95.00	75.00	75.00	75.00	75.00	75.00	75.00
DL-Methionine	0.30	0.37	Lard	4.74	--	--	--	--	--	--
Wheat starch	63.50	35.79	Safflower oil	0.26	4.63	4.67	4.69	4.80	4.91	5.04
Cellulose	5.00	6.18	Sunflower oil	--	20.37	19.15	17.95	15.49	13.04	10.53
Choline bitartrate	0.20	0.25	MaxEPA	--	--	1.18	2.36	4.71	7.05	9.43
AIN[76] minerals	3.50	4.32								
AIN[76] vitamins	1.00	1.24								
CaH_2PO_4	1.50	1.85								
Total	95.00	75.00	Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

Chemie, Brussels, Belgium; conc. 25 mg/ml). The release rate of this pump was 1 μl/h. Autopsy was performed 343, 344 or 345 days after the last injection of azaserine. The animals were anaesthetized with ether and exsanguinated by cannulating the abdominal aorta. Blood was collected in heparin-containing tube centrifuged at 1700 g for 20 min and stored at -80°C until analysis. The pancreas and liver were excised an weighed. About one-third of the pancreas of 2 animals per cage was snap-frozen in liquid nitrogen and stored at -80°C until fatty acid or prostaglandin analysis. The remaining two-thirds of these pancreata plus a other pancreata and all livers were fixed in 10% neutral buffered formalin. Livers and pancreata of BrdU-treated animals were fixed for 24 hours in formalin followed by 72 hours in 70% ethanol. The organs were processed for microscopy by conventional methods, step-sectioned at 5 μm and collected on organosilane-coated slides. Parallel sections were stained with haematoxylin and eosin (H&E) or with a monoclonal antibody against BrdU (Organon Technics, the Netherlands) and examined by light microscopy. In the H&E stained slides all pancreatic lesions were identified as acidophilic or basophilic Atypical Acinar Cell Foci (AACF), localized carcinoma (carcinoma *in situ*; CIS) or invasive carcinomas according to the criteria of Longnecker (31) and Rao *et al.* (32). Basophilic AACF were not scored because of insufficient yield. The area of the acidophilic AACF was determined by using an intra-ocular grid as described before (33). In slides stained for BrdU the Labeling Index (LI) was expressed as the ratio of brown stained BrdU-positive cells to blue stained normal cells. To select a random sample of acinar cells, only nuclei that were located beneath the crossings of the horizontal and vertical lines in a 20x20 intra-ocular grid at high power magnification (400x) were counted. In normal pancreatic tissue at least 1000 nuclei per pancreas were counted. A mean of 8.8 AACF per pancreas was evaluated (without discrimination between focus size) and a mean of 133 nuclei per AACF was counted.

Analytical procedures. Fatty acids. Pancreatic microsomes were prepared by homogenizing 100-200 mg pancreatic tissue in 0.1 M Tris-KCl buffer, pH 7.4. Subsequently, the homogenate was centrifuged at 10,000 g for 30 min and the supernatant was centrifuged at 105,000 g for 60 min. The microsomal pellet was resuspended in 300 μl buffer and stored at -30°C until fatty acid analysis. Total lipids were extracted from 50 μl aliquots of pancreatic microsomes or from 100 μl aliquots of blood plasma as described by Folch *et al.* (34). Fatty acid composition was determined by GLC. The samples were eluted on a capillary BD23 column (J&W Scientific) after saponification with NaOH in methanol and transmethylation of the fatty acids with borontrifluoride in methanol.

Prostaglandins. Pancreatic tissue (100-200 mg) was homogenized in 0.1 M phosphate-buffered saline (pH 7.4) containing 15% methanol and was applied to Sep-pak C-18 columns (J.T. Baker Inc., Phillipsburg, NJ, USA). After washing with 6 ml 15% methanol/phosphate-buffered saline and 6 ml petroleum ether, the samples were eluted with 6 ml methanol. After evaporation of the methanol under N_2, the samples were dissolved in 1.0 ml potassium phosphate buffer (1.0 M; pH 7.4) and subsequently analyzed by using enzyme immunoassay kits for PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and TXB_2 (Cascade Biochem Ltd, Reading, United Kingdom).

Statistics. Food and energy intake and body and pancreatic weights were statistically evaluated by 2-way analysis of variance followed by Dunnetts test. Prostaglandin levels were evaluated by analysis of variance followed by student's *t* test. The number of pancreatic lesions was evaluated by 2-sample *t* test or by one-way analysis of variance followed by linear trend tests with orthogonal contrasts. The number of tumour bearing animals (incidence) was analyzed by Pearson χ^2-test. Fatty acid compositions were evaluated by 2-way analysis of variance using percentage of dietary MaxEPA and carcinogen-treatment as factors, and by 1-way analysis of variance followed by linear trend tests with orthogonal contrasts.

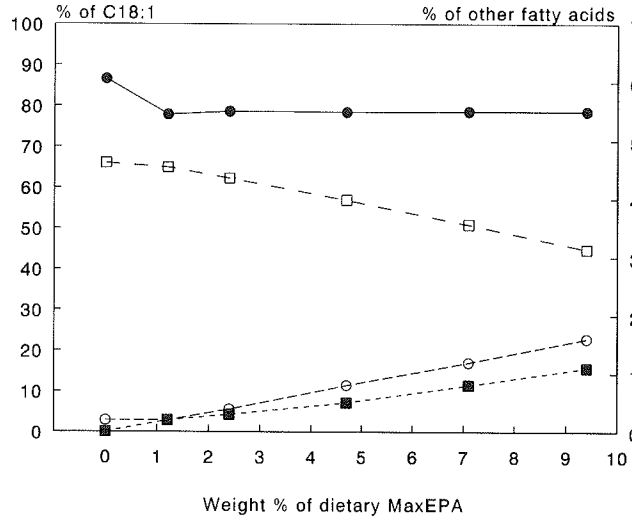

Figure 1. Percentage of selected dietary fatty acids. ●, linoleic acid; □, oleic acid; ○ ,eicosapentaenoic acid; ■ ,docosahexaenoic acid.

Results

Food consumption and body and organ weights. Mean food consumption of rats maintained on a LF diet was significantly ($P<0.001$) higher than that in rats maintained on a HF diet. However, due to a higher energy content of the HF diet, mean caloric intake was similar among LF and HF groups (Table 3).

Mean body weight gain over the study showed no significant differences among the groups (Fig. 2). Mean body weights of animals fed a HF diet were significantly higher relative to animals fed a LF diet ($P<0.05$; Table 4). Azaserine treatment caused a consistent significant increase in both absolute and relative pancreas weights in all groups ($P<0.001$) in comparison with saline-treated controls. Both absolute and relative liver weights were significantly higher in animals kept on a HF diet than in LF controls ($P<0.01$ and $P<0.05$, respectively).

Microscopy. Feeding a HF diet significantly enhanced tumour growth in comparison with the LF control diet, as reflected by a significantly higher number of tumour bearing animals ($P<0.001$), number of AACN ($P<0.01$) and total number of carcinomas ($P<0.05$). Including MaxEPA in the high fat diet resulted in a significant dose-related linear increase in number of AACN with both a diameter of 0.5 - 1.0 mm as well as a diameter of 1.0 -

3.0 mm ($P<0.01$). No such effect was seen on the number of adenomas and carcinomas nor on the number of tumour bearing animals (Table 5 and Fig. 3).

Cell proliferation. Labeling index in normal acinar cells was low (below 1%, Fig. 4) and similar in all groups. The mean LI in AACN from animals fed the LF, the HF and the HF diet including 9.4% MaxEPA was 28.4±3.2, 24.8±1.7 and 15.5±1.2, respectively. Cell proliferation was significantly higher in AACN in comparison to normal tissue ($P<0.001$). A high level of MaxEPA in the HF diet caused a significant ($P<0.05$) decrease of the LI in AACN in comparison with AACN from both the HF diet without MaxEPA and the LF diet.

Table 3 Food and energy consumption[a]

	Food	Energy
LF	15.4±0.3***	238.7
HF/0.0% MaxEPA	12.6±0.2	248.2
HF/1.2% MaxEPA	12.1±0.2	238.4
HF/2.4% MaxEPA	12.1±0.2	238.4
HF/4.7% MaxEPA	12.8±0.3	252.2
HF/7.1% MaxEPA	12.9±0.3	254.1
HF/9.4% MaxEPA	12.4±0.2	244.3

[a]Food intake in g/day per animal; Energy intake in KJ/day.
Statistics: analysis of variance, ***$P<0.001$

Fatty acid analyses. Fatty acid profiles of blood plasma and pancreatic microsomes are presented in Tables 6 and 7. Statistics are only presented on oleic acid (OA; C18:1), linoleic acid (LA; C18:2), arachidonic acid (AA; C20:4), eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6). Additionally, for reasons of clarity, the profiles of OA, LA, AA, EPA and DHA are depicted in Fig. 5.

In blood plasma the levels of AA (C20:4) and DHA (C22:6) were significantly higher in saline-treated *versus* azaserine treated rats ($P<0.001$). In pancreatic microsomes the level of OA (C18:1) was significantly higher ($P<0.05$) and the levels of AA (C20:4), EPA (C20:5) and DHA (C22:6) were significantly lower ($P<0.001$, $P<0.05$ and $P<0.001$, respectively) in saline-treated rats in comparison with azaserine-treated rats.

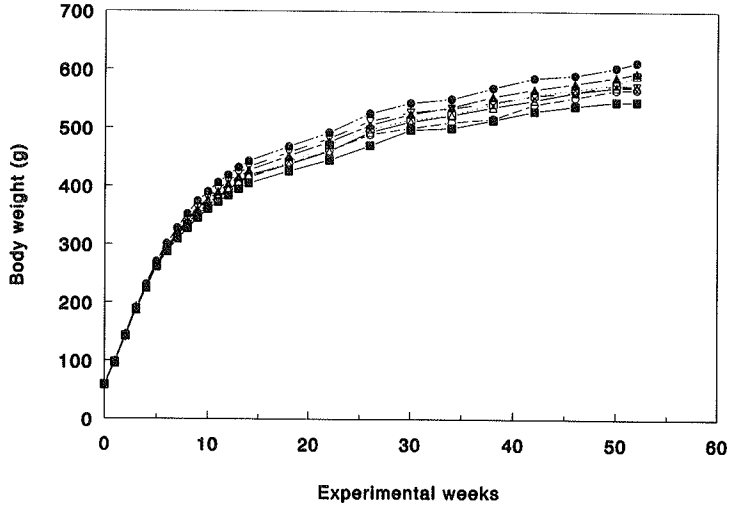

Figure 2. Body weight gain of azaserine-treated rats maintained on a low fat diet or a high fat diet containing increasing levels of MaxEPA for 12 months. ■, LF; ▽, HF/0.0% MaxEPA; ○, HF/1.2% MaxEPA; △, HF/2.4% MaxEPA; ♦, HF/4.7% MaxEPA; ●, HF/7.1% MaxEPA; □, HF/9.4% MaxEPA.

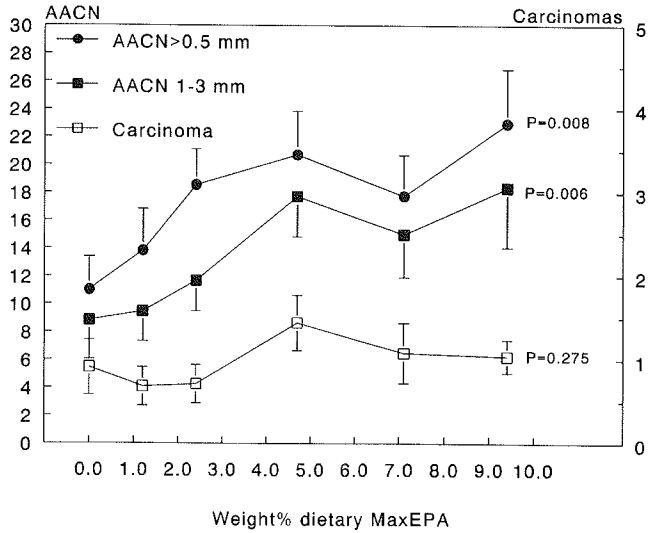

Figure 3. Number of (pre)neoplastic lesions in the pancreas of azaserine-treated rats maintained on a high fat diet containing increasing levels of MaxEPA for 12 months. Statistics: Analysis of variance followed by linear trend tests (orthogonal contrasts).

Table 4 Body and organ weight at autopsy[a]

Diet group[b]		N	Absolute weight (g)			Relative weight (g/kg)	
			Body wt[c]	Pancreas wt[d]	Liver wt[e]	Pancreas wt[d]	Liver wt[e]
LF	sal	5	546±22	1.07±0.06	12.2±1.0	1.97±0.12	22.3±1.2
	aza	25	546±10	1.47±0.06	14.0±0.4	2.69±0.10	25.7±0.5
HF/0.0% MaxEPA	sal	4	588±24	1.07±0.08	15.1±0.8	1.81±0.06	25.6±0.4
	aza	22	571±12	1.80±0.14	14.6±0.5	3.13±0.19	25.4±0.6
HF/1.2% MaxEPA	sal	5	584±36	0.93±0.07	14.6±0.9	1.58±0.06	24.9±0.3
	aza	21	570±13	1.57±0.10	14.7±0.5	2.76±0.16	25.8±0.6
HF/2.4% MaxEPA	sal	5	566±31	1.26±0.07	14.6±1.3	2.23±0.11	25.6±1.3
	aza	23	591±17	1.79±0.18	15.7±0.6	2.98±0.21	26.5±0.5
HF/4.7% MaxEPA	sal	4	632±41	1.32±0.20	16.3±1.2	2.17±0.44	25.8±0.6
	aza	24	595±16	1.96±0.15	15.7±0.6	3.28±0.22	26.2±0.5
HF/7.1% MaxEPA	sal	5	637±51	1.04±0.09	16.5±1.5	1.68±0.22	25.9±0.8
	aza	23	613±14	1.78±0.11	16.4±0.5	2.87±0.15	26.7±0.5
HF/9.4% MaxEPA	sal	4	643±53	1.04±0.11	18.8±1.9	1.65±0.25	29.1±0.8
	aza	22	572±17	1.95±0.15	16.2±0.7	3.37±0.20	28.1±0.8

[a]Values are means ± SEM; [b]LF, low fat; HF, high fat; sal, saline-treated; aza, azaserine-treated
Statistics: analysis of variance + Dunnetts tests: [c]$P<0.05$ (LF *versus* HF); [d]$P<0.001$ (sal *versus* aza); [e]$P<0.01$ (LF *versus* HF).

Table 5 Incidence and number of pancreatic (pre)neoplastic lesions in azaserine-treated rats maintained on a low fat diet or a high fat diet including 0.0, 1.2, 2.4, 4.7, 7.1 or 9.4 wt% MaxEPA for 12 months.

Mean no. of (pre)neoplastic lesions[a]								
	LF	HF/ wt% MaxEPA						p[c]
		0.0%	1.2%	2.4%	4.7%	7.1%	9.4%	
No. of rats	25	22	21	23	24	23	21	
Incidence (%)[b]	1 (4)[1]	9 (41)	9 (43)	10 (43)	14 (58)	13 (56)	14 (67)	
AACN (0.0<Ø<1.0 mm)	0.76±0.23[2]	11.00±2.38	13.81±3.00	18.54±2.55	20.71±3.12	17.75±2.93	22.96±3.92	0.008
AACN (1.0<Ø<3.0 mm)	0.68±0.48[3]	8.82±2.78	9.46±2.14	11.67±2.21	17.71±2.89	14.99±3.08	18.35±4.28	0.006
Adenoma (Ø>3.0 mm)	0.16±0.16	0.59±0.46	0.55±0.23	0.29±0.11	0.79±0.25	0.25±0.11	0.74±0.35	0.780
Carcinoma *in situ*	0.04±0.04	0.36±0.20	0.46±0.22	0.50±0.21	0.76±0.21	0.92±0.35	0.78±0.19	0.068
Adenocarcinoma	0.00±0.00	0.55±0.17	0.23±0.09	0.21±0.09	0.68±0.18	0.17±0.10	0.26±0.09	0.357
Total carcinoma	0.04±0.04[3]	0.91±0.33	0.68±0.23	0.71±0.23	1.44±0.33	1.08±0.36	1.04±0.20	0.275

[a]Values are means ± SEM; [b]Incidence, the percentage of tumour-bearing rats; [c]*P* for linear trend tests among HF groups (orthogonal contrasts). Incidence of tumours is significantly lower in the LF group in comparison with the HF groups ([1]$P<0.001$; Pearson χ^2-test). Number of lesions is significantly lower in the LF group in comparison with the HF groups ([2]$P<0.001$; [3]$P<0.05$; analysis of variance followed by Student's *t* test).

In blood plasma and pancreas the levels of OA (C18:1) decreased and the levels of EPA (C20:5) and DHA (C22:6) increased, parallel to the dietary supply of these fatty acids. Levels of LA (C18:2) increased and AA (C20:4) decreased in both blood plasma and pancreas of saline- and azaserine-treated rats. Significant linear increases were observed in levels of LA (C18:2; $P<0.001$), EPA (C20:5; $P<0.001$) and DHA (C22:6; $P<0.001$) and significant linear decreases were observed in levels of OA (C18:1; $P<0.01$) and AA (C20:4; $P<0.001$; except for C20:4 in pancreas of saline-treated rats: $P=0.926$).

LA (C18:2) levels in microsomes from tumours were significantly lower in all diet groups measured ($P<0.05$, at least), whereas AA (C20:4) levels were significantly elevated in tumours from animals fed 2.4% and 4.7% MaxEPA in comparison to non-tumorous pancreas from azaserine-treated rats ($P<0.01$). No differences were found in EPA and DHA profiles, except for the DHA level in tumours from the group fed 2.4% MaxEPA, which was significantly higher in comparison with non-tumorous tissue ($P<0.001$). The profiles of LA (C18:2), AA (C20:4), EPA (C20:5) and DHA (C22:6) in pancreatic tumours are depicted in Figure 6.

Prostaglandins. Pancreatic 6-keto-$PGF_{1\alpha}$- and $PGF_{2\alpha}$-levels were significantly lower in saline-treated rats maintained on the high fat diet containing 4.7% and 9.4% MaxEPA, in comparison to saline-treated rats maintained on the high fat diet without MaxEPA ($P<0.05$ and $P<0.01$, for the respective prostaglandins). In azaserine-treated rats $PGF_{2\alpha}$-levels were significantly lower ($P<0.05$) in pancreata from animals fed 9.4% MaxEPA in comparison to those from animals fed the high fat diet without MaxEPA. Pancreatic $PGF_{2\alpha}$- and TXB_2-levels were inversely related to the percentage of dietary MaxEPA in both saline- and azaserine-treated rats. Parts of all grossly visible pancreatic rat tumours were analyzed for prostaglandins. Microscopic examination of the grossly visible pancreatic lesions indicated that the number of adenocarcinomas in the group given high fat without MaxEPA was high enough ($N=3$) to justify statistical analysis. PGE_2-, TXB_2-, $PGF_{2\alpha}$-, but not 6-keto-$PGF_{1\alpha}$-levels in pancreatic adenocarcinomas were significantly elevated ($P<0.001$) in comparison with non-tumorous pancreas from azaserine-treated rats (Fig. 7).

Discussion

The results of the present study demonstrated a strong enhancing effect of a high fat (25 wt%) diet including 5 wt% LA on pancreatic carcinogenesis in azaserine-treated rats. Moreover, including various amounts of fish oil (MaxEPA) in the high fat diet resulted in a significant dose-related linear increase in the number of putative preneoplastic AACN with a diameter between 0.5 and 3.0 mm. It appeared that long-chain PUFA of the ω-3

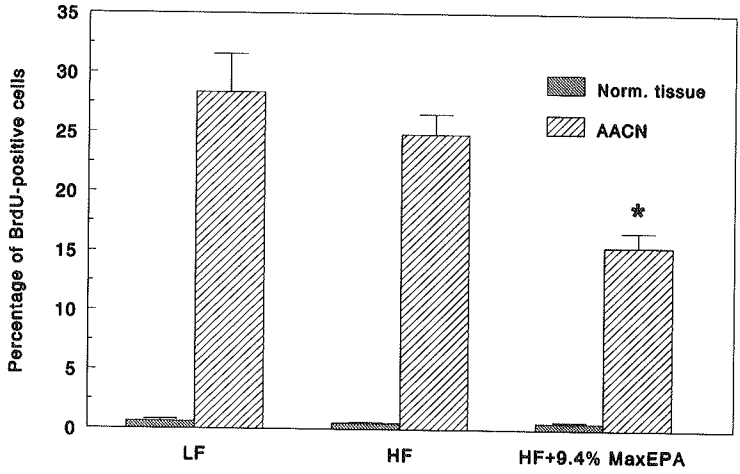

Figure 4. Labeling index of normal and preneoplastic pancreatic tissue (AACN) in rats maintained on a 5 wt% fat AIN[76]-based diet, a 25 wt% fat AIN[76]-based diet or a 25% fat AIN[76]-based diet containing 9.4 wt% MaxEPA for 12 months. Statistics: analysis of variance followed by Student's *t* tests. *$P<0.05$.

series from fish oil in the diet are readily taken up by the rat, resulting in increased concentrations in both plasma and pancreas, concomittant with increasing concentration in the feed. The presence of MaxEPA in the diet significantly decreased cell proliferation in AACN and influenced the metabolism of linoleic acid, arachidonic acid and hence prostaglandin synthesis substantially.

Mean terminal body weights were significantly lower in rats maintained on a LF diet in comparison to rats maintained on a HF diet, pointing to a difference in energy intake. A low energy intake has frequently been related to inhibition of the carcinogenic process. However, to ascribe the presently observed differences between the development of tumours in the HF and the LF group to a difference in energy intake is most probably not a valid explanation, since mean body weight gain of the HF and LF groups were similar during the whole study, except at autopsy. This observation, in combination with the finding that caloric intake was similar among all groups led to the conclusion that differences in energy intake in this study do not account for the observed differences in tumorigenic response. The observed differences in tumour response must be ascribed to the level and type of fat in the diet. The presently used HF diet (25 wt% fat, including 5 wt% LA) was reported to promote the growth of AACN significantly, in comparison with

Table 6 Fatty acid composition of plasma of untreated and azaserine-treated rats maintained on a high fat diet containing increasing levels of MaxEPA for 12 months[a,b].

	Percentage of dietary MaxEPA											
	0.0%		1.2%		2.4%		4.7%		7.1%		9.4%	
Fatty acid	saline treated	aza treated	saline treated	aza treated	saline treated	aza treated	saline treated	aza treated	saline treated	aza treated	saline treated	aza treated
C14:0	0.1±0.1	0.3±0.1	0.2±0.1	0.2±0.0	0.3±0.0	0.1±0.0	0.3±0.0	0.2±0.0	0.5±0.0	0.1±0.0	0.5±0.0	0.4±0.1
C16:0	10.1±0.1	13.8±0.5	11.7±0.2	13.5±0.2	12.1±0.2	16.1±1.2	12.8±0.4	15.3±0.2	13.2±0.1	17.0±0.1	13.4±0.2	17.8±0.7
C16:1*trans*	0.5±0.0	0.9±0.1	0.8±0.0	0.9±0.0	0.6±0.0	0.9±0.1	0.7±0.1	0.8±0.0	0.5±0.0	0.7±0.0	0.4±0.0	0.6±0.0
C16:1*cis*	0.2±0.0	0.3±0.1	0.4±0.0	0.3±0.0	0.4±0.0	0.4±0.0	0.5±0.0	0.7±0.1	0.6±0.1	1.1±0.1	0.6±0.0	1.3±0.3
C18:0	14.4±0.7	14.5±0.3	13.3±0.3	13.2±0.2	13.2±0.4	16.2±1.1	13.2±0.5	14.3±0.2	13.0±0.5	12.3±0.3	13.1±0.3	12.5±0.4
C18:1	27.4±2.1	28.8±1.9	27.8±0.7	28.3±1.0	26.7±1.6	23.3±1.0	24.4±1.1	21.1±0.4	18.9±0.8	23.4±0.9	16.7±0.3	19.4±0.7[c]
C18:2	13.8±0.6	13.4±0.6	14.0±0.8	15.0±0.2	15.2±0.5	14.1±0.8	15.5±0.2	14.9±0.1	16.8±0.8	16.0±0.3	17.3±0.2	17.2±0.1[c]
C18:3 (ω-6)	0.4±0.0	0.5±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.4±0.0	0.4±0.0	0.4±0.0	0.4±0.0
C20:0	0.2±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.0±0.0
C20:1	0.4±0.0	0.3±0.0	0.4±0.0	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.1±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.2±0.0
C20:2	0.4±0.0	0.4±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.2±0.0
C20:3	0.4±0.0	0.4±0.0	0.6±0.0	0.5±0.0	0.7±0.0	0.8±0.0	1.0±0.1	0.9±0.0	1.0±0.1	0.9±0.0	1.1±0.1	0.9±0.0
C20:4	30.7±2.0	26.3±2.4	25.0±1.0	23.3±1.1	24.0±1.1	22.2±1.4	22.5±0.8	24.3±0.6	23.5±1.4	18.8±1.1	23.1±0.6	17.6±0.4[c]
C20:5	0.0±0.0	0.0±0.0	0.7±0.1	0.8±0.0	1.5±0.0	1.3±0.1	2.9±0.1	2.6±0.0	5.6±0.4	4.0±0.2	6.6±0.7	6.2±0.3[c]
C22:6	0.6±0.0	0.0±0.0	4.0±0.2	3.1±0.1	4.2±0.2	3.4±0.3	5.4±0.2	4.0±0.1	5.9±0.3	4.4±0.2	7.0±0.1	5.2±0.1[c]

[a]Values are mean percentages of total fatty acids ± SEM (N=3); aza, azaserine
[b]Statistics: 2-way analysis of variance or 1-way analysis of variance followed by linear trend tests (orthogonal contrasts)
[c]Statistically significant linear trend (P<0.001, except for C18:1: P<0.01)

Table 7 Fatty acid composition of pancreatic microsomes of untreated and azaserine-treated rats maintained on a high fat diet containing increasing levels of MaxEPA for 12 months[a,b].

	Percentage of dietary MaxEPA											
	0.0%		1.2%		2.4%		4.7%		7.1%		9.4%	
Fatty acid	saline treated	aza treated	saline treated	aza treated	saline treated	aza treated	saline treated	aza treated	saline treated	aza treated	saline treated	aza treated
C14:0	0.4±0.0	0.2±0.0	0.5±0.0	0.3±0.0	0.6±0.1	0.3±0.1	0.9±0.1	0.5±0.0	1.2±0.0	0.6±0.0	1.2±0.0	0.5±0.0
C16:0	9.1±0.7	13.5±0.8	13.6±1.0	12.4±1.0	13.0±0.6	14.5±1.0	16.3±1.0	13.0±1.1	17.9±1.4	15.5±0.8	17.0±0.7	15.5±0.8
C16:1*trans*	1.0±0.1	0.9±0.0	1.1±0.1	0.7±0.0	1.4±0.2	0.8±0.1	1.2±0.1	0.7±0.0	1.1±0.1	0.7±0.0	0.9±0.0	0.6±0.0
C16:1*cis*	0.3±0.0	0.4±0.0	0.4±0.0	0.3±0.0	0.6±0.1	0.5±0.0	0.9±0.1	0.6±0.1	1.4±0.2	1.0±0.1	1.6±0.1	1.0±0.1
C18:0	5.6±0.5	7.4±0.6	7.5±0.6	7.7±0.4	7.0±0.1	7.7±0.3	7.9±0.9	8.3±1.1	8.6±0.6	8.5±0.3	7.3±0.8	8.8±0.7
C18:1	65.3±1.0	51.3±2.8	57.2±1.9	52.4±1.3	56.9±1.6	48.4±1.0	50.5±5.6	45.6±2.7	45.5±2.8	46.7±2.0	40.4±3.2	35.6±2.0[c]
C18:2	15.4±0.2	13.6±0.4	15.0±0.3	14.7±0.5	15.6±0.1	15.2±0.3	17.0±0.6	17.8±0.2	17.0±0.2	17.9±0.3	18.8±0.3	20.7±0.5[c]
C18:3 (ω-6)	1.0±0.2	0.2±0.0	1.6±0.4	0.2±0.0	1.6±0.1	0.1±0.0	1.1±0.4	0.3±0.1	1.5±0.1	0.3±0.1	1.0±0.2	0.2±0.0
C20:0	0.5±0.1	0.2±0.0	0.0±0.0	0.2±0.1	0.8±0.1	0.2±0.0	0.6±0.2	0.2±0.0	0.7±0.1	0.3±0.0	0.4±0.1	0.2±0.0
C20:1	0.4±0.0	0.7±0.1	0.0±0.0	0.5±0.1	0.6±0.0	0.6±0.1	0.3±0.1	0.4±0.0	0.0±0.0	0.7±0.1	0.3±0.0	0.6±0.0
C20:2	0.0±0.0	0.3±0.0	0.0±0.0	0.2±0.0	0.0±0.0	0.3±0.1	0.2±0.0	0.0±0.0	0.0±0.0	0.3±0.0	0.1±0.0	0.2±0.0
C20:3	0.0±0.0	0.7±0.3	0.0±0.0	0.4±0.2	0.0±0.0	0.5±0.1	0.3±0.0	0.5±0.0	0.0±0.0	0.9±0.2	0.0±0.0	0.5±0.1
C20:4	1.8±0.5	8.6±1.4	3.1±0.5	5.8±0.5	2.0±0.3	6.1±0.4	3.4±0.8	4.2±1.2	2.3±0.4	4.0±0.8	1.8±0.3	4.1±0.7[c]
C20:5	0.0±0.0	0.0±0.0	0.2±0.1	0.3±0.0	0.4±0.0	0.9±0.1	1.3±0.3	2.1±0.7	1.4±0.3	2.5±0.3	1.7±0.3	3.0±0.6[c]
C22:6	0.0±0.0	0.0±0.0	0.5±0.2	1.1±0.0	0.4±0.1	1.2±0.1	1.0±0.2	1.7±0.5	1.0±0.2	1.9±0.1	1.1±0.1	2.3±0.3[c]

[a]Values are mean percentages of total fatty acids ± SEM; aza, azaserine (*N*=3)
[b]Statistics: 2-way analysis of variance or 1-way analysis of variance followed by linear trend tests (orthogonal contrasts)
[c]Statistically significant linear trend (P<0.001, except for C18:1: P<0.01, and for C20:4 in saline-treated rats: P=0.926)

a low fat control diet (18,29).

In a number of studies the chemopreventive effect of dietary fish oil has been reporte in experimental mammary gland carcinogenesis (19-22, 35, 36), colon carcinogenesis (23 26) and pancreatic carcinogenesis (27, 28). To investigate the effects of fish oil (containing high concentrations of ω-3 fatty acids), most investigators used mixtures of corn oil and fish oil or fish oil as such. Most of the studies which had the intention to vary ω-3 fatty acids in the diet, had an experimental design also resulting in a variation i dietary ω-6 fatty acid levels. Moreover, in those studies where fish oil was used as sole lipid source, the level of ω-6 fatty acids frequently was a deficient one. When mixtures o corn oil and fish oil were used, LA levels were adequate, but remained in a number of cases below 4%. This is of particular relevance in studies concerning the effects of fish o on mammary gland carcinogenesis, because Ip *et al.* (16) demonstrated a positive association between dietary LA and tumour burden up to a threshold level of around 4.4% of dietary LA. This observation has led to the hypothesis that amounts of dietary LA below this level will result in less enhancing effects. The effects of dietary fish oil may thus be obscured, since the effects of ω-6 and ω-3 fatty acids are believed to be antagonistic.

In colon carcinogenesis the influence of LA has not been proven beyond doubt, but it has been found that high fat diets containing corn oil, safflower oil, beef tallow or lard enhance colon tumorigenesis, whereas coconut oil and olive oil do not, indicating that fatty acid composition of the diet is also one of the determining factors in colon carcinogenesis (12). O'Connor *et al.* (27) reported that 20% MO caused a significant inhibition of the growth of azaserine-induced AACN in rat pancreas as compared to a 20% CO diet. In a subsequent study (28), they varied the ω-3:ω-6 ratio from 0.01 up to 7.0 by mixing CO with MO and observed a significant decrease in development of AACN with increasing ratio of ω-3:ω-6. They acknowledged that their dietary regimen also implied a variation of dietary LA from 0.6% up to 12.0%, which is within the range where growth of AACN is significantly increased (4.4% to 8.5%; ref. 16). In a previous study (18), we varied the dietary LA levels from 2.0% to 15.0%, without influencing the chain length of the fatty acids, and observed an inverse rather than a positive dose-response relationship between LA and AACN development. In order to minimize the number of variables and their possible confounding effects, it seems of paramount importance to keep LA in the diet at a constant level when investigating the effects of other variables such as dietary fish oil. The results of the present study demonstrate an enhancing rather than an inhibitory effect of MaxEPA on growth of AACN whereas no significant effect on development of pancreatic carcinomas was found. The present contradictory results in comparison to those of other studies (27,28) can, in our view, be ascribed to our study

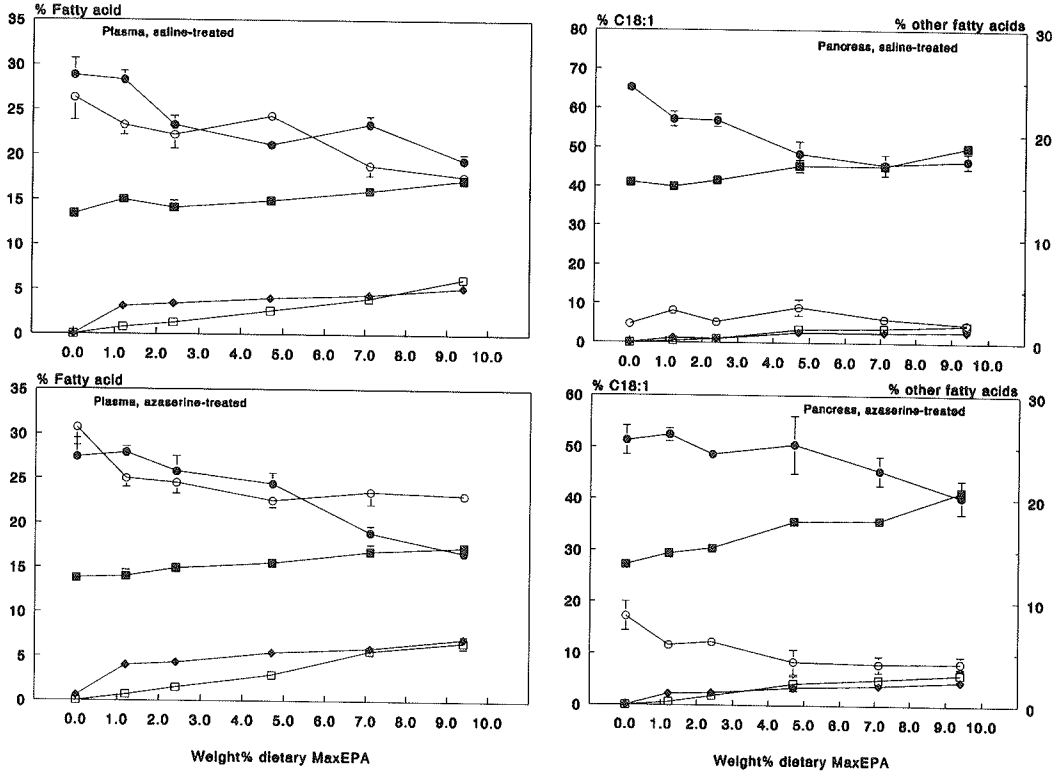

Figure 5. Fatty acid profiles in plasma and pancreatic microsomes of saline- and azaserine-treated rats as a function of dietary MaxEPA. ●, C18:1; ■, C18:2; ○, C20:4; □, C20:5; ♦, C22:6. Values are means ± SEM (N = 3). Statistics: Analysis of variance followed by linear trend tests (orthogonal contrasts). Significant linear trend for C18:1 ($P<0.01$), C18:2 ($P<0.001$), C20:4 ($P<0.001$; except C20:4 in saline treated rat pancreas: $P=0.926$), C20:5 ($P<0.001$), C22:6 ($P<0.001$).

design in which dietary LA is kept constant. The present results also emphasize that an enhancing effect on growth of early stages in pancreatic carcinogenesis (AACN), does not necessarily lead to more carcinomas. Consequently, a long-term (12-15 months) study is needed to establish the modulating effects of dietary or other factors on pancreatic carcinogenesis. Short-term (4-6 months) studies may be suitable to study certain steps in pancreatic carcinogenesis, but a long-term study is needed to find out the implications for the development of pancreatic carcinomas. The observation that after 12 but not after 6 months (29) an enhancing effect of fish oil on the growth of AACN is seen, suggests that fish oil modulates pancreatic carcinogenesis in azaserine-treated rats only after a rather

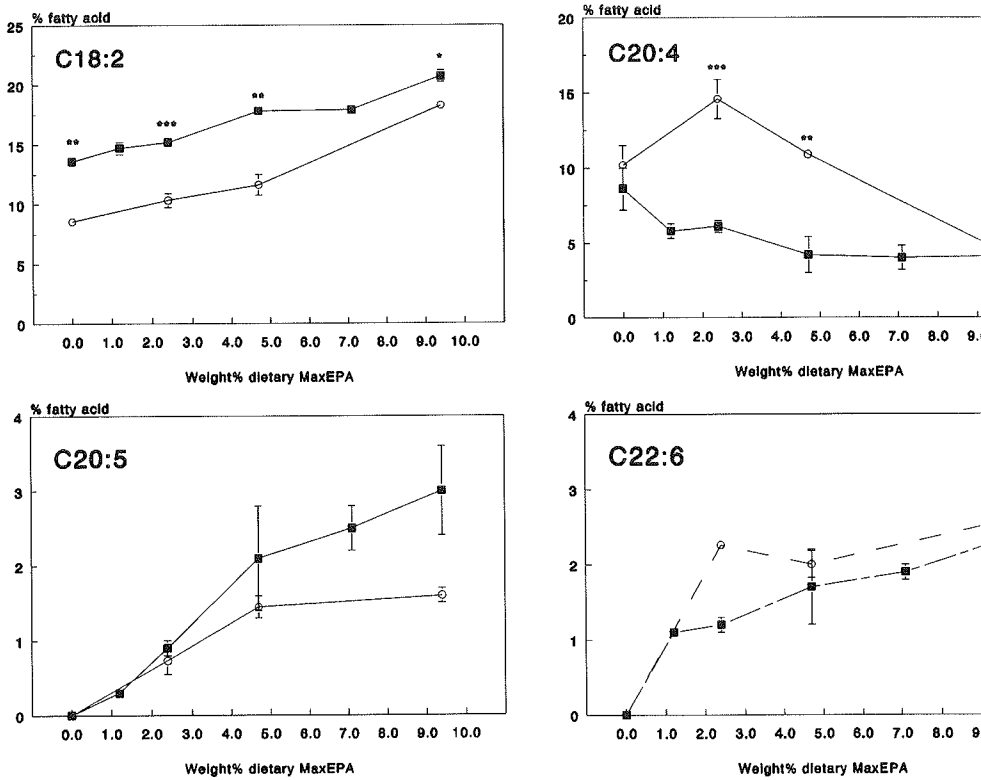

Figure 6. LA (C18:2), AA (C20:4), EPA (C20:5) and DHA (C22:6) levels in microsomes of pancreas tumours (○) and non-tumorous pancreas (■) of rats maintained on a high fat diet containing increasing levels of MaxEPA.

long period of feeding. Moreover, it can not be excluded that prolonged feeding of diets high in MaxEPA for over 12 months finally also will modulate development of pancreati carcinomas.

The significantly higher LI of the acidophilic AACN *versus* normal acinar tissue, as observed in the present study, is in agreement with the previously observed high growth potential of these putative preneoplastic lesions (32). Although MaxEPA caused a dose-related increase in number of AACN, the LI of AACN in the pancreas of animals maintained on the HF diet including 9.4% MaxEPA was significantly lower than the LI AACN present in the pancreas of rats fed the HF diet without MaxEPA. However, when only the LI of the AACN with a diameter over 0.5 mm were compared, the significant difference between the HF and the HF/MaxEPA group disappeared. On the other hand, t

LI of AACN smaller than 0.5 mm observed in the HF/MaxEPA group was significantly lower than that of the HF group, suggesting that MaxEPA influenced cell proliferation mainly in small AACF, whereas cell proliferation of larger lesions is not influenced any more. It seems that the effect of dietary MaxEPA depends on the stage of the carcinogenic process: at an early stage MaxEPA inhibits cell proliferation, leading to a decreased growth of AACF (28), whereas at a later stage MaxEPA enhances rather than inhibits growth of AACN (29). Furthermore, it appears that MaxEPA has no effect on the development of carcinomas, which occurs at a late stage of the carcinogenic process.

The main compositional changes in fatty acids in blood plasma and pancreas as a result of increasing MaxEPA in the diet were a decrease in OA (C18:1) and increases in EPA (C20:5) and DHA (C22:6). An increasing level of dietary MaxEPA caused a shift in the ratio of EPA:DHA from equal or higher than 1 towards an EPA:DHA-ratio of equal or less than 1, both in plasma and pancreas. An explanation for this interesting observation may be formation of DHA from EPA. This newly formed DHA accompanied by DHA from the diet accumulates, resulting in higher DHA levels than EPA levels in plasma and tissue (37). Furthermore, the presently observed fatty acid profiles show that feeding MaxEPA causes a decrease of AA (C20:4) levels in both blood plasma (saline- and azaserine-treated rats) and pancreatic tissue (azaserine-treated rats), which can be ascribed to either replacement of AA by EPA (37) or to an inhibition of the LA-converting enzyme δ^6-desaturase by EPA and/or DHA (38). Evidence in favour of the latter process is given by the observed increase in plasma and pancreatic levels of LA in MaxEPA fed animals, although the amount of LA in the diet was kept constant.
The differences between fatty acid profiles in pancreatic tumours and non-tumorous tissue indicate an accelerated turnover of LA to AA in tumour tissue, which is reflected in consistently lower LA levels accompanied by higher AA levels, pointing to an altered fatty acid metabolism in neoplastic tissue.

LA may give rise to PGs of the 2-series via AA. It has been demonstrated that some of these PGs stimulate cell proliferation ($PGF_{2\alpha}$; ref. 39) or have immunosuppressive properties (PGE_2; ref. 40) and hence may either promote tumour growth or disturb inhibition of tumour development. ω-3 PUFA may inhibit the formation of PGs derived from LA and AA, by either inhibiting the conversion of LA to AA or inhibiting the actual PG formation via cyclooxygenase. The dose-related decrease of TXB_2 and $PGF_{1\alpha}$ in non-tumorous pancreatic tissue of rats maintained on a high fat diet containing 0.0%, 4.7% or 9.4% MaxEPA, together with the increased LA levels in plasma and non-tumorous pancreas, demonstrate that ω-3 fatty acid influence LA/AA metabolism as expected. The decreased PG-levels in AACN-containing pancreatic tissue do not correlate with the increased mean number of AACN when dietary MaxEPA is increased, suggesting no

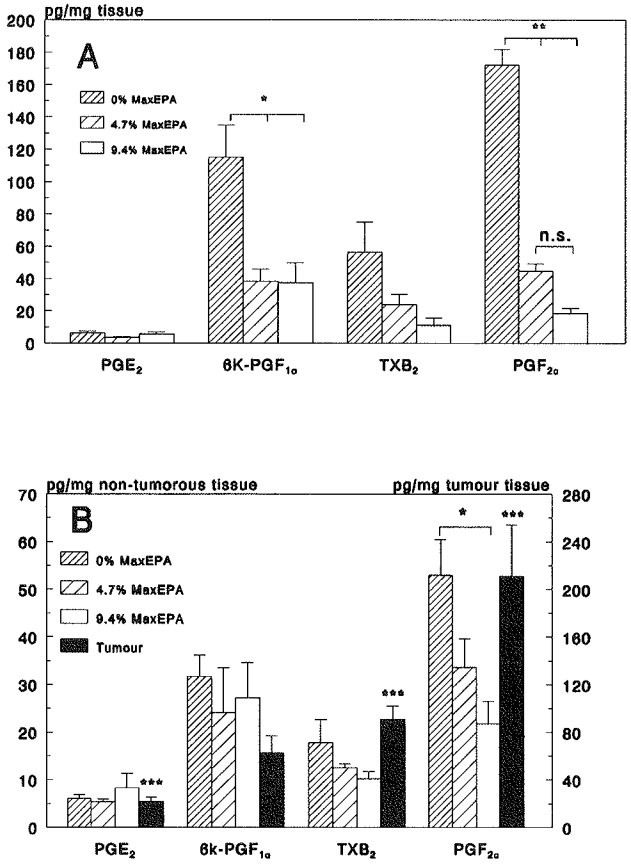

Figure 7. Prostaglandin levels in pancreas of (A) saline-treated rats and (B) azaserine-treated rats. Statistics analysis of variance followed by Student's *t* test. *P<0.05, **P<0.01, ***P<0.001.

strong relationship between growth of preneoplastic acinar lesions and prostaglandins of the 2-series. PG levels are elevated, however, in pancreatic carcinomas indicating that they may play a role in the development of AACN to ultimate pancreatic carcinomas.
From the present results it can be concluded that (i) dietary MaxEPA has a dose-related enhancing effect on the development of AACN, but not on development of carcinomas in the pancreas of azaserine-treated rats consuming diets with 25 wt% fat and 5 wt% LA and (ii) dietary MaxEPA inhibits the conversion of LA to AA, as well as the conversion of AA to TXB_2 or $PGF_{2\alpha}$ in non-tumorous pancreatic tissue and (iii) PGs may play a role in the growth/development of pancreatic adenocarcinomas, but probably less so in the growth of AACN.

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Effects of dietary fish oil (MaxEPA) on N-nitrosobis (2-oxopropyl)amine-induced pancreatic carcinogenesis in hamsters[1]

M.J. Appel and R.A. Woutersen[2], TNO Nutrition and Food Research Institute, Division of Toxicology, Department of Pathology, P.O. Box 360, 3700 AJ, Zeist (The Netherlands)

Submitted to Carcinogenesis

Abstract

In the present study the putative chemopreventive effect of dietary fish oil (MaxEPA) on BOP-induced pancreatic carcinogenesis in hamsters has been investigated. Groups of hamsters were maintained on a semi-purified low fat (LF; 5 wt%) diet or on semi-purified high fat (HF; 25 wt%) diets containing 2 wt% linoleic acid (LA) and including 0.0, 1.2, 2.4, 4.7, 7.1 or 9.4 wt% MaxEPA.

The number of borderline lesions (BLL[3]) was significantly higher ($P<0.05$) in the HF groups containing 1.2, 2.4 or 9.4 wt% MaxEPA in comparison with the HF group without MaxEPA. The number of malignant pancreatic lesions were similar in all groups.

An increase in dietary MaxEPA resulted in significant alterations of the fatty acid profiles of both blood plasma and pancreatic microsomes. In general, significant increases of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) levels were accompanied by decreases of arachidonic acid (AA) levels in both plasma and pancreatic microsomes. The linoleic acid (LA) level decreased in plasma and increased in pancreatic microsomes with increasing dietary MaxEPA. In microsomes from pancreatic ductular

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: BLL, borderline lesion; LF, low fat; HF, high fat; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LA, linoleic acid; AA, arachidonic acid; OA, oleic acid; PUFA, polyunsaturated fatty acids; BOP, N-nitrosobis(2-oxopropyl)amine; LI, labeling index; CDC, cystic ductal complex; TDC, tubular ductal complex; AACF, atypical acinar cell foci; PG, prostaglandin; TXB_2, thromboxane B_2; HPOP, N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine; MO, menhaden oil.

adenocarcinomas LA levels remained similar, whereas AA, EPA and DHA levels were consistently higher in comparison with microsomes from non-tumorous pancreas of BOP-treated hamsters.

Prostaglandin (PG) E_2 and $PGF_{2\alpha}$ levels were higher in pancreas from BOP-treated hamsters than in pancreas from saline-treated hamsters. The pancreatic levels of PGE_2 ($P<0.05$), 6-Keto-$PGF_{1\alpha}$ ($P<0.01$) and $PGF_{2\alpha}$ ($P<0.05$) decreased significantly with increasing dietary MaxEPA. The levels of PGE_2 ($P<0.001$), 6-Keto-$PGF_{1\alpha}$ ($P<0.05$), $PGF_{2\alpha}$ ($P<0.001$) and thromboxane (TX) B_2 ($P<0.001$) in pancreatic adenocarcinomas were higher than in non-tumorous pancreas of BOP-treated hamsters.

BOP-treatment caused a significant increase ($P<0.001$) in the Labeling Index (LI) of acinar cells in comparison with acinar cells of untreated controls. The LI in centroacinar and ductular cells was not affected by BOP-treatment. Neither type nor amount of dietary fat influenced the LI in cystic ductular complexes (CDC), tubular ductular complexes (TDC) or BLL. The LI of TDC was significantly higher than that of CDC ($P<0.001$) and lower than that of BLL ($P<0.001$).

It is concluded that (i) dietary fish oil has a slight enhancing rather than an inhibitory effect on BOP-induced pancreatic carcinogenesis in hamsters and (ii) dietary fish oil dose-dependently inhibits the conversion of LA to AA and of AA to certain PGs and (iii) the consistent increase in acinar cell proliferation after BOP-treatment indicates a possible role for acinar cells in BOP-induced pancreatic carcinogenesis in the hamster.

Introduction

In the last two decades research into the modulating effects of dietary fat on experimental carcinogenesis has been extensive. The modulating effects of dietary fat has been demonstrated in animal models for cancer of the breast, colon and pancreas (1-6). Two types of polyunsaturated fatty acids (PUFA), ω-6 PUFA and ω-3 PUFA, are believed to be involved in dietary fat-modulated carcinogenesis. ω-6 PUFAs (mainly in the form of linoleic acid; LA) have tumour promoting properties, whereas ω-3 PUFAs inhibit tumour growth. From the studies of Ip *et al.* (7) and Roebuck *et al.* (8), it was concluded that threshold levels of dietary LA of 4.4% and 4.4-8.5% were needed to elicit a maximum response in development of DMBA-induced mammary tumours and azaserine-induced pancreatic tumours, respectively. In experimental colon carcinogenesis, where dose-effects of dietary LA have not been investigated in detail, polyunsaturated fat, but not saturated fat, promotes tumour growth, indicating a possible role for LA (9). In a previous study we could not confirm the promoting effects of increasing levels of dietary LA on pancreatic

carcinogenesis, either in azaserine-treated rats or in N-nitrosobis(2-oxopropyl)amine (BOP)-treated hamsters (10). In the latter study, hamsters fed the lowest level of dietary LA (2 wt%) exhibited the largest tumour response.

In studying the effects of dietary ω-3 fatty acids from fish oil on carcinogenesis it is of paramount importance to keep, as far as possible, all variables that may influence the carcinogenic process, at a constant level. The present study was designed to investigate the effects of dietary fish oil (MaxEPA) on pancreatic carcinogenesis in hamsters using a high fat diet containing a fixed level of linoleic acid (2 wt% LA) in combination with a variable level of fish oil.

Materials and Methods

Animals. Two hundred eighty 4-week old male Syrian golden hamsters were obtained from Harlan-CPB, Austerlitz, The Netherlands. The hamsters were kept in macrolon cages, 5 animals per cage, on a softwood bedding and under standard laboratory conditions. During the initiation phase the hamsters were fed a standard laboratory chow. At 5, 6 and 7 weeks of age 210 hamsters received a s.c. injection of 20 mg N-Nitrosobis(2-oxopropyl)amine (BOP; Nacalai Tesque Inc., Kyoto, Japan) per kg body wt, which was dissolved freshly in 0.9% NaCl-solution. Seventy control animals received injections with 0.9% NaCl-solution only. Directly after the third injection the animals were randomly allocated to 7 groups of 40 animals each (ten 0.9% NaCl-treated controls plus 30 BOP-treated animals).

Diets. One week after the last injection the animals were maintained on an AIN^{76}-based diet containing either 5 or 25 wt% fat. One group received an AIN^{76}-based diet including 5 wt% lard (Best Food, The Netherlands; LF), containing a marginal (0.61 wt%) but nutritionally adequate level of linoleic acid (LA; ref. 11). The high fat (HF) experimental groups received a 25 wt% fat diet containing 2 wt% LA and including 0.0, 1.2, 2.4, 4.7, 7.1 or 9.4 wt% (0, 2.5, 5, 10, 15 and 20 en%) MaxEPA. The HF diets were compounded by mixing high linoleic safflower oil (Unilever, Vlaardingen, The Netherlands) with high oleic sunflower oil (Contined, Bennekom, The Netherlands) and MaxEPA (fish oil; Seven Seas, Hull, United Kingdom). The safflower oil, the sunflower oil and the MaxEPA contained 0.55 g/kg, 0.44 g/kg and 1.80 g/kg vitamin E, respectively. α-Tocopherol was added to all diets as extra antioxidant to a level of 0.450 g/kg. The composition of the AIN^{76}-based diets and the fatty acid compositions of the oils are summarized in Tables 1 and 2. The diets were prepared monthly and stored at -20°C until use. The animals were fed daily to minimize oxidation of the polyunsaturated fatty acids. Peroxide values (as measured by means of the A.O.C.S. official method in terms of milliequivalents peroxide per kg) of the HF/9.4% MaxEPA containing diet, stored at -20°C for 3 months, were below 1.0 and remained below 1.0 when exposed to air at room temperature for 24 hours. Longer periods of exposure to air at room temperature caused a rapid increase of the peroxide value. The profile of the dietary fatty acids of interest are depicted in Figure 1. Food consumption was measured daily during the first 3 months and on 7 consecutive days per month during the remainder of the study. The animals were weighed weekly during the first 3 months of the study and monthly, thereafter.

Table 1 Percentage fatty acid composition of the dietary lipids[a]

Fatty acid	Lard	SA	SO	MaxEPA
C14:0	1.8	0.1	0.1	7.1
C16:0	25.6	7.0	3.8	17.5
C16:1	2.9	0.1	0.1	9.9
C18:0	14.2	2.6	4.0	4.2
C18:1	43.1	13.1	82.6	12.9
C18:2 (ω-6)	8.7	76.0	7.7	4.2
C18:3 (ω-3)	0.6	0.4	0.1	--
C20:0	0.2	0.3	0.3	2.5
C20:1	0.8	0.2	0.3	4.4
C20:4 (ω-6)	--	--	--	1.6
C20:5 (ω-3)	--	--	--	18.2
C22:0	0.1	0.2	0.9	--
C22:1	--	--	--	1.1
C22:4 (ω-6)	--	--	--	1.2
C22:6 (ω-3)	--	--	--	14.9
Total	98.0	99.9	100.0	99.7
ω-3/ω-6 (ratio)	0.07	0.01	0.01	4.7

[a]SA, Safflower oil; SO, Sunflower oil (Trisun); MaxEPA, fish oil

Monitoring and autopsy. Three days before autopsy 5 saline-treated control hamsters and 5 BOP-treated hamsters from the LF, the HF/0.0% MaxEPA and the HF/9.4% MaxEPA groups had an Alzet osmotic pump (Alza Corp., Palo Alto, USA, model 2001) implanted s.c., containing 200 μl of a BrdU solution (Sigma Chemie, Brussels, Belgium; conc. 25 mg/ml). The release rate of this pump was 1 μl/h. Autopsy was performed 372, 373 or 374 days after the last injection of BOP. The animals were anaesthetized with ether and exsanguinated by cannulating the abdominal aorta. Blood was collected in heparin-containing tubes, centrifuged at 1700 g for 20 min and stored at -80°C until analysis. The pancreata were excised and weighed. About one-third of the pancreas of 2 animals per cage was snap-frozen in liquid nitrogen and stored at -80°C until fatty acid or prostaglandin analysis. The remaining two-thirds of these pancreata plus all other pancreata were fixed in 10% neutral buffered formalin. Pancreata of BrdU-treated animals were fixed for 24 hours in formalin followed by 72 hours in 70% ethanol. The organs were processed for microscopy by conventional methods, step-sectioned at 5 μm and collected on organosilane-coated slides. Parallel sections were stained with haematoxylin and eosin (H&E) or with a monoclonal antibody against BrdU (Organon Technics, the Netherlands) and examined by light microscopy. To examine centroacinar cells, parallel sections of hamster pancreas were stained with haematoxylin, a monoclonal

Table 2 Weight percentage composition of the AIN[76]-based diets.

Premix				LF	HF/ wt% MaxEPA					
Diet components	LF	HF			0.0%	1.2%	2.4%	4.7%	7.1%	9.4%
Casein	20.00	25.00	Premix	95.00	75.00	75.00	75.00	75.00	75.00	75.00
DL-Methionine	0.30	0.37	Lard	4.74	--	--	--	--	--	--
Wheat starch	63.50	36.79	Safflower oil	0.26	0.33	0.37	0.42	0.53	0.63	0.73
Cellulose	5.00	6.18	Sunflower oil	--	24.67	23.45	22.23	19.77	17.30	14.85
Choline bitartrate	0.20	0.25	MaxEPA	--	--	1.18	2.35	4.70	7.07	9.42
AIN[76] minerals	3.50	4.32	Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0
AIN[76] vitamins	1.00	1.24								
CaH_2PO_4	1.50	1.85	C20:5+C22:6	0.0	0.0	0.39	0.74	1.56	2.34	3.12
Total	95.00	75.00	C18:2	0.61	2.15	2.14	2.13	2.12	2.11	2.09

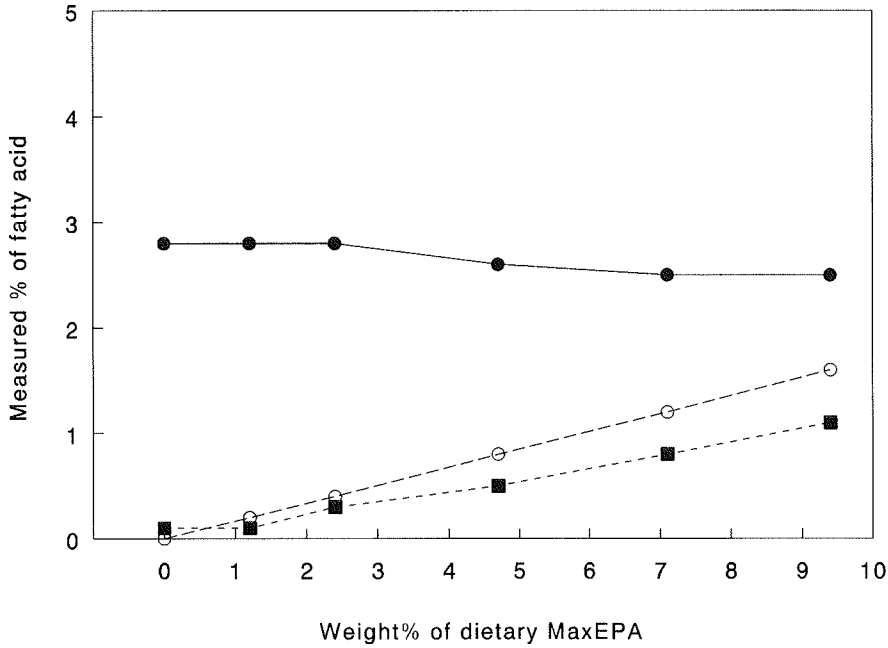

Figure 1. Percentages of selected fatty acids in the diets containing MaxEPA. ●, LA; ○, EPA; ■, DHA.

antibody against BrdU and a polyclonal antibody against cytokeratin filaments (Dakopatts, Glostrup, Denmark), which was made visible with an alkaline phosphatase-conjugated second antibody. The antibody against cytokeratins has been reported to bind specifically to ductular and centroacinar cells (12).

Pancreatic lesions were classified as cystic ductular complexes (CDC), tubular ductular complexes (TDC), borderline lesions (BLL), carcinomas *in situ* and ductular adenocarcinomas, according to the criteria of Meijers *et al.* (13) and Pour and Wilson (14).

Labeling Index. In slides stained for BrdU the Labeling Index (LI) was expressed as the ratio of brown stained BrdU-positive cells to blue stained normal cells. To select a random sample of acinar cells, only nuclei were counted that were located beneath the crossings of the horizontal and vertical lines in a 20x20 intra-ocular grid at high power magnification (400x). In normal pancreatic acinar tissue from saline-treated hamsters and phenotypically normal acinar tissue from BOP-treated hamsters, 344±35 nuclei per pancreas were counted. The LIs in normal ductal/ductular tissue and in centroacinar cells were determined by counting 350±53 and 673±167 nuclei per pancreas, respectively. A mean of 5 CDC (220±67 nuclei per CDC), a mean of 5 TDC (239±83 nuclei per TDC) and a mean of 1.7 BLL (172±67 nuclei per BLL) per hamster were scored.

Analytical procedures. Fatty acids. Pancreatic microsomes were prepared by homogenizing 100-200 mg pancreatic tissue in 0.1 M Tris-KCl buffer, pH 7.4. Subsequently, the homogenate was centrifuged at 10,000 g for 30 min and the supernatant was centrifuged at 105,000 g for 60 min. The microsomal pellet was resuspended in 300 μl buffer and stored at -30°C until fatty acid analysis. Total lipids were extracted from

50 μl aliquots of pancreatic microsomes or from 100 μl aliquots of blood plasma as described by Folch *et al.* (15). Fatty acid composition was determined by GLC. The samples were eluted on a capillary BD23 column (J&W Scientific) after saponification with NaOH in methanol and transmethylation of the fatty acids with borontrifluoride-methanol.

Prostaglandins. Pancreatic tissue (100-200 mg) was homogenized in 0.1 M Phosphate Buffered Saline (PBS; pH 7.4) containing 15% methanol and applied to Sep-pak C-18 columns (J.T. Baker Inc, Phillipburg, NJ, USA). After washing with 6 ml 15% methanol/PBS and 6 ml petroleum ether, the samples were eluted with 6 ml methanol. After evaporation of the methanol under N_2, the samples were dissolved in 1.0 ml buffer and subsequently analyzed by using enzyme immunoassay kits for PGE_2, $PGF_{2\alpha}$, 6-Keto-$PGF_{1\alpha}$ and TXB_2 (Cascade Biochem Ltd, Reading, UK).

Statistics. Food and energy intake and body and pancreatic weights were statistically evaluated by two-way analysis of variance followed by Dunnetts test, prostaglandin levels were evaluated by analysis of variance followed by Student's *t* test, the number of pancreatic lesions was evaluated by two-way analysis of variance or by one-way analysis of variance followed by linear trend tests with orthogonal contrasts. The number of tumour-bearing animals (incidence) was analyzed by χ^2-test. Fatty acid compositions were evaluated by two-way analysis of variance using percentage of dietary MaxEPA and carcinogen-treatment as factors, and by one-way analysis of variance followed by linear trend tests with orthogonal contrasts.

Results

Food intake and body and pancreas weights. Food consumption was significantly higher in animals fed the LF diet ($P<0.01$) in comparison with animals fed the HF diets (Table 3). However, due to the difference in caloric content of the diets (LF, 15.9 MJ/kg and HF, 19.8 MJ/kg) the mean energy intake was significantly lower ($P<0.001$) in the LF group than in the HF groups.

Table 3 Food and energy consumption[a]

	Food	Energy
LF	5.2±0.1**	80.5***
HF/0.0% MaxEPA	4.9±0.1	96.2
HF/1.2% MaxEPA	5.0±0.1	98.2
HF/2.4% MaxEPA	4.7±0.1	92.4
HF/4.7% MaxEPA	4.8±0.1	94.4
HF/7.1% MaxEPA	4.7±0.1	93.5
HF/9.4% MaxEPA	4.8±0.1	93.9

[a]Food intake in g/day per animal; Energy intake in KJ/day.
Statistics: analysis of variance, **P<0.01; ***P<0.001.

Table 4 Body and pancreatic weights at autopsy[a,b]

	Body wt (g)				Absolute pancreas wt (g)				Relative pancreas wt (g/kg)			
Diet group	Controls	(N)	BOP-treated	(N)	Controls	(N)	BOP-treated	(N)	Controls	(N)	BOP-treated	(N)
LF/0.0% MaxEPA	127.1±4.4*	(8)	149.1±4.7	(15)	0.406±0.03	(8)	0.453±0.04	(15)	3.20±0.2	(8)	3.09±0.3	(15)
HF/0.0% MaxEPA	168.3±2.6	(8)	146.9±4.6	(11)	0.467±0.05	(8)	0.449±0.03	(11)	2.78±0.3	(8)	3.11±0.3	(11)
HF/1.2% MaxEPA	167.7±6.0	(7)	151.4±4.1	(16)	0.494±0.08	(7)	0.473±0.04	(16)	2.92±0.4	(7)	3.15±0.3	(16)
HF/2.4% MaxEPA	153.6±6.2	(9)	142.4±8.0	(9)	0.411±0.04	(9)	0.523±0.06	(9)	2.76±0.4	(9)	3.74±0.5	(9)
HF/4.9% MaxEPA	166.4±3.1	(9)	147.1±3.9	(10)	0.456±0.04	(9)	0.436±0.04	(10)	2.76±0.3	(9)	2.97±0.3	(10)
HF/7.1% MaxEPA	156.7±6.7	(10)	147.7±2.6	(24)	0.477±0.04	(10)	0.441±0.04	(23)	2.93±0.3	(10)	2.96±0.3	(23)
HF/9.4% MaxEPA	152.2±7.2	(9)	150.6±4.6	(21)	0.507±0.05	(8)	0.503±0.04	(21)	3.42±0.5	(8)	3.36±0.3	(21)

[a]Values are means ± SEM; Relative pancreas wt, Absolute pancreas wt per kg body wt; [b]Statistics: analysis of variance + Dunnetts tests; *$P<0.05$

Table 5 Number of pancreatic lesions in BOP-treated hamsters maintained for 12 months on a low fat diet or a high fat diet containing increasing levels of MaxEPA.

(Pre)neoplastic lesions	No. of lesions						
	LF	HF/ wt% MaxEPA					
		0.0%	1.2%	2.4%	4.7%	7.1%	9.4%
No. of animals	28	33	27	28	30	30	29
Tumour incidence (%)[a]	8 (29)	9 (27)	5 (19)	6 (21)	5 (17)	8 (27)	7 (24)
Borderline lesion	27	10	34*	36*	18	13	37*
Carcinoma *in situ*	3	4	1	1	3	0	3
Ductular adenocarcinoma	6	5	4	6	4	9	7
Total no. of carcinomas	9	9	5	7	7	9	10

[a]Tumour incidence, number (%) of animals bearing one or more carcinomas (*in situ*) in the pancreas; statistics (HF data): 1-way analysis of variance followed by Student's *t* tests; *$P<0.05$ in comparison with the HF/0.0% MaxEPA group; statistics (tumour incidence): Pearson χ^2-tests

Body and pancreas weights (Table 4) were similar among all groups, except for the mean body weight in the control animals maintained on a LF diet, which was significantly lower ($P<0.05$).

Microscopy. The incidence of pancreatic lesions was similar among all groups (Table 5). No differences were observed in number of pancreatic lesions in animals fed a LF diet in comparison to animals fed a HF diet. Increasing MaxEPA in the HF diet resulted in a significantly ($P<0.05$) higher number of borderline lesions in the groups receiving 1.2%, 2.4% or 9.4% MaxEPA, but not in the groups receiving 4.7% or 7.1% MaxEPA, in comparison with the HF group without MaxEPA. The numbers of carcinomas *in situ*, ductular adenocarcinomas or total carcinomas were similar in all groups.

Fatty acids. Fatty acid composition of blood plasma and pancreatic microsomes are presented in Tables 6 and 7. A graphic presentation of LA, AA, EPA and DHA levels in plasma and pancreas is given in Figure 2. In plasma of both saline- and BOP-treated hamsters significant linear increases were seen in EPA and DHA with increasing dietary MaxEPA ($P<0.001$). In plasma of saline-treated hamsters OA ($P<0.001$), LA ($P<0.05$) and AA ($P<0.001$) had significantly decreased. In plasma of BOP-treated animals AA had decreased significantly ($P<0.001$), whereas OA and LA remained constant. In pancreatic microsomes OA had decreased (saline-treated; $P<0.05$) or remained constant (BOP-treated), LA had increased ($P<0.001$), AA had decreased (saline-treated; $P<0.01$) or remained constant (BOP-treated) and EPA and DHA had increased significantly ($P<0.01$) in both saline and BOP-treated hamsters, with increasing MaxEPA in the diet.

AA, EPA and DHA levels were significantly ($P<0.001$) higher in microsomes from pancreatic adenocarcinomas in comparison with microsomes from non-tumorous pancreas of BOP-treated hamsters. LA levels in microsomes from pancreatic adenocarcinomas were not different from those in microsomes from non-tumorous pancreas of BOP-treated hamsters (Figure 3).

Prostaglandins. PGE_2- and $PGF_{2\alpha}$- levels in non-tumorous pancreatic tissue of BOP-treated hamsters were significantly higher ($P<0.01$ and $P<0.05$, respectively) in comparison with normal pancreatic tissue from saline-treated hamsters in the 3 diet groups measured (Figure 4). Significant linear decreases in PG-levels with increasing levels of dietary MaxEPA were seen in saline-treated hamsters with PGE_2 ($P<0.05$), 6-Keto-$PGF_{1\alpha}$ ($P<0.01$ and $PGF_{2\alpha}$ ($P<0.05$), and in BOP-treated hamsters with 6-Keto-$PGF_{1\alpha}$ ($P<0.01$) and $PGF_{2\alpha}$ ($P<0.05$). The number of pancreatic tumours analyzed for prostaglandins was too small ($N=4$) to discriminate between diet groups. Significantly higher levels of PGE_2 ($P<0.001$) 6-Keto-$PGF_{1\alpha}$ ($P<0.05$), $PGF_{2\alpha}$ ($P<0.001$) and TXB_2 ($P<0.001$) were found in pancreatic carcinomas than in non-tumorous pancreas of BOP-treated hamsters (values of all diet groups combined; Figure 5).

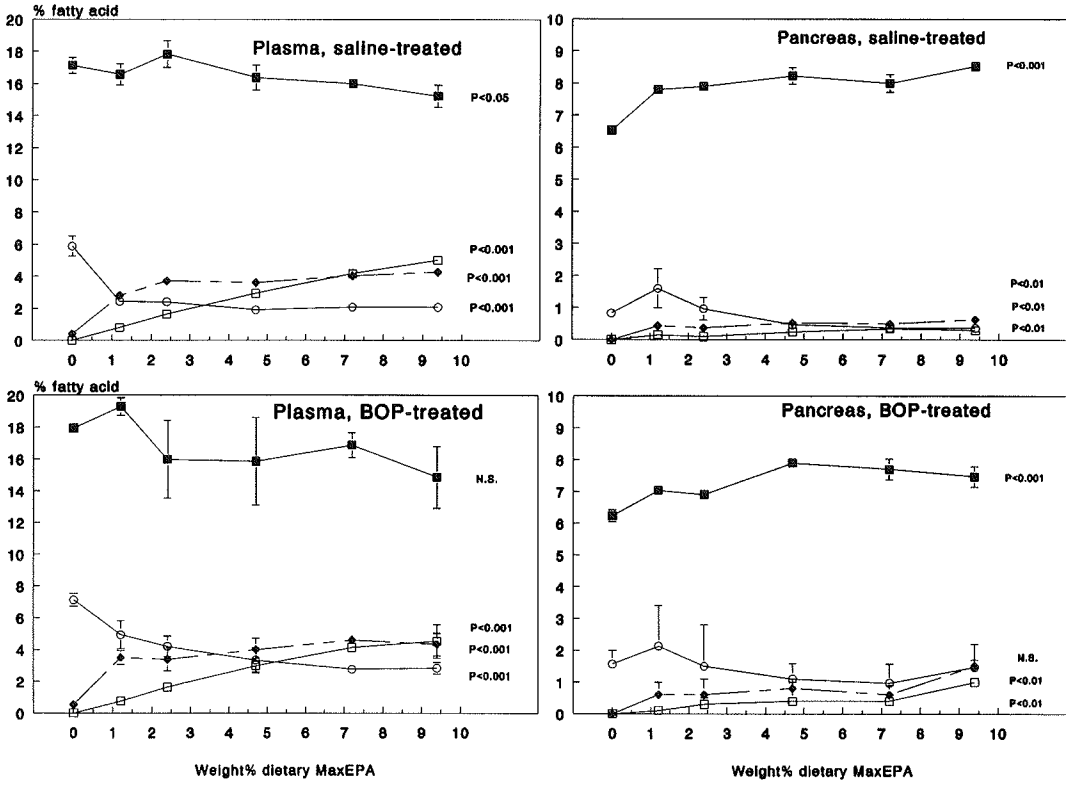

Figure 2. Percentages of selected fatty acids in plasma and pancreatic microsomes of saline- and BOP-treated hamsters maintained on a HF diet including increasing levels of MaxEPA for 12 months. ■, LA; ○, AA; ♦, EPA; □, DHA. Statistics: linear trend test.

Cell proliferation. LIs in ductular and centroacinar pancreatic cells were similar in saline and BOP-treated animals. The LI in acinar cells increased from 1.52±0.22 in saline-treated to 6.07±0.90 in BOP-treated hamsters ($P<0.01$). Feeding MaxEPA caused no significant differences in LIs of either of the cell types examined (Figure 6). All BOP/BrdU-treated hamsters showed numerous pancreatic CDC and TDC and most of them exhibited BLL in their pancreata. The LIs in CDC, TDC and BLL were not influenced by either the type or the amount of fat in the diet. Since no differences in the LIs were observed between the LF, HF and HF/9.4 wt% MaxEPA groups, the data for each pancreatic lesion were pooled (Figure 7). The LI in TDC was significantly ($P<0.01$) higher than the LI in CDC, whereas the LI in BLL was significantly higher than the LIs of both

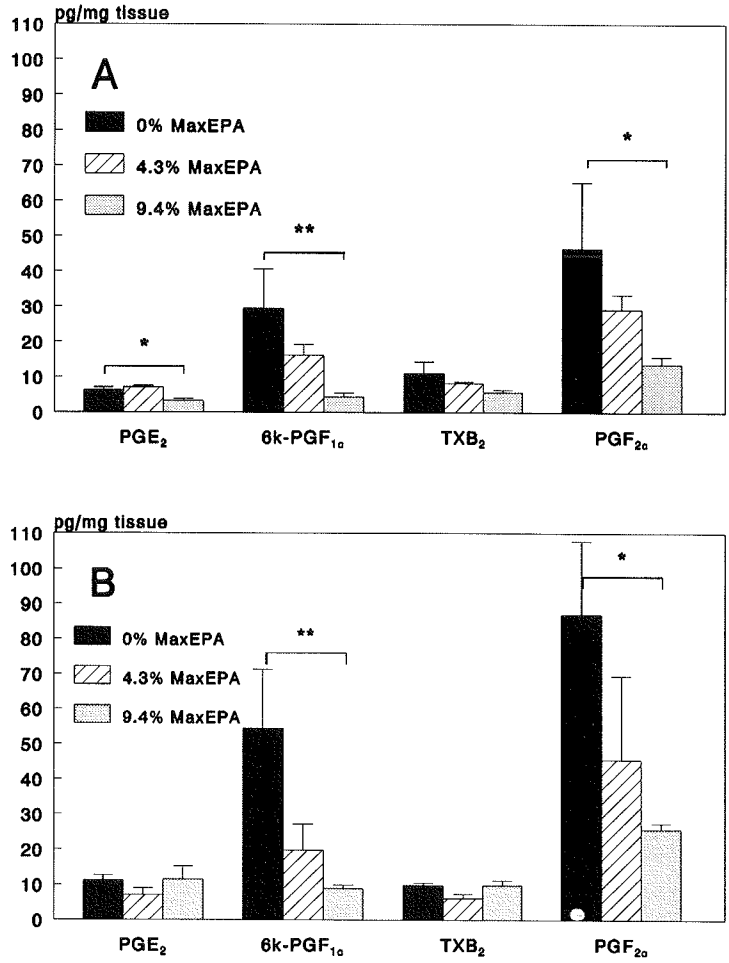

Figure 4. Prostaglandin levels in non-tumorous pancreas of saline-treated (A) and BOP-treated (B) hamster maintained on a high fat diet containing increasing levels of MaxEPA. Statistics: linear trend test $^{*}P<0.05$, $^{**}P<0.01$.

Table 7 Fatty acid composition of pancreas from untreated and BOP-treated hamsters maintained for 12 months on a high fat diet containing increasing levels of MaxEPA[a,b]

	Percentage of dietary MaxEPA											
	0.0%		1.2%		2.4%		4.7%		7.1%		9.4%	
Fatty acid	saline treated	BOP treated	saline treated	BOP treated	saline treated	BOP treated	saline treated	BOP treated	saline treated	BOP treated	saline treated	BOP treated
C14:0	0.3±0.0	0.6±0.1	0.3±0.1	0.5±0.0	0.8±0.1	0.6±0.1	1.0±0.0	0.9±0.1	0.9±0.1	1.2±0.1	1.2±0.2	1.8±0.2
C16:0	11.2±0.3	12.1±1.3	12.4±1.2	13.5±2.1	12.2±0.5	11.9±2.6	12.7±1.5	14.0±1.2	13.4±1.2	15.2±2.1	13.8±0.4	18.0±0.9
C16:1*trans*	0.8±0.1	1.2±0.1	0.7±0.1	1.1±0.1	0.8±0.0	132±0.1	0.6±0.1	1.0±0.0	0.8±0.1	1.1±0.2	0.7±0.0	1.0±0.2
C16:1*cis*	0.4±0.1	0.6±0.0	0.4±0.1	0.7±0.1	0.5±0.1	1.2±0.3	0.6±0.0	1.5±0.3	1.0±0.0	2.0±0.3	1.3±0.1	2.0±0.4
C18:0	4.3±0.3	6.0±0.8	6.2±0.9	6.2±1.8	5.4±1.0	4.4±1.8	5.5±0.1	4.9±1.0	5.6±0.8	5.5±1.4	6.3±0.3	7.2±0.9
C18:1	74.6±0.2	68.5±3.3	68.7±3.3	66.5±6.0	70.3±2.1	70.2±6.6	69.8±1.3	66.3±3.0	67.7±2.9	62.9±6.1	65.7±0.8	56.7±2.6[c]
C18:2	6.5±0.0	6.2±0.2	7.8±0.2	7.0±0.1	7.9±0.1	6.9±0.1	8.2±0.3	7.9±0.2	8.0±0.3	7.7±0.3	8.5±0.2	7.5±0.3[c,d]
C18:3 (ω-6)	0.3±0.1	1.4±0.2	0.6±0.1	1.0±0.3	0.4±0.2	0.6±0.4	0.4±0.0	0.7±0.2	0.5±0.2	1.2±0.5	0.4±0.1	1.5±0.4
C20:0	0.2±0.0	0.4±0.0	0.2±0.0	0.3±0.2	0.2±0.1	0.1±0.0	0.2±0.1	0.2±0.0	0.4±0.2	0.5±0.4	0.3±0.1	0.8±0.0
C20:1	0.3±0.0	0.4±0.1	0.3±0.0	0.3±0.0	0.3±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.5±0.1	0.5±0.2	0.6±0.0	0.4±0.0
C20:2	0.1±0.0	0.0±0.0	0.0±0.0	0.3±0.0	0.0±0.0	0.2±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
C20:3	0.1±0.0	0.0±0.0	0.3±0.1	0.0±0.0	0.2±0.1	0.0±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.0±0.0	0.1±0.0	0.1±0.0
C20:4	0.8±0.0	1.6±0.4	1.6±0.6	2.1±1.2	1.0±0.3	1.5±1.3	0.5±0.0	1.1±0.5	0.4±0.1	1.0±0.6	0.4±0.1	1.5±0.2[c]
C20:5	0.0±0.0	0.0±0.0	0.2±0.1	0.1±0.0	0.1±0.0	0.3±0.2	0.3±0.1	0.4±0.6	0.4±0.2	0.4±0.2	0.3±0.1	1.0±0.1[c,d]
C22:6	0.0±0.0	0.0±0.0	0.4±0.1	0.6±0.4	0.4±0.1	0.6±0.5	0.5±0.1	0.8±0.3	0.5±0.1	0.6±0.3	0.6±0.1	1.5±0.7[c,d]

[a]Values are mean percentages of total fatty acids ± SEM (*N*=3)
[b]Statistics: 2-way analysis of variance or 1-way analysis of variance followed by linear trend tests (orthogonal contrasts)
[c]Statistically significant linear trend (Saline-treated hamsters; OA:P<0.05; LA:P<0.001; AA, EPA and DHA:P<0.01)
[d]Statistically significant linear trend (BOP-treated hamsters; LA:P<0.001, EPA and DHA:P<0.01)

partly, to elevated caloric intake in hamsters fed HF diets. In the present study food intake was significantly lower ($P<0.01$), but energy intake was significantly higher ($P<0.001$) in hamsters maintained on the HF diets than those maintained on the LF diet. This indicates that hamsters tend to adjust their food intake according to caloric need, but not as efficient as rats, who adjust their food intake with near-100% efficiency (17).

In many studies in which fish oil has been reported to have inhibitory effects on experimental carcinogenesis, the study design is inaccurate with respect to the composition of the dietary lipids. In many studies fish oil is mixed with corn oil to vary ω-3 fatty acids in the diets or given as such, leading to simultaneous variation of LA and EFA deficiency, respectively (9,18,19). Using the azaserine-rat model for pancreatic carcinogenesis O'Connor *et al.* (20) found inhibitory effects of menhaden oil (MO) on the growth of atypical acinar cell foci (AACF). However, the way in which they increased the dietary MO from 0% to 20% resulted in an estimated simultaneous decrease in dietary LA from 12% to 0.6%. Such levels of dietary LA are in the range found to promote the development of both mammary tumours (7) and pancreatic AACF (8), indicating that it is very important to keep ω-6 PUFA levels constant, when the effects of ω-3 PUFA are studied. We were not able to reproduce the results of O'Connor *et al.* (20) in a 6-month study, using a fixed dietary LA level in the azaserine-rat model (17). Moreover, after 12 months we found a strong enhancing effect of dietary fish oil on pancreatic tumour development in azaserine-treated rats (21). The present results with BOP-treated hamsters also indicate an enhancing rather than an inhibitory effect of MaxEPA on pancreatic carcinogenesis.

The decrease of LA and AA in plasma after feeding MaxEPA points to an efficient replacement of these fatty acids by EPA and DHA, without influencing the conversion of LA to AA significantly. Although dietary supply of LA was constant, LA increased and AA decreased in pancreatic microsomes, pointing to an inhibition of LA metabolism. One of the mechanisms by which dietary PUFAs may influence tumour growth, is modulation of the LA/AA cascade to prostanoids, thromboxanes and leukotrienes. The rate limiting enzyme in this pathway is δ^6-desaturase, which converts LA to γ-linolenic acid. Unlike the rat, the hamster is a slow converter of LA, in terms of desaturase activity (22), which explains the overall lower levels of AA in hamster plasma and pancreas than in rat plasma and pancreas (17). In this respect hamsters are much more akin to humans, indicating that the hamster model may be more adequate than the rat model for studying the effects of PUFA on pancreatic cancer.

Microsomes from pancreatic adenocarcinomas contained more AA than microsomes from non-tumorous pancreatic tissue, indicating an accelerated turnover of LA to AA. Moreover, the levels of EPA and DHA in tumour tissue were also higher than in non-

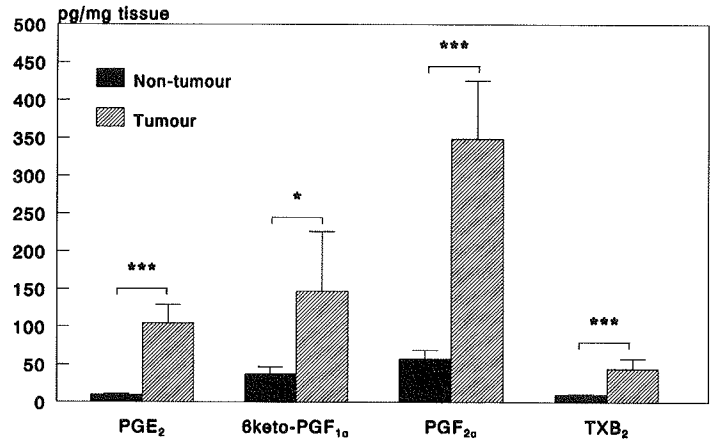

Figure 5. Prostaglandin levels in non-tumorous and tumorous pancreatic tissue of BOP-treated hamsters.

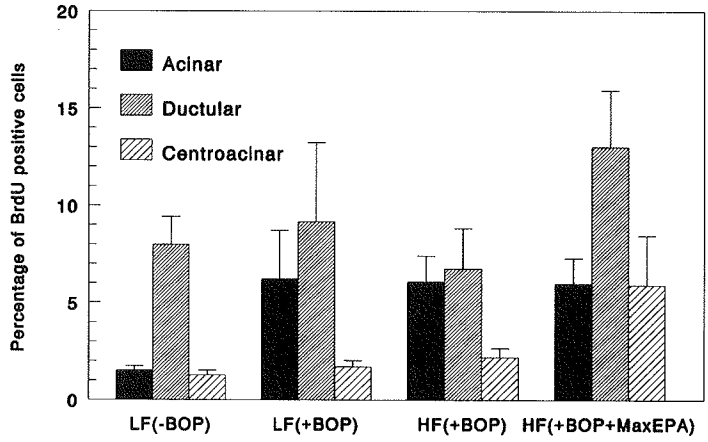

Figure 6. Labeling Index in pancreatic cells of saline- or BOP-treated hamsters maintained on a LF diet, a HF diet or a HF diet including 9.4 wt% MaxEPA for 12 months.

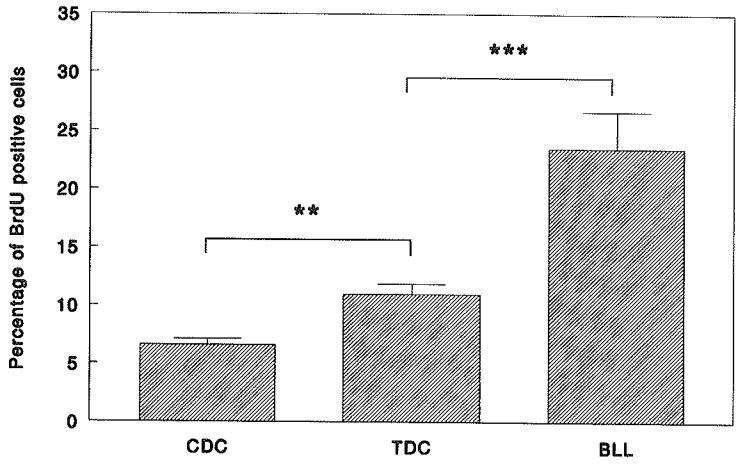

Figure 7 Labeling Index in BOP-induced pancreatic ductular lesions in hamsters. CDC, cystic ductal complex; TDC, tubular ductal complex; BLL, borderline lesion. Statistics: analysis of variance; $^{**}P<0.01$, $^{***}P<0.001$.

tumorous tissue, indicating a more effective incorporation of the ω-3 PUFA in tumour tissue. Both observations point to an altered fatty acid metabolism in neoplastic pancreatic tissue.

Including MaxEPA in the diet caused a significant dose-related decrease in prostaglandin content of pancreatic tissue. This is in agreement with the physiological properties of ω-3 PUFA, which include modulation of the conversion of AA to prostanoic and thromboxanes via cyclooxygenase (23). BOP-treatment caused a significant rise in the concentration of certain prostaglandins in 'normal' as well as in tumorous pancreatic tissue, indicating a possible role of these eicosanoids in tumour development. The observation that increasing MaxEPA in the diet had an enhancing rather than an inhibitory effect on the development of pancreatic tumours in hamsters, whereas at the same time dietary fish oil caused a reduction in prostaglandin levels, is not in agreement with the suggested promoting effect of prostaglandins of the 2-series on carcinogenesis (23). The ductular tumours in the hamster pancreas are highly immunogenic, resulting in a considerable infiltrate of lymphocytes in and around the tumours. Lymphocytes produce large amounts of eicosanoids and hence may have confounded the results (22).

BOP-treatment caused no effect on cell proliferation in phenotypically normal ductula and centroacinar cells, whereas it enhanced proliferation of acinar cells. Both these

observations confirm previous findings (24). Kokkinakis and Subbarao (25) found a five-fold increase in pancreatic DNA synthesis in hamster pancreas after treatment with N-nitroso(2-hydroxypropyl)(2-oxopropyl)amine (HPOP) and an increase in ^{3}H-thymidine LI in acinar cells, 9 days after carcinogen treatment. The normal pancreatic acinar cell population is rather stable, i.e. a population with low cell turnover, intended to maintain the tissue in its physiological state. Carcinogen-induced hyperplasia is a common phenomenon, which usually lasts for several days after treatment (26). Radiation-induced cell proliferation has been reported to last for up to 5 weeks in treated mouse skin (27). In the present and a previous study (24), the proliferative effects of BOP were present for at least 1 year. In this respect, it is tempting to speculate on a possible role of the acinar cell in the development of BOP-induced ductular tumours in hamster pancreas. Genotoxic effects, in the form of DNA-adducts have been demonstrated in pancreatic acinar cells of BOP-treated hamsters (28,29). If sustained hyperplasia in itself is a carcinogenic hazard by providing relatively good conditions for propagating critical changes in DNA (27), it seems justifiable to presume that acinar cells are somehow involved in pancreatic carcinogenesis of BOP-treated hamsters.

From the results of the present study it can be concluded that (i) ω-3 PUFA from fish oil inhibit the conversion of LA to AA in pancreas, but not in plasma and (ii) ω-3 PUFA from fish oil inhibit dose-dependently the formation of certain prostaglandins from AA and (iii) BOP-treatment enhances proliferation of pancreatic acinar cells in hamsters and (iv) dietary fish oil has a slightly enhancing rather than an inhibitory effect on BOP-induced pancreatic carcinogenesis in hamsters.

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General discussion and conclusions

The azaserine/rat model: short-term *versus* long-term studies

During the last 10 years substantial experience has been acquired at the TNO Nutrition and Food Research Institute with both the azaserine-rat and the BOP-hamster model for experimental pancreatic carcinogenesis. Using these models, the effects of dietary components such as coffee, alcohol, micronutrients and saturated and unsaturated fat on pancreatic tumour development have been investigated (1-7).

In the short-term version (4-6 months) of the rat model, the number and size of putative preneoplastic atypical acinar cell nodules (AACN) are used as parameters to detect possible modulating effects on the development of ultimate pancreatic tumours. This short-term assay is widely used in experimental pancreatic carcinogenesis because it provides results within a few months at relatively low costs. Since several short- and long-term studies have been conducted with the azaserine-rat model at TNO during the last 10 years, it was considered worthwhile to evaluate the results in order to determine the predictive value of the short term study, by comparing the results of short-term studies with those of long-term studies. Table 1 summarizes the effects of dietary factors on growth of pancreatic acidophilic AACN as observed in a short-term study and the effects on development of acidophilic AACN (with a diameter over 1 mm), adenomas and adenocarcinomas as observed in long-term studies with the azaserine-rat model. All studies were performed at the TNO Nutrition and Food Research Institute under similar experimental conditions.

The parameter 'area of pancreas occupied by focus tissue', used in Table 1 represents a reliable calculation of the pancreatic burden of AACN. From the data in Table 1 it is clear that consistent correlation between short- and long-term data only exists with vitamin C, vitamin E, unsaturated fat and fat with 5% LA. Some correlation exists with coffee and saturated fat and a correlation is absent with alcohol, β-carotene, selenium and fish oil. Moreover, the results of the studies described in chapters 6 and 7 emphasize that an enhancing effect of fish oil (MaxEPA) on growth of early stages in pancreatic carcinogenesis (AACN), does not necessarily lead to more carcinomas. It can be concluded that a long-term study is needed to establish the modulating effects of dietary or

other factors on pancreatic carcinogenesis in rats. Short-term studies are suitable to study the effects of modulators on early steps of pancreatic carcinogenesis, but long-term studie are needed to find out the implications for the development of pancreatic tumours.

Table 1 Comparison of the short-term and long-term effects of dietary components on azaserine-induced pancreatic carcinogenesis in rats[1].

Diet component	Short-term effect	Long-term effects		
	Area of pancreas occupied by focus tissue	AACN (Ø>1mm)	Adenoma	Carcinoma
Coffee	0	-	+	0
Alcohol	+	0	0	0
β-carotene	0	-	-	-
Vitamin E	0	0	0	0
Vitamin C	-	-	-	-
Selenium	0	-	-	-
Saturated fat (lard)	0	0	0	+
Unsaturated fat (corn oil)	0	+	+	+
Fat containing 5 wt% LA	+	+	0	+
Fish oil (MaxEPA)	0	+	0	0

[1]0, no significant effect; +, significant increase, -, significant decrease in comparison with appropriate cont groups.

The role of cell proliferation in pancreatic carcinogenesis

One of the main characteristics of tumour growth is increased cell proliferation. The studies described in chapter 3 show that cell proliferation correlates positively with the development of (pre)neoplastic pancreatic lesions in both rats and hamsters. Carcinogen-induced hyperplastic acinar or ductular lesions in the exocrine pancreas, which are generally considered to be putative preneoplastic, exhibit invariably a higher cell proliferation than both normal acinar and ductular tissue, as well as basophilic AACN, which are not considered to be related to the development of pancreatic tumours. The measurement of number and size represents a growth parameter of acidophilic AACN.

Cell proliferation of AACN as a parameter of growth is only informative if it can be modulated similarly to the modulation of number and size of AACN. Cell proliferation in putative preneoplastic lesions in rat pancreas can be modulated by dietary raw soya flour (8) and dietary fish oil (9). Dietary fish oil caused a decrease in cell proliferation accompanied by an increase in the size of azaserine-induced pancreatic AACN in rats. These rather contradictory observations are difficult to explain. One possible explanation could be that the acinar cells of AACN become hypertrophic in MaxEPA fed animals. Fatty acids from fish oil (mainly DHA) have been reported to cause cell swelling *in vitro* (10). Incorporation of ω-3 PUFA into acinar cell membranes may cause a similar effect. However, a consequence of this hypothesis would be that determination of number, size and volume of AACN, is not in all cases a reliable parameter for the measurement of growth response of pancreatic AACN to exogenous factors.

Another and probably more plausible explanation, may be the existence of subpopulations of AACN which cannot be distinguished in H&E stained slides but exhibit different growth potentials. This implies that determination of mean cell proliferation in the total population of AACN is inadequate to detect potential modulation of cell proliferation by dietary or other factors. When cell proliferation was directly correlated with the size of the AACN, the observed inconsistency was less pronounced. Incorporation of a cell proliferation parameter in future studies is needed to validate the method. Providing the method is further validated, measurement of cell proliferation in AACN may be a valuable parameter in short-term (4-6 months) studies with the azaserine-rat model.

The LIs in BOP-induced ductular lesions were consistently lower (CDC) or higher (TDC and BLL) than those in normal ductular or centroacinar cells, indicating that cell proliferation is a parameter that correlates positively with (pre)neoplastic development in hamster pancreas. In the BOP-hamster model size is not used as a parameter to classify the ductular pancreatic lesions. The criteria for classification of ductular adenocarcinomas in the pancreas of BOP-treated hamsters are adequate (11), but those for borderline lesions (cellular atypia, inflammation and fibrosis) are, apart from cellular atypia, not exclusive for (pre)neoplastic development. Many BOP-induced ductular lesions showing high LIs, appeared to be borderline lesions (BLL) in H&E stained tissue slides. Therefore, determination of cell proliferation would significantly facilitate diagnosis of BOP-induced putative (pre)neoplastic pancreatic BLL. The presence of a high cell proliferation in BLL may, provided that the classic characteristics of neoplastic transformation are present, justify classification of BLL as carcinomas *in situ*.

The consistent and sustained hyperplasia induced by BOP in hamster acinar pancreatic cells, may form a carcinogenic hazard in itself (12) and strongly points to involvement of acinar cells in the development of pancreatic ductular tumours of BOP-treated hamsters.

In conclusion, measuring cell proliferation using the presently described protocol with osmotic pumps and BrdU, results in relevant additional information on the potential modulating effects of (dietary) factors on pancreatic carcinogenesis and hence should be incorporated in future studies.

Cholecystokinin, dietary fat and pancreatic carcinogenesis

One of the most important pancreatropic stimulatory hormones is cholecystokinin (CCK; Ref. 13). In rats the pancreatic secretion and growth is basically regulated by the intraduodenal activity of proteolytic enzymes (mainly trypsin). High trypsin activities down-regulate the CCK release, and low trypsin activities stimulate CCK release. Inhibition of trypsin activity will result in increased release of CCK, followed by (over)stimulation of pancreatic secretion leading to increased pancreatic growth (14). This negative feedback regulation has been demonstrated in rats, mice and chicken, but not in several other species like dog or monkey (15). The relevance of inhibition of proteases fo human pancreatic function and pathology is not clear: high activity of duodenal proteases has been reported to suppress pancreatic exocrine secretion in humans (13), but inhibition of more than 95% of the intraduodenal trypsin activity had no effect on pancreatic secretion (16).

In BOP-treated hamsters injections with CCK resulted either in enhancing effects (17) no effect (18) or inhibitory effects on pancreatic carcinogenesis (19), indicating that the role of CCK in pancreatic carcinogenesis in the hamster remains controversial. However, is beyond doubt that CCK is involved in azaserine-induced pancreatic carcinogenesis in rats. When CCK or its analogue caerulein is injected subcutaneously, a dramatic increase in pancreatic weight and in number and size of AACN (20-24) and in number of pancreatic adenocarcinomas (23,25) has been observed. The effects of CCK on pancreatic carcinogenesis could largely be blocked by lorglumide, a specific CCK-receptor antagonist, whereas the effects of a high fat diet were not influenced by lorglumide. Furthermore, CCK, but not a high fat diet, enhanced pancreatic weight (chapter 4). From these results we concluded that CCK and dietary fat enhance pancreatic carcinogenesis vi different mechanisms.

Laboratory chow and pancreatic carcinogenesis

The results from experimental studies with chemically induced colon tumours, generally show a protective effect of dietary fibre on tumour development. However, the

source of fibre is very important: pure cellulose is protective in most studies, whereas pectins have no or an enhancing effect. Moreover, the amount of dietary fat is also crucial, since it has been demonstrated that a complex interaction between dietary fat and dietary fibre exists in colon carcinogenesis (26). The results from studies on experimental mammary gland carcinogenesis in rats point generally to a protective effect of dietary fibre, irrespective of the percentage of fat in the diet (27).

In rodent studies it appeared that natural ingredient diets, containing mixtures of whole-plant-derived products, generally protect rodents against carcinogens in comparison with semi-purified diets containing only highly refined components of whole foods. The lab chow used at TNO contained 11.5% indigestible dietary fibre, which is 2.7 times higher than the percentage of dietary fibre (4.3%) in the purified diet (28). The observed inhibitory effect of the cereal-based lab chow *versus* the AIN[76] semi-purified diet on pancreatic carcinogenesis (chapter 4) might be caused by the high fibre content of the lab chow. Further research is needed to confirm this hypothesis.

Oleic acid and pancreatic carcinogenesis

Inherent to our study design described in chapter 5, incorporation of LA in the diet took place at the expense of OA. Low levels of LA concurred with high levels of OA, and vice versa. The diet groups showing a relatively high incidence of (pre)neoplastic lesions in the pancreas of both rats and hamsters contained a high percentage of OA, which raises the question whether OA has a specific promoting effect on pancreatic carcinogenesis. In some studies OA has been associated with tumour promoting effects. Dayton *et al.* (29) observed more DMBA-induced mammary tumours in rats fed a 20% high-oleic safflower oil diet than in rats fed a diet containing 20% coconut oil. Chan *et al.* (30) found a positive correlation between both LA and OA in the diet and MNU-induced mammary tumours. Recently, Khoo *et al.* (31) studied the effect of dietary OA on pancreatic fatty acid profile and on the development of AACN in azaserine-treated rats, and observed an enhancing effect of OA on pancreatic carcinogenesis. However, foci were absent in the group treated with azaserine alone and in the other groups the number of foci observed per pancreas was unusually low in comparison with the numbers of foci observed by other workers in this field (4-6, 32). Since OA cannot be metabolized to LA or other long chain PUFA and PGs, the findings by Khoo *et al.* (31) are hard to explain mechanistically. However, absence of a concentration-related effect of LA on pancreatic carcinogenesis observed in the present studies, might be due to the presence of a high concentration of OA in the diets containing low concentrations of LA. Moreover, saturated fats such as lard and beef tallow contain 40% - 50% OA which may be responsible for the promoting

however, have used levels of dietary LA inadequate to meet essential fatty acid requirements. Moreover, using diets high in PUFA, especially ω-3 PUFA, requires strict monitoring of the formation of lipid peroxides.

O'Connor *et al.* (53) reported that 20% menhaden oil (MO) caused a significant inhibition of the growth of azaserine-induced AACN in rat pancreas as compared to a 2(corn oil (CO) diet. In a subsequent study (54), they varied the ω-3:ω-6 ratio from 0.01 u to 7.0 by mixing CO with MO and observed a significant decrease in development of AACN with increasing ratio of ω-3:ω-6. They acknowledged that their dietary regimen also implied a variation of dietary LA from 0.6% up to 12.0%, which is within the range where growth of AACN is significantly increased (4.4% to 8.5%; Ref. 41). We varied th dietary LA levels from 2.0% to 15.0% in a high fat diet, keeping the percentage of C-18 carbon fatty acids in the lipids between 92% and 94% and observed a negative rather tha a positive dose-response relationship between LA and AACN development (42). In order to minimize the number of variables and their possible confounding effects, it seems of paramount importance to keep LA in the diet at a constant level when investigating the effects of other variables such as fish oil. The results described in chapters 5 and 6 demonstrated an enhancing rather than an inhibitory effect of MaxEPA on growth of AACN whereas no effect on development of pancreatic carcinomas was found. The present contradictory results in comparison to those of other studies (53,54) can, in our view, be ascribed to our study design in which dietary LA is kept constant. The observation that after 12 but not after 6 months (55) fish oil enhanced growth of AACN, suggests that fish oil modulates pancreatic carcinogenesis in azaserine-treated rats after a rather long feeding period. Moreover, it cannot be excluded that feeding of diets high in MaxEPA for over 12 months will also modulate development of pancreatic carcinomas.

Feeding fish oil to BOP-treated hamsters did not result in a chemopreventive effect o pancreatic carcinogenesis. Evidence to the contrary was found: a significant increase in th number of borderline lesions in animals maintained on fish oil was observed (chapter 8). The enhancing effects of fish oil on pancreatic carcinogenesis in BOP-treated hamsters a in accordance with the enhancing effects observed on pancreatic carcinogenesis in azaserine-treated rats.

Several mechanisms have been proposed for the effects of ω-3 PUFA on carcinogenesis. Some studies indicate that fish oil attenuates the immune response, by counteracting the immunosuppression caused by for example cyclosporin (56) or ω-6 PUFA (57). Intercellular communication, which modulates cell growth and differentiation may also play a role in tumour promotion. Metabolic cooperation based on passage of lo molecular weight molecules through gap junctions, is blocked by tumour promoters like 12-O-tetradecanoylphorbol-13-acetate (TPA) or phenobarbital (58). Intercellular

communication is inhibited by unsaturated, but not by saturated fat (59). Most, if not all, cancer cells have dysfunctional gap junctional intercellular communication, but it is not known whether this defect can be modulated by dietary fat (60). The growth promoting effects of unsaturated fat on tumour cells may also be caused by formation of lipid oxidation products, such as epoxides and peroxides. Fatty acid hydroperoxides have been reported to activate cell proliferation (61). In addition, various antioxidants (like selenium, β-carotene and vitamin E), which are effective scavengers of lipid peroxiradicals, have been reported to inhibit chemically induced mammary carcinogenesis in animals fed diets rich in PUFA (62). We observed significant chemopreventive effects of β-carotene and selenium, but not of vitamin E, on dietary fat-promoted azaserine-induced pancreatic carcinogenesis in rats (7). However, we did not observe any protective effect of either of the tested antioxidants in the BOP-hamster model (63). A direct association between the effects of antioxidants on the formation of lipid peroxiradicals and carcinogenesis is difficult to prove. Moreover, the inhibitory effects of ω-3 PUFA on tumour growth are in contrast to their susceptibility to lipid peroxidation. An interesting point of view is given by de Vries and van Noorden (64), who hypothesized that increased radical formation by ω-3 PUFA, as a result of elevated lipid peroxidation in tumour cells (65,66), may result in damage of the tumour tissue, hence leading to chemoprevention. Lipid peroxides can cause breaks in DNA strands, resulting in cell death (67). In this respect, rapidly dividing cancer cells may be relatively sensitive to the cytotoxic effects of lipid peroxides. We did not observe any differences in lipid peroxide content, measured as thiobarbituric acid reactive substances (68), in pancreatic homogenates of rats maintained on HF diets with or without fish oil.

We conclude that dietary fish oil, under our experimental conditions, enhances rather than inhibits pancreatic carcinogenesis.

Fatty acid metabolism, prostaglandins and pancreatic carcinogenesis

The composition of the lipid bilayer is of utmost importance for the properties of the biological membrane, and consequently for the function of the cell. Membrane lipids regulate membrane fluidity, stability and permeability, as well as the kinetics and thermodynamic characteristics of enzymes and other proteins. Consistent lipid changes expressed in tumours are not commonly found, and at present the relationship between altered membrane lipids and neoplasia is not clear (69). Changes in membrane structure and function, metabolic processes that take place in the cell membranes and in the cytoplasm of the cell, utilizing liberated membrane constituents may very well be

influenced by alteration of the fatty acid profile of the cell membrane. Our data clearly demonstrate that feeding increasing levels of LA, EPA or DHA to rats or hamsters caused a dose-related alteration of the composition of fatty acids in both plasma and microsomes of normal pancreatic cells as well as of pancreatic tumour cells.

In azaserine-treated rats the conversion of LA to AA was inhibited in blood plasma, whereas in BOP-treated hamsters the opposite effect was seen. Irrespective of carcinogen-treatment the AA concentration in rat plasma was 3-4 times higher than that in hamsters, which is in accordance with the observations of Horrobin (70), who stated that rats conver LA to AA very efficiently, which is most probably caused by the relatively high elongation and desaturation ability of rat liver in comparison with, for example, hamster liver. The significant dose-response relationship observed between the amount of LA in the diet and the AA concentration in the plasma of rats but not of hamsters may also be related to the difference in hepatic metabolic activity between these two species.

Rats and hamsters incorporate the ω-3 PUFA similarly and dose-relatedly, both in plasma and in pancreatic microsomes of non-tumorous tissue. However, EPA and DHA were incorporated more efficiently in ductular tumours induced by BOP in hamster pancreas than in acinar tumours induced in rat pancreas by azaserine. A portion of the dietary EPA was rapidly converted to DHA in both rat and hamster plasma and pancreas, resulting in a shift in the EPA:DHA ratio from equal or higher than 1 towards an EPA:DHA ratio of equal or less than 1. Such accumulation of newly formed DHA combined with dietary DHA has been reported previously by Mathias and Dupont (71). Our observation that rats maintained on a LF or a HF diet without MaxEPA exhibited similar AA concentrations in blood plasma and pancreas, indicates that an increase in amount of LA and unsaturated fat in the diet do not influence the conversion of LA to AA (chapter 5). However, when LA was provided to rats in the same amount (i.e. 5 wt%) in combination with increasing amounts of MaxEPA (containing EPA and DHA), an increase in LA concentrations accompanied by a decrease in AA levels in blood plasma and also, but to a lesser extent, in pancreas was observed. This apparently reduced conversion of LA to AA can be ascribed to either replacement of AA by EPA (71) or to an inhibition of the LA-converting enzyme δ^6-desaturase by EPA and/or DHA (72). Evidence in favour of the latter process is given by the observed increase in plasma and pancreatic levels of LA in MaxEPA fed animals, although the amount of LA in the diet was kept constant. The effects of MaxEPA on fatty acid profiles in hamster pancreas were similar to those in rats. In hamster plasma, however, the decrease in AA was not paralleled by an increase in LA, suggesting that the ω-6 PUFA are replaced more effectively by the ω-3 PUFA in hamsters than in rats.

In microsomes from pancreatic carcinomas from both rats and hamsters AA-levels

were higher and LA-levels were lower than in microsomes from non-tumorous pancreatic tissue. These differences between fatty acid profiles in pancreatic tumours and non-tumorous tissue indicate an accelerated turnover of LA to AA in tumour tissue, pointing to an altered fatty acid metabolism in neoplastic tissue.

It is hypothesized that the promoting effect of a high fat diet on carcinogenesis is the result of an accelerated formation of AA and subsequently of PGs (73). The ω-6 PUFA, particularly LA may give rise to PGs of the 2-series via AA. It has been demonstrated that some of these PGs may stimulate cell proliferation ($PGF_{2\alpha}$; Ref. 74) or have immunosuppressive properties (PGE_2; Ref. 75) and hence may either enhance tumour growth or disturb inhibition of tumour development.

Quantitative determination of PG-levels in pancreatic tissue of rats and hamsters did not show any difference, with increasing dietary LA levels, indicating that AA and PGs are not formed in direct proportion to dietary or tissue LA. The metabolic pathway of LA to PGs seems to be strictly regulated and becomes saturated at dietary LA levels below 2 wt% in both rats and hamsters. However, when fish oil was incorporated in the experimental diets the prostaglandin content of pancreatic tissue of both rats and hamsters was significantly altered. In rats, $PGF_{2\alpha}$- and TXB_2-levels, and in hamsters PGE_2-, 6-keto-$PGF_{1\alpha}$-, and $PGF_{2\alpha}$-levels were inversely related to the percentage of MaxEPA in the diet. It has been demonstrated that ω-3 fatty acids compete with ω-6 fatty acids for conversion to prostaglandins via cyclooxygenase (73), which is confirmed by the present findings.

PG levels in the acinar carcinomas in rat pancreas as well as in the ductular carcinomas in hamster pancreas were significantly elevated in comparison with non-tumorous tissue, which is in accordance with the observed differences in fatty acid profiles in pancreatic tumours. These data suggest that accelerated LA/AA metabolism with subsequently accelerated PG-production may play a role in chemically induced pancreatic carcinogenesis in both rat and hamsters, at a rather late stage of the carcinogenic process.

Conclusions

The data presented in the present thesis lead to the following conclusions:

1. The promoting effects of CCK and dietary fat on pancreatic carcinogenesis in azaserine-treated rats are most probably mediated via different mechanisms.

2. Routine measurement of cell proliferation may (i) add relevant information on modulation of growth of putative preneoplastic pancreatic lesions in both the

azaserine-rat model and the BOP-hamster model and (ii) facilitate classification of (pre)neoplastic pancreatic lesions in the BOP-hamster model, provided that the method is properly validated.

3. Dietary ω-3 PUFA from fish oil inhibit the conversion of LA to AA in blood plasma (rats) and in pancreatic microsomes (rats and hamsters).

4. Dietary ω-3 PUFA from fish oil, but not ω-6 PUFA (LA), influence the content of prostaglandins of the 2-series in the pancreas.

5. Accelerated production of prostaglandins of the 2-series may play a role in experimental pancreatic carcinogenesis in both rats and hamsters, but probably not in the growth of early putative preneoplastic pancreatic lesions.

6. Dietary LA has an inhibitory, rather than a promoting effect on chemically-induced pancreatic carcinogenesis in both rats and hamsters.

7. Dietary fish oil has an enhancing, rather than an inhibitory effect on chemically-induced pancreatic carcinogenesis in both rats and hamsters.

Although the dietary fat content is an important dietary factor in pancreatic carcinogenesis the specific role of chain length and degree of unsaturation of the fatty acids remains obscure and controversial. Consequently, for the time being it is not justifiable to further specify the recommendation of the Netherlands Nutrition Council to reduce fat intake.

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Summary

In the Netherlands pancreatic cancer is the 8th (men) and 11th (women) leading cause of death from cancer. Every year 1500 people die from this disease. Diagnosis of pancreatic cancer is usually late due to lack of symptoms and treatment at this late stage of the disease is virtually impossible. Prognosis is poor, mainly due to metastatic spread. Three months after diagnosis 50% of the patients has died. This percentage increases to 92% and 97% after 1 and 5 years, respectively. Until diagnostic tools have been improved and more effective treatments have been developed, primary prevention is the most effective way to deal with this fatal form of cancer, and should always have high priority.

The pancreas consists of: (i) the endocrine pancreas (the islets of Langerhans) which releases hormones like insulin and glucagon into the blood and (ii) the exocrine pancreas which produces and excretes pancreatic juice, containing mainly bicarbonate and proteolytic enzymes. The pancreatic juice is excreted into a finely branched network of ductules and ducts, which empty into the small intestine.

To investigate pancreatic carcinogenesis, we used two animal models, in which tumours of the exocrine pancreas are induced with specific carcinogens. When rats are injected i.p. with azaserine, putative preneoplastic atypical acinar cell nodules (AACN) develop after 2 to 4 months and acinar adenocarcinomas after 6 to 15 months in the pancreas. When hamsters are treated s.c. with N-nitrosobis(2-oxopropyl)amine (BOP), putative preneoplastic cystic ductular complexes (CDC) and tubular ductular complexes (TDC) develop within 4 months and borderline lesions (BLL) and ductular adenocarcinomas can be found in the exocrine pancreas after 6-15 months.

Dietary fat has been found to enhance pancreatic carcinogenesis in azaserine-treated rats and BOP-treated hamsters. However, the mechanism by which dietary fat exerts this effect is unknown. The gut hormone cholecystokinin (CCK) is an important regulatory hormone in pancreatic function and growth and has also been found to promote azaserine-induced pancreatic carcinogenesis in rats. It has been demonstrated that ω-6 polyunsaturated fat has stronger tumour promoting properties than saturated fat. This led to the hypothesis that linoleic acid (C18:2ω-6), the major polyunsaturated fatty acid (PUFA) of the ω-6 family, is responsible for this effect. It has also been hypothesized that dietary ω-3 polyunsaturated fat (fish oil) is capable of inhibiting carcinogenesis, by counteracting the enhancing effects of linoleic acid on tumour development. The inhibitory

effects of fish oil have been attributed to eicosapentaenoic acid (C20:5ω-3) and docosahexaenoic acid (C22:6ω-3). One of the postulated mechanisms by which a diet hig in LA is able to promote tumour development is an acceleration of prostaglandin production, whereas, on the contrary, EPA and DHA have been shown to inhibit prostaglandin formation.

The main objective of the studies described in the present thesis is to elucidate the mechanism by which dietary fat promotes experimental pancreatic carcinogenesis in rats and hamsters. Studies have been designed and performed in order to answer the followin questions:

1. Do dietary fat and CCK exert their enhancing effect on pancreatic carcinogenesis via similar mechanism?
2. What is the biological role of dietary LA in pancreatic tumour promotion?
3. What are the chemopreventive and/or anti-tumour effects of ω-3 PUFA?
4. What are the effects of the type of fat on fatty acid metabolism and on the conversion of arachidonic acid (C20:4ω-6) to prostaglandins?

It is not clear whether ductular cells or (dedifferentiated) acinar cells are the progenitors of the ductular adenocarcinomas in the hamster pancreas. In the study described in chapter 3 pancreatic cell proliferation has been determined quantitatively of normal acinar cells, normal ductular cells, AACN cells (rats) and cells from ductular lesions (hamsters) at various time intervals over a 52 week period. Basal proliferation in normal acinar or ductular cells was relatively low, whereas cell proliferation had significantly increased in AACN and in TDC and BLL. Moreover, treatment with BOP caused a prolonged increase in the proliferation of acinar cells in hamster pancreas, whic remained present for at least 52 weeks after carcinogen-treatment. This finding, in combination with the frequently observed acinar cells in the lining epithelium of TDC, indicates that acinar cells may be involved in the development of ductular adenocarcinomas in the pancreas of BOP-treated hamsters. It is concluded that, based on the observations in chapter 3, the Labeling Index (LI) of AACN may be a useful parameter for assessing modulation of growth of AACN in rat pancreas by dietary or oth factors. In chapters 6 and 7, however, studies have been described which do not support this conclusion: feeding fish oil to rats resulted in an increase in number and size of AACN accompanied by a decrease in cell proliferation in AACN. The inconsistency of these results was less pronounced when the LI was correlated with the size of the AACN Further research is needed to validate and establish the significance of the LI-parameter.

Cell proliferation in normal pancreatic acinar, centroacinar or ductular cells, as well a

in ductular cells from BOP-induced pancreatic lesions was not modulated by dietary fish oil. The consistently elevated LI in BLL (chapters 3 and 8) indicates that cell proliferation in ductular lesions in hamster pancreas may provide a useful parameter to discriminate between BLL with a low and a high growth potential. The presence of high cell proliferation in a BLL may justify reclassification of such lesions as carcinomas *in situ*.

A high level of dietary fat (20 wt% - 25 wt%) has been shown to promote the development of chemically induced pancreatic tumours in rats and hamsters. Dietary components like proteins, carbohydrates and fats stimulate the release of the gut hormone cholecystokinin (CCK), which stimulates pancreatic acinar cells to excrete their contents. It has been demonstrated that CCK promotes the development of azaserine-induced pancreatic tumours in the rat. In chapter 4 a study has been described in which we investigated whether CCK and dietary fat promote pancreatic carcinogenesis by similar mechanisms. The results from this study indicate that CCK, but not dietary fat, increased pancreatic weight and that lorglumide, a specific CCK receptor antagonist, largely inhibited the enhancing effects of CCK, but not of dietary fat, on pancreatic carcinogenesis. Based on these results we concluded that it is unlikely that the promoting effect of dietary fat on pancreatic carcinogenesis in rats is mediated via CCK.

Apart from the amount of fat, the type of fat is an important factor in the process of tumour promotion. The studies described in chapter 4 showed that, apart from polyunsaturated fat, saturated fat (lard) also promotes azaserine-induced pancreatic carcinogenesis in rats. In chapters 5 to 8 of this thesis the effects of dietary linoleic acid (ω-6 PUFA) and dietary fish oil (ω-3 PUFA) on pancreatic carcinogenesis in rats and hamsters have been described. Moreover, the involvement of the 2-series prostaglandins, which are the biologically active metabolites of linoleic acid, have been studied. The results described in chapter 5 show that 4 wt% and 2 wt% of linoleic acid in a high fat diet caused the strongest enhancing effects on pancreatic carcinogenesis in rats and hamsters, respectively. At higher levels, the effects of dietary linoleic acid were inhibitory rather than promoting. In chapters 6 and 7 it has been described that increasing percentages of fish oil in a high fat diet containing a fixed level of 5 wt% linoleic acid, resulted in a dose-related increase in number and size of AACN in the pancreas of rats. No effect was observed on the number of adenomas and carcinomas. From the study described in chapter 8 it appears that fish oil has no chemopreventive effect on pancreatic carcinogenesis in hamsters maintained on high fat diets including 2 wt% linoleic acid and containing increasing levels of fish oil.

In both rats and hamsters the fatty acid profiles of blood plasma and microsomes from non-tumorous pancreas showed an accurate reflection of the dietary fatty acid profiles: proportional increases in linoleic acid, eicosapentaenoic acid and docosahexaenoic acid

Samenvatting

De pancreas (alvleesklier) is opgebouwd uit kliercellen (de eilandjes van Langerhans), die hormonen als insuline en glucagon maken en aan het bloed afgeven, en uit kliercellen die pancreassap/verteringsenzymen maken die, via een netwerk van klierbuisjes in één centrale afvoergang uitgescheiden worden in de dunne darm.

Pancreaskanker komt weliswaar niet zo frequent voor, maar is wel fataal! Pancreaskanker wordt vaak te laat ontdekt, omdat vroege symptomen afwezig zijn. Als de diagnose wordt gesteld is de tumor bijna altijd al uitgezaaid. De prognose van de ziekte is daarom ook zeer slecht: 50% van de patiënten sterft binnen 2 tot 3 maanden nadat de diagnose gesteld is. Eén jaar na het stellen van de diagnose is nog 8% van de patiënten in leven en de 5-jaars overleving is minder dan 3%. Totdat er methoden zijn ontwikkeld die een vroege diagnose of een adequate behandeling mogelijk maken, lijkt preventie de enige effectieve manier om deze vorm van kanker te bestrijden.

Eén van de factoren die bij het ontstaan van pancreaskanker een rol speelt is vet in de voeding. Om de rol van vet in de ontwikkeling van pancreaskanker bij proefdieren te bestuderen hebben we gebruik gemaakt van twee diermodellen. In beide modellen wordt een chemische stof gebruikt om tumoren op te wekken die ontstaan in de kliercellen (acinaire tumoren bij de rat) of in de afvoergangen (ductulaire tumoren bij de hamster). Bij de rat resulteert injectie met azaserine na enkele maanden in groepjes afwijkende kliercellen, die een verhoogde delingscapaciteit vertonen. Deze snel groeiende zogenaamde "Atypical Acinar Cell Nodules" (AACN) worden beschouwd als voorstadia van de acinaire tumoren. Bij de hamster wordt N-nitrosobis(2-oxopropyl)amine (BOP) gebruikt. Injectie van hamsters met BOP resulteert in afwijkende klierbuisjes (ducts), die uiteindelijk tot ductulaire tumoren kunnen leiden. De pancreastumoren die bij de mens voorkomen zijn meestal van het ductulaire type.

Van voedingsvet is aangetoond dat het de groei van pancreastumoren in azaserine-behandelde ratten en BOP-behandelde hamsters bevordert. Het mechanisme achter dit effect is echter nog onbekend. Het darmhormoon cholecystokinine, dat vrijkomt na het eten van b.v. vet, regelt in belangrijke mate de functie en de groei van de pancreas. Cholecystokinine heeft bovendien een stimulerend effect op de groei van azaserine-geïnduceerde pancreastumoren bij ratten. Het is meermalen beschreven dat ω-6 meervoudig onverzadigde vetzuren de tumorgroei sterker stimuleren dan verzadigde vetzuren. Hieruit volgde de hypothese dat linolzuur, het belangrijkste ω-6 meervoudig onverzadigde vetzuur, verantwoordelijk is voor dit effect. Het is ook beschreven dat ω-3

meervoudig onverzadigde vetzuren uit visolie in het rantsoen van proefdieren de groei van tumoren kan remmen. Deze "visvetzuren" zouden dit effect hebben doordat zij het bevorderende effect van linolzuur tegengaan. Het stimulerende effect van linolzuur wordt mogelijk veroorzaakt door een verhoogde produktie van bepaalde prostaglandines (PGs). Visvetzuren kunnen de vorming van deze PGs remmen en zo de tumorgroei tegengaan.

De belangrijkste doelstelling van de studies beschreven in dit proefschrift is het ophelderen van het mechanisme waarlangs voedingsvet de groei van pancreastumoren stimuleert. Er werden een aantal studies uitgevoerd die tot doel hadden antwoord te krijgen op de volgende vragen:

1. Vindt de bevordering van de groei van pancreastumoren door voedingsvet en cholecystokinine plaats via hetzelfde mechanisme?
2. Wat is de biologische rol van linolzuur in de bevordering van de groei van pancreastumoren?
3. Wat is de biologische rol van visvetzuren in de modulatie van de groei van pancreastumoren?
4. Wat zijn de effecten van het type vet op het vetzuurmetabolisme en op de omzetting van arachidonzuur in prostaglandines?

Het is nog onduidelijk of de ductulaire lesies in de hamsterpancreas ontstaan uit ductulaire cellen of uit (veranderde) acinaire cellen. Kwantitatieve bepaling van de celdelingsactiviteit van normale acinaire cellen, normale ductulaire cellen, acinaire lesies (rat) en van ductulaire lesies (hamsters) in de pancreas van ratten en hamsters is beschreven in hoofdstuk 3. Een kenmerk van veel tumoren is dat ze snel groeien en dus een hoge celdelingsactiviteit vertonen. De celdelingsactiviteit van (voorstadia van) tumoren kan op een eenvoudige wijze kwantitatief worden bepaald. Uit de in hoofdstuk 3 beschreven resultaten blijkt dat acinaire en ductulaire cellen onder normale omstandigheden betrekkelijk weinig delen en dat de celdeling in de acinaire en ductulaire lesies significant hoger is. Bovendien blijkt dat acinaire cellen van met BOP behandelde hamsters ook een verhoogde celdeling vertonen. Deze verhoogde celdeling blijft minstens 52 weken aanwezig. Deze bevinding, gecombineerd met het feit dat acinaire cellen vaak worden aangetroffen in ductulaire lesies, wijst erop dat de acinaire cel mogelijk betrokken is bij het ontstaan van ductulaire tumoren in de hamsterpancreas en mogelijk ook een rol speelt bij het ontstaan van ductulaire pancreastumoren bij de mens. De celdelingsactiviteit zou een goede parameter kunnen zijn om de effecten van voedings- en andere factoren op de groei van acinaire lesies te meten. De resultaten van de experimenten beschreven in de hoofdstukken 6 en 7 tonen echter aan dat het voorkomen van grote acinaire pancreaslesies

niet altijd gecorreleerd is met een hoge celdelingsactiviteit: ratten die een rantsoen kregen waaraan visolie was toegevoegd hadden grotere acinaire lesies, maar een lagere celdelingsactiviteit dan ratten die geen visolie kregen. Verder onderzoek naar de waarde van het bepalen van de celdelingsactiviteit in dit type studies is nodig.

In de pancreas van de hamster beïnvloedde visolie in het rantsoen noch de celdelingsactiviteit van normale acinaire of ductulaire cellen, noch die van ductulaire lesies.

De celdelingsactiviteit in de voorstadia van de ductulaire tumoren was consequent hoger dan van alle andere celtypen in de hamsterpancreas (hoofdstukken 3 en 8). Dit laat zien dat celdelingsactiviteit in de pancreas van BOP-behandelde hamsters een bruikbare parameter is om voorstadia van tumoren te classificeren.

Een hoog vetgehalte in het rantsoen (20%-25%) bevordert de ontwikkeling van pancreastumoren bij ratten en hamsters. Voedingscomponenten zoals eiwitten, koolhydraten en vetten stimuleren de afgifte van het darmhormoon cholecystokinine, dat de pancreas stimuleert tot de vorming van pancreassap en de uitscheiding hiervan in de dunne darm. Het is aangetoond dat toediening van cholecystokinine bij azaserine-behandelde ratten de ontwikkeling van pancreastumoren bevordert. De resultaten van het onderzoek beschreven in hoofdstuk 4 tonen aan dat de bevorderende werking van cholecystokinine en van voedingsvet zeer waarschijnlijk niet via hetzelfde mechanisme tot stand komt.

Naast de hoeveelheid vet speelt ook de samenstelling van het vet mogelijk een belangrijke rol bij het proces van de tumorpromotie. **Verzadigd** vet (reuzel) in het rantsoen heeft een bevorderend effect op het ontstaan van pancreastumoren bij de rat (hoofdstuk 4). **Meervoudig onverzadigd** vet heeft echter een nog veel sterker modulerend effect op de tumorgroei bij proefdieren. In de hoofdstukken 5 t/m 8 van dit proefschrift is beschreven wat de effecten zijn van linolzuur en visolie in het rantsoen op de ontwikkeling van pancreastumoren bij ratten en hamsters en wat de rol hierin is van prostaglandines, die uit linolzuur kunnen worden gevormd. Bij hamsters en ratten die een rantsoen kregen met respectievelijk 2% en 4% linolzuur werd het sterkste tumor-bevorderend effect gevonden. Hogere concentraties linolzuur in een hoog vet rantsoen bleken eerder een remmend dan een promoverend effect te hebben op de ontwikkeling van pancreastumoren bij zowel ratten als hamsters.

Uit het onderzoek beschreven in de hoofdstukken 6 en 7 blijkt dat een toenemend percentage visolie in een hoog vet rantsoen een dosis-afhankelijke toename van het aantal preneoplastische acinaire lesies in de pancreas van ratten tot gevolg heeft. Op het aantal adenomen en carcinomen had visolie echter geen effect. Er kon ook geen remmend effect van visolie worden aangetoond op de ontwikkeling van pancreastumoren bij hamsters.

In zowel ratten als hamsters is het vetzuurpatroon in bloedplasma en pancreas een goede afspiegeling van het vetzuurpatroon in het rantsoen. De gehaltes linolzuur en visvetzuren nemen toe in plasma en pancreas wanneer het linolzuur- of het visoliegehalte in het rantsoen wordt verhoogd. Het arachidonzuurgehalte in plasma van de rat nam toe met stijgende linolzuurconcentraties in het rantsoen. Het arachidonzuurgehalte in ratteplasma was in alle gevallen 3-4 maal hoger dan in hamsterplasma. Hoge gehaltes aan visvetzuren in plasma en pancreas gingen over het algemeen gepaard met hoge linolzuurgehaltes en lage arachidonzuurgehaltes. Een mogelijke verklaring hiervoor is dat visvetzuren de omzetting van linolzuur in arachidonzuur remmen. Linolzuur was verlaagd en arachidonzuur verhoogd in pancreastumoren van ratten, terwijl er geen verschil was in gehaltes van de visvetzuren. In tumoren van de hamsterpancreas bleef het linolzuurgehalte gelijk, terwijl de arachidonzuur- en de visvetzuurgehaltes verhoogd waren t.o.v. normaal pancreasweefsel.

In normaal pancreasweefsel van ratten en hamsters had het percentage linolzuur in het rantsoen geen invloed op het gehalte aan bepaalde prostaglandines (PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ and thromboxaan (TX) B_2). Een visolie-bevattend rantsoen had bij ratten na 6 maanden een verlagend effect op de gehaltes van $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ en TXB_2, maar niet op het gehalte PGE_2 in de pancreas (hoofdstuk 6). Een visolie-bevattend rantsoen resulteerde na 12 maanden in een verlaging van de gehaltes $PGF_{2\alpha}$ en 6-keto-$PGF_{1\alpha}$ in de pancreas van ratten (hoofdstuk 7) en in een verlaging van de gehaltes PGE_2, $PGF_{2\alpha}$ en 6-keto-$PGF_{1\alpha}$ in de pancreas van hamsters (hoofdstuk 8). In pancreastumoren van hamsters waren de gehaltes PGE_2 (hoofdstuk 8), $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ en TXB_2 (hoofdstukken 4 en 8) significant verhoogd t.o.v. normaal pancreasweefsel.
In pancreastumoren van ratten waren de gehaltes PGE_2, $PGF_{2\alpha}$ en TXB_2 (hoofdstuk 7) significant verhoogd t.o.v. normale pancreas. Deze resultaten wijzen erop dat prostaglandines betrokken zijn bij de groei van pancreastumoren bij ratten en hamsters, maar niet bij de ontwikkeling van voorstadia van deze tumoren.

Uit de resultaten van de in dit proefschrift beschreven experimenten kan worden geconcludeerd dat:

1. Het promoverende effect van voedingsvet op pancreaskanker in azaserine-behandelde ratten waarschijnlijk niet plaats vindt via cholecystokinine.
2. Bepaling van celproliferatie een belangrijk hulpmiddel is bij de classificatie van ductulaire lesies in de hamsterpancreas. Validatie van deze methode is echter noodzakelijk.
3. Meervoudig onverzadigde vetzuren afkomstig uit visolie (ω-3 vetzuren) de omzetting

van linolzuur in arachidonzuur in bloedplasma (ratten) en in de pancreas (ratten en hamsters) remmen.

4. Meervoudig onverzadigde vetzuren afkomstig uit visolie (ω-3 vetzuren) het gehalte van de 2-serie prostaglandines in de pancreas van ratten en hamsters verlagen, terwijl linolzuur (ω-6 vetzuren) hier geen effect op heeft.
5. Een verhoogde produktie van de 2-serie prostaglandines mogelijk een rol speelt bij de uitgroei van pancreastumoren van ratten en hamsters, maar waarschijnlijk niet bij het ontstaan van preneoplastische pancreaslesies.
6. In tegenstelling tot de heersende opvatting linolzuur in het rantsoen eerder een remmend dan een bevorderend effect heeft op de ontwikkeling van chemisch-geïnduceerde pancreastumoren bij ratten en hamsters.
7. In tegenstelling tot de heersende opvatting visolie in het rantsoen eerder een bevorderend dan een remmend effect heeft op de ontwikkeling van chemisch-geïnduceerde pancreastumoren bij ratten en hamsters.

Hoewel het vetgehalte in de voeding een belangrijke rol speelt bij de ontwikkeling van pancreaskanker, blijft het specifieke effect van de ketenlengte en mate van onverzadigdheid van de vetzuren onduidelijk. Dientengevolge is het niet verantwoord om het huidige advies van de gezondheidsraad om minder vet te eten nader te specificeren.

Curriculum vitae

Marko Appel werd op 16 juli 1963 in Dordrecht geboren. Na het voltooien van het VWO in 1983 en de militaire dienstplicht in 1984, werd begonnen met de studie milieuhygiëne aan de Landbouwuniversiteit Wageningen. Het doctoraal examen, met als hoofdvakken Toxicologie (Prof. J. Koeman) en Biochemie (Prof. C. Veeger) werd behaald in 1990. Op 1 oktober 1990 trad hij in dienst van het Integraal Kankercentrum Midden Nederland (IKMN) als assistent in opleiding. Gedurende het 4-jarige onderzoek was hij gedetacheerd bij de afdeling Pathologie van de divisie Toxicologie van TNO-Voeding te Zeist. Vanaf 1 januari 1995 is hij op een door de Nederlandse Kankerbestrijding gefinancierd project verbonden aan de afdeling Pathologie van de divisie Toxicologie van TNO-Voeding.

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