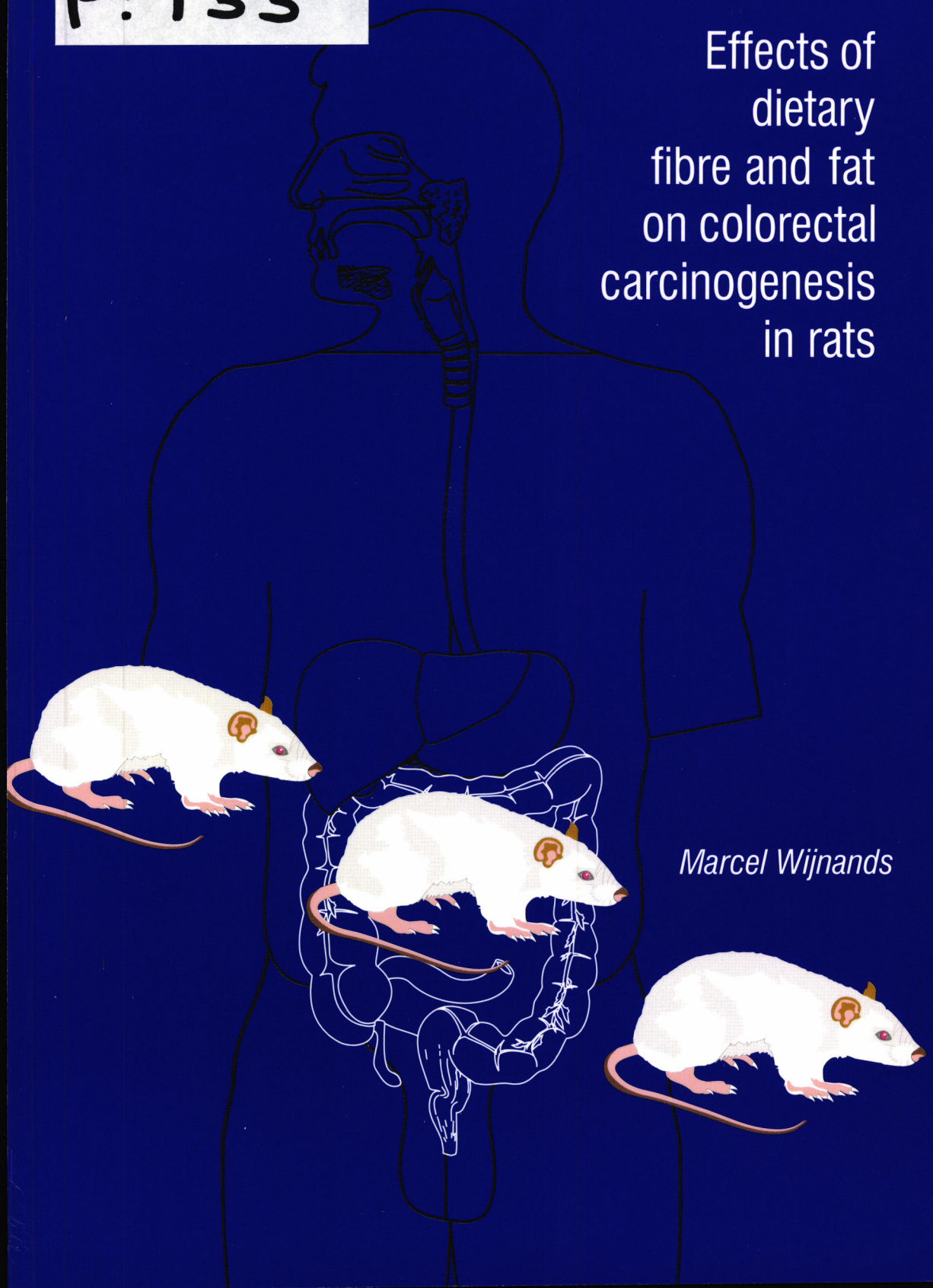
Effects of dietary fibre and fat on colorectal carcinogenesis in rats



Marcel Wijnands

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Effects of dietary fibre and fat on colorectal carcinogenesis in rats

De invloed van vezel en vet in de voeding op het ontstaan van kanker in de dikke darm bij ratten

(met een samenvatting in het Nederlands)

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Utrecht
op gezag van de Rector Magnificus, Prof. Dr. W.H. Gispen,
ingevolge het besluit van het College voor Promoties
in het openbaar te verdedigen op
donderdag 25 september 2003 des middags te 14.30 uur

door

Marcel Victor Wilhelmus Wijnands

geboren op 5 november 1957, te Heerlen

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Contents

List of abbreviations

AC	aberrant crypt(s)
ACF	aberrant crypt focus/foci
AIN	American Institute of Nutrition
ANOVA	analysis of variance
AOM	azoxymethane
BIT	benzyl isothiocyanate
BrdU	bromodeoxyuridine
CA2	carbonic anhydrase II
CDK4	cyclin-dependent kinase 4
cDNA	complementary DNA
COX-2	cyclooxygenase 2
CUR	curcumin
CYP1A1	cytochrome P 450 1A1
DHA	docosahexaenoic acid
DMH	1,2-dimethylhydrazine
DNA	deoxyribonucleic acid
EPA	eicosapentaenoic acid
F344	Fischer 344
FO	fish oil
FOS	fructo-oligosaccharides
GALT	gut associated lymphoid tissue
GOS	galacto-oligosaccharides
LC	low cellulose
LF	low fat
LGOS	low galacto-oligosaccharides
LI	labelling index
LRS	low resistant starch
HC	high cellulose
HE	haematoxylin and eosin
HF	high fat
HFO	high fish oil
HGOS	high galacto-oligosaccharides

HRS	high resistant starch
MF	medium fat
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
NCP	non-cellulose polysaccharides
NDO	non-digestible carbohydrates
ODC	ornithine decarboxylase
PBS	phosphate-buffered saline
PUFA	polyunsaturated fatty acids
RNA	ribonucleic acid
RS	resistant starch
RT-PCR	reverse transcription polymerase chain reaction
RUT	rutin
SCFA	short chain fatty acids
TIMP-1	tissue inhibitor of metalloproteinase 1
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling
WB	wheat bran
% w/w	percentage by weight

Chapter 1

General introduction

Etiology of colorectal cancer

Colorectal cancer is one of the major causes of cancer death in many Western countries, second or third after breast cancer, lung cancer and prostate cancer. In the etiology of colorectal cancer many factors have been identified. It is generally accepted that the diet is the most important one, which is supported by a vast amount of data from epidemiological and animal model studies. A wide variety of dietary components have been investigated for their effects on colorectal carcinogenesis: whole products such as meat, dairy products, vegetables, fruit, coffee and tea, or individual components such as certain types of fibre, fat, vitamins and minerals, as single components or in combinations to study possible interactions. In addition, several drugs with assumed anticancer potency have been studied, for example aspirin and piroxicam.

Some individuals have an increased risk of getting the disease in case there is a family history of colorectal cancer (Bonaïti-Pellié, 1999; Fuchs *et al.*, 1994; Kune *et al.*, 1987). A specific disease is known as Familial Adenomatous Polyposis. This is a rare dominantly inherited increased susceptibility to colon cancer in which individuals develop hundreds of mainly colonic polyps as a result of multiple genetic alterations (Bodmer *et al.*, 1989; Cho and Vogelstein, 1992).

Many people who regularly drink alcohol and smoke cigarettes are probably aware of the fact that their liver, heart and lungs may be injured by these life-style factors, but other organ systems can also be affected. Results of epidemiological studies show that alcohol drinking and tobacco smoking are associated with an increased risk of colorectal cancer (Cope *et al.*, 1991; Giovannucci *et al.*, 1994, 1995; Kikendall *et al.*, 1989; Kono *et al.*, 1990; Kune and Vitetta 1992; Newcomb *et al.*, 1995; Potter *et al.*, 1999; Sandler *et al.*, 1993a; Wu *et al.*, 1987).

The incidence of colorectal cancer in Africa and Asia is considerably lower than in Europe and North America. Although genetic differences may be involved, it has been shown that diet plays an important role. In Africa and Asia the diet is characterised by a low level of fat and a high level of fibre, whereas in the Western countries the diet is generally high in fat and low in fibre. The conception that a high-fat/low-fibre diet increases the risk of colorectal cancer (and other diseases) and that a high fibre intake is inversely related to this disease, is confirmed by epidemiological studies and widely accepted (Giovannucci *et al.*, 1992; Hill, 1998;

Howe *et al.*, 1992; Sandler *et al.*, 1993a; Smigel, 1992; Trock *et al.*, 1990; West *et al.*, 1989).

We conducted a series of experimental animal studies to investigate the effects of dietary fibre and fat on chemically induced colorectal tumours and their precursor lesions. These experiments are described in this thesis.

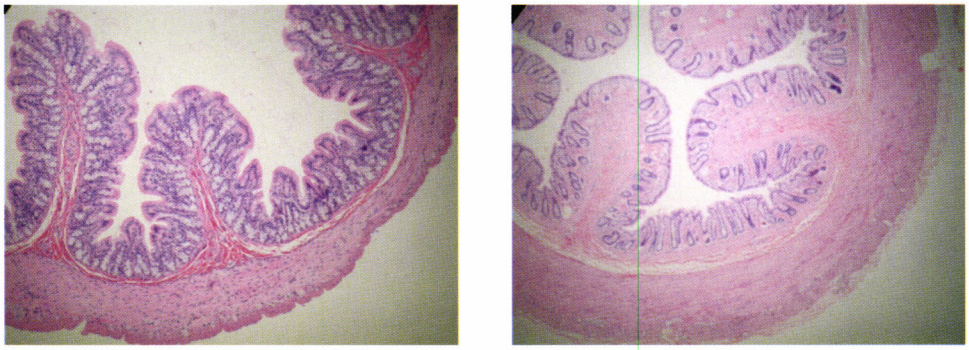
The colon

Foodstuffs are pre-digested in the stomach and subjected to digestion in the small intestines by enzymes originating from the small intestinal mucosa and the pancreas. The majority of nutrients are absorbed in the small intestines. The large intestines form the last part of the gastrointestinal tract and anatomically consist of the caecum, the colon and the rectum. They harbour populations of bacteria, which can further process certain substrates in the intestinal content by fermentation. Absorption also takes place in this part of the gut, mainly of water, electrolytes, some vitamins and short chain fatty acids (SCFA). Histologically, the colon has the following layers (Junqueira *et al*, 1977; Young and Heath, 2000) (Fig.1.1.a):

- Mucosal layer, composed of an epithelial lining, lamina propria and lamina muscularis mucosae. The lamina propria is a connective tissue layer containing blood and lymph vessels, nerves and the 'gut associated lymphoid tissue' (GALT), which consists of focal organised aggregates as well as scattered individual cells of the immune system. The lamina muscularis mucosae is a continuous layer of smooth muscle separating the mucosa from the submucosa.
- Submucosa, which is a connective tissue layer containing blood and lymph vessels, nerves and GALT.
- Tunica muscularis, divided in a circular internal muscle layer and a longitudinal external muscle layer.
- Tunica serosa, composed of loose connective tissue with blood and lymph vessels and adipose tissue, and a simple squamous covering mesothelium.

The epithelial surface is increased by the presence of crypts (glands or crypts of Lieberkühn). A single layer of columnar epithelial cells, consisting mainly of

colonocytes and mucus producing goblet cells or mucocytes, lines the crypts. The histological features of the colon and rectum are basically comparable. Towards the anus the muscle layers gradually become thicker, but there is no strictly identifiable point of transition from the colon to the rectum (Fig.1.1.b). Because of the lack of a clear distinction between colon and rectum, the fact that tumours can occur in either of them and have a similar morphology, the tumours described in this thesis are referred to as 'colorectal tumours'. The epithelial layer consists of a dynamic population of cells, which are renewed every few days. Renewal takes place by division of stem cells situated at the base of the crypts. New cells move up towards the luminal side and eventually are exfoliated into the lumen.

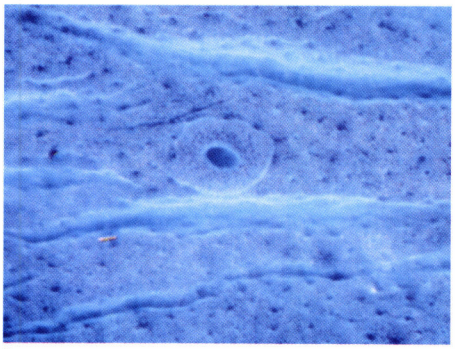


a b

Figure 1.1. Normal colon (a) and rectum (b) of a rat. HE, 25x.

The epithelial cells with their relatively high cell turnover are in direct contact with the intestinal content, which may contain potentially harmful constituents. Due to the absorption of fluid, the transit time slows down and the concentration of chemicals increases. The specific physiology of the colon may explain why this part of the body is at a relatively high risk for cancer.

Colorectal carcinogenesis is believed to be a multistage process. Normal mucosa becomes hyperproliferative and dysplastic; at this stage aberrant crypt foci (ACF) can be identified. From there adenomas may develop, some of which may grow and undergo malignant transformation, ultimately leading to invasive cancer (O'Brien *et al.*, 1990; Winawer *et al.*, 1991). Adenomas are circumscribed areas of dysplastic epithelium that distort the adjacent normal mucosal architecture by compression, but the epithelium is confined by the basement membrane (Fig.1.2.e).



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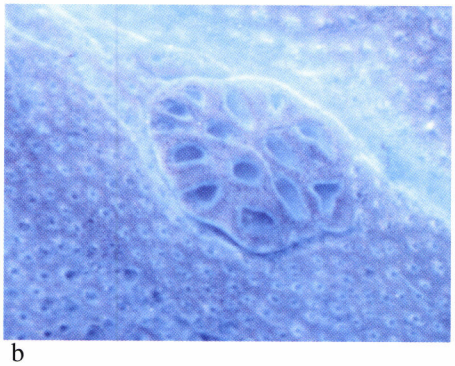

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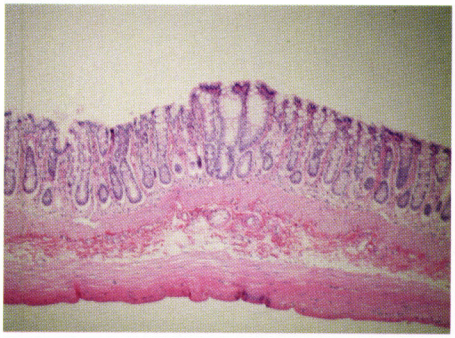

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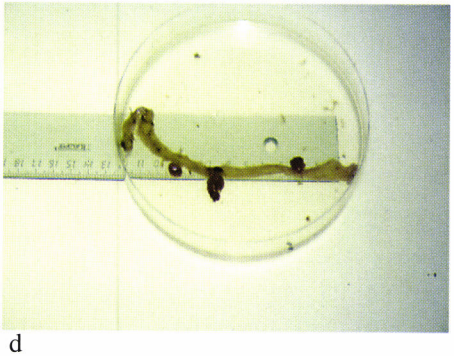

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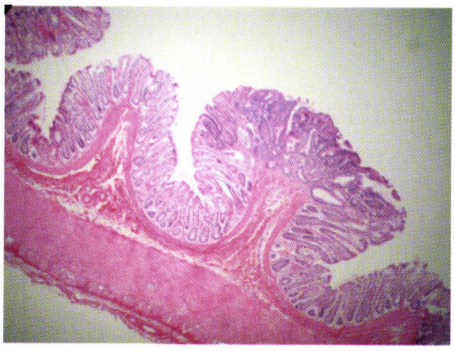

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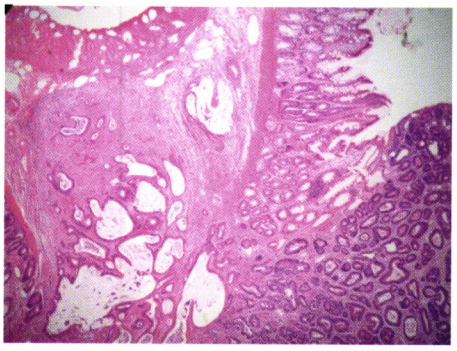

f

Figure 1.2. ACF and colorectal tumours in a rat.
a. Single AC. Methylene blue, low magnification.
b. ACF. Methylene blue, low magnification.
c. ACF. HE, 40x.
d. Several colontumours.
e. Small colon adenoma. HE, 25x.
f. Colon adenocarcinoma, infiltrating into the submucosa. HE, 25x.

Adenocarcinomas are characterised by invasion of tumour cells through the basement membrane into the lamina propria or submucosa (Whiteley *et al.*, 1996) (Fig.1.2.f). The carcinogenesis concept is associated with a cascade of genetic alterations, characteristic for each stage of the process (Vogelstein *et al.*, 1988). There are also indications for the possibility that carcinomas sometimes arise *de novo* (Jaskiewicz *et al.*, 1998; Konishi *et al.*, 1999; Maskens, 1976; Maskens and Dujardin-Loits, 1981). Nauss *et al.* (1984) observed a significant association between sessile adenocarcinomas but not polypoid adenomas with lymphoid aggregates.

ACF have received a lot of attention since Bird (1987) has first described them. ACF do not occur spontaneously in rats (McLellan and Bird, 1988b), but are abundant within a few weeks after treatment with carcinogens such as 1,2-dimethylhydrazine (DMH) or azoxymethane (AOM). They can also be found in humans, with a higher prevalence in patients with colorectal polyps or cancer (Pretlow *et al.*,1991; Takayama *et al.*, 1998). ACF can be viewed easily by microscopic examination of unsectioned colons stained with methylene blue. Aberrant crypts (AC) stain more darkly and are larger than the surrounding crypts, may protrude slightly above the mucosal surface and often demonstrate a slit-like orifice (Fig.1.2.a). Multiple aberrant crypts together form a focus: ACF (Fig. 1.2.b). Histologically, ACF demonstrate a heterogeneous morphology, ranging from just elongation and widening of the crypts (Fig.1.2.c) to dysplastic proliferation (Bouzourene *et al.*, 1999; Fenoglio-Preiser and Noffsinger, 1999; Di Gregorio *et al.*, 1997). They may show branching glands, mucin depletion and frequent mitoses. Mitotic activity, in normal crypts confined to the base, may extend to the upper part of the crypt. The monoclonality of ACF in humans was demonstrated by Siu *et al.* (1999). The questions whether ACF are precancerous lesions, what role they have in the carcinogenic process and whether they are relevant biomarkers with predictive value for the development of colorectal cancer, have inspired many investigators to undertake a variety of animal studies resulting in a huge number of publications. Interesting overviews have been published by Bird (*et al.*, 1989, 1995, 2000) and Fenoglio-Preiser and Noffsinger (1999), but there are many more. The general view is that ACF are preneoplastic lesions.

Animal models for colorectal cancer

The basic concept of the animal models for colorectal cancer is relatively simple: groups of animals are exposed to a carcinogen known to induce colorectal tumours. Next, the various groups are treated differently with respect to their diet or any other variable. Finally, the effect of the variable on the development of colorectal tumours and their precursor lesions is studied. The rat is the most suitable animal species because the development of colorectal cancer is comparable to that in humans (Hamilton *et al.*,1982; Madara *et al.*, 1983) and the rat is more sensitive than the mouse for the induction of colorectal tumours by carcinogens (Tudek *et al.*, 1989). Usually only males are used because they are more prone to develop intestinal tumours than females (Berman *et al.*, 1979). The results of such an experiment are usually expressed as the tumour incidence (percentage of animals with one or more tumours), multiplicity (number of tumours per tumour-bearing animal) and size. In addition, many other parameters may be studied to get insight into the mechanisms responsible for inter-group differences in tumour yield, if present. An example of a rat colon with some tumours is presented in Fig. 1.2.d.

Rodent models for colorectal cancer are extensively described (Berman *et al.*, 1979; Lamont and O'Gorman, 1978; Lindström *et al.*, 1978; Rogers and Nauss, 1985; Sunter *et al.*, 1978). Chemicals used for the induction of colorectal tumours are for example N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), DMH and AOM. DMH is metabolised in the liver; one of its metabolites is AOM. In the last decade, AOM seems to be the most chosen carcinogen. It is more potent than DMH. Only 2 or 3 subcutaneous injections are sufficient to induce colorectal tumours. This provides an advantage, because it allows a short injection protocol, which makes it easier to distinguish the initiation phase from the promotion phase of carcinogenesis.

Effects of dietary fibre and fat on the development of colorectal cancer

Dietary fibre

In the past decades dietary fibre has been defined in different ways. In the classical definition dietary fibre is regarded as 'non-starch polysaccharides and

lignin of plant cell walls'. These components have in common that they are not susceptible to enzymatic digestion in the small intestines, but may or may not be subjected to bacterial fermentation in the large intestines. However, this view has been adjusted later because more substances appeared to fit in this definition.

Most fibres are polymers composed of a few up to several hundred thousand monosaccharides. Some fibres, such as cellulose and hemicellulose, are insoluble. Pectins, beta-glucans, gums, resistant starches and others are soluble in (hot) water.

Cellulose is mainly found in roots and leafy vegetables. It is a long linear polymer of many (up to 10,000) β-1,4 linked glucose molecules. It can be hydrolysed by cellulase producing bacteria that are abundant in the gastrointestinal tract of herbivores, but are lacking or only remotely present in the human gut. Starches are also polymers of glucose units and consist of amylose and amylopectin. Amylose is a linear polymer of glucose linked by α-1,4 glucosidic bonds. Amylopectin is a branched polymer of glucose linked by α-1,4 and α-1,6 glucosidic bonds. It can readily be hydrolysed by salivary, pancreatic and intestinal enzymes. Starches can be found in, for example, potatoes. Resistant starch (RS) is the portion of starch which escapes enzymatic digestion in the small intestines and reaches the large intestines (Cummings and Bingham, 1987; Gur and Asp, 1994; McBurney, 1991; Southgate, 1998; Van Munster and Nagengast, 1993; Van Munster *et al.*, 1994). There are three types of RS: physically entrapped starch, uncooked starch granules and retrograded starch, which is cooked and cooled. It has been estimated that up to 10% of dietary starch may reach the colon. Wheat bran (WB) is the grind husk of wheat. Basically, it is what remains after the starch is removed from the grain. Like all cereals, it is a heterogeneous mixture of cellulose, hemicellulose and other compounds. Hemicellulose is a heterogeneous group of polysaccharides consisting of sugars other than glucose. They can be branched to a high degree and typically each molecule contains 50-200 sugar units. Lignin is not a polysaccharide and is insoluble and not fermentable.

Food processing may alter the physico-chemical properties of fibre, and, therefore, its physiological function. It has been shown that certain oligo-saccharides derived from whey (so originating from animals) also can be included within the definition of dietary fibre (Wijnands *et al.*, 1999; Chapter 2 of this thesis). Possibly, the group of substances regarded as dietary fibre will be extended in the future.

The general hypotheses of the protective effects of dietary fibre have been reviewed in various papers (Hague *et al.*, 1996; McIntyre *et al.*, 1993; Schweitzer and Würsch, 1984) and are recapitulated below:

- Bulking effect: high-fibre diets cause an increased stool mass. The fibre itself is the greatest contributor to bulking if it is non-fermentable. Fermentable fibres can have water binding capacity and stimulate bacterial growth which also contribute to an increased faecal weight. The bulking effect leads to dilution and adsorption of (co)carcinogens and toxicants and in a rapid transit of the gut contents, resulting in decreased exposure of the intestinal mucosa to harmful substances.
- Dietary fibre can be fermented to a greater or less extent by anaerobic bacteria in the large intestines. The main fermentation products are SCFA, such as acetate, propionate and butyrate. SCFA reduce the colonic pH, which results in reduced ammonia concentration, decreased solubility of bile acids and inhibition of the formation of cytotoxic secondary bile acids. High concentrations of secondary bile acids have been associated with an increased colon cancer risk. Butyrate has been shown to suppress colorectal tumour formation, to protect the colonic epithelium from dysplastic change, to stimulate cell differentiation, to induce apoptosis, and to decrease proliferation of colonic cells *in vivo* and *in vitro*. Propionate has been shown to inhibit proliferation and enhance differentiation of colon cancer cell lines.
- Dietary fibre can influence the number and ratio of intestinal bacteria and the activity of bacterial enzymes capable of transforming primary into secondary bile acids, or enzymes such as β-glucuronidase, nitroreductase and azoreductase, which have been implicated in the etiology of colorectal cancer.

Dietary fat

After pre-digestion in the stomach, dietary fat is further digested in the small intestines. Primary bile acids produced in the liver are excreted in the duodenum and emulsify the mixture of dietary lipids, which makes them readily accessible for further digestion by pancreas lipase and facilitates their absorption (Stryer, 1975). Uptake of the digested lipids takes place in the small intestines. In the ileum most

bile acids are absorbed (enterohepatic cycle), but a small fraction enters the large intestines and may be transformed into secondary bile acids by bacteria.

Dietary fat is generally considered a promoter of carcinogenesis (Reddy, 1995; Whittemore, 1990; Zhao, 1991), but interestingly, the effect of fat on carcinogenesis depends on the type of fat. Caygill *et al.* (1996) found that mortality data for breast and colorectal cancer in 24 European countries correlated with the consumption of animal, but not vegetable, fat, and there was an inverse correlation with fish oil consumption. Willett *et al.* (1990) did similar observations in a prospective study among women. Populations such as Eskimos, consuming large amounts of fat derived from marine oil containing ω-3 polyunsaturated fatty acids (PUFA), specifically, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have low incidences of (colorectal) cancer (Blot *et al.*, 1975). In animal model studies, promoting effects on colorectal cancer were found for corn oil and safflower oil, but not for olive oil, coconut oil and fish oil (Reddy and Maeura, 1984; Reddy and Sugie, 1988). The development of chemically induced mammary gland tumours in rats was inhibited by dietary perilla oil as opposed to dietary soybean and safflower oil (Hirose *et al.*, 1990).

The explanation for the promoting effect of high-fat diets has been, and still is, an issue of debate. Fat has a high caloric content, which justifies the question whether the promoting effect of fat is due to a specific action or to a caloric effect, since high energy intake has consistently been associated with an increased colon cancer risk (Benito *et al.*, 1991; Lyon *et al.*, 1987; Peters *et al.*, 1992). Furthermore, it has been shown that overweight is associated with an increased risk of colorectal cancer, whereas calorie restriction by reduced food intake as well as physical activity have a preventive effect (Caygill *et al.*, 1998; Giacosa *et al.*, 1999; Kritchevsky, 1993). Slattery *et al.* (1997) showed that the energy source in high-risk diets was not relevant. Thus, the high caloric content of fat is not the only enhancing factor in carcinogenesis.

Another important factor is that certain types of dietary fat give rise to an increased concentration of colonic secondary bile acids (Reddy and Maeura, 1984). Bile acids are consistently found in higher concentration in colorectal cancer patients than in controls. They can cause DNA damage and enhance proliferation of colorectal epithelial cells. In animal studies bile acids are positively correlated with the incidence of colorectal tumours, cell proliferation or nuclear aberrations.

Therefore, secondary bile acids are considered promoters for colorectal cancer (Cheah, 1990; Reddy, 1995).

Despite a high caloric contribution to the diet, certain fat types, such as fish oil (rich in ω-3 fatty acids) and olive oil (rich in ω-9 fatty acids) have been shown to inhibit colorectal cancer, most probably through an influence on the arachidonic acid metabolism and by inhibition of prostaglandin synthesis (Bartolí *et al.*, 2000; Reddy, 1995).

Objectives of this thesis

In this thesis, studies are described regarding the effects of different types and levels of dietary fibre and fat on the occurrence of chemically induced ACF and colorectal tumours in rats. To get insight into the mechanisms underlying the effects a series of analyses was performed: weight and pH of caecal content, the concentration of caecal SCFA, proliferation and apoptosis of colonocytes and in adenomas, the concentration of faecal bile acids, faecal output and DNA adduct formation. In addition, in one experiment curcumin (CUR), rutin (RUT) and benzyl isothiocyanate (BIT) were used as model substances along with WB, to study alterations in expression of a selection of genes implicated to be involved in colorectal carcinogenesis.

The main questions addressed were:

- Does the effect of dietary fibre depend on the type of fibre?
- Does the effect of dietary fat depend on the type of fat?
- Does the combination of fibre and fat influence the effect of the separate factors?
- Do the effects occur during the initiation or the promotion phase?
- Is the occurrence of ACF positively correlated with the occurrence of colorectal tumours, or in other words, are ACF suitable as biomarker for colorectal cancer?
- What are the mechanisms underlying the effects of fibre and fat?

To answer these questions the following experiments were conducted:

1 DMH-treated Wistar rats were fed diets with low or high levels of either a non-fermentable fibre source (cellulose) or a fermentable fibre source (galacto-oligosaccharide, GOS, derived from whey), combined with different levels of dietary fat **(Chapter 2)**.

2 In AOM-treated F344 rats, the effects of diets low or high in GOS on the development of ACF and colorectal tumours were studied. The study was designed such that distinction could be made between possible effects being exerted during the initiation or the promotion phase **(Chapter 3)**.

3 The effects of diets with low or high levels of either a non-fermentable fibre source (cellulose) or a fermentable fibre source (Novelose 330), combined with different levels of dietary fat (soya oil), on the development of ACF and colorectal tumours were studied in AOM-treated F344 rats. **(Chapter 4)**.

4 AOM-treated F344 rats were fed diets with low or high levels of sunflower oil or with a high level of fish oil. The fish oil diet was fed only during the initiation phase. **(Chapter 5)**.

5 The effects of diets with WB, CUR, RUT or BIT on the development of ACF and colorectal tumours were studied in AOM-treated F344 rats. ACF were counted after 7, 15 and 26 weeks. Tumours were scored after 26 weeks and 8 months. In addition, the expression was studied of a selection of genes thought to be involved in colorectal carcinogenesis **(Chapter 6)**.

Finally, a case of a rare spontaneous rectum carcinoma in a Wistar rat is described in an **Appendix**. This case report illustrates that the tumour yield in the animal model used for the studies described in this thesis was not confounded by so-called background pathology. It also emphasises that there is no need to use saline-treated controls in this particular animal model.

Chapter 2

A comparison of the effects of dietary cellulose and fermentable galacto-oligosaccharides, in a rat model of colorectal carcinogenesis: fermentable fibre confers greater protection than non-fermentable fibre in both high and low fat backgrounds

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Abstract

The objective of this experiment was to compare the effects of diets with either a non-fermentable fibre source (cellulose) or a fermentable fibre source (galacto-oligosaccharide, GOS), combined with different levels of dietary fat, on the development of colorectal cancer. Male Wistar rats were fed AIN[76]-based diets with either a low or high level of cellulose, or a low or high level of GOS, for 9 months. The fat content of the diets was low, medium or high. All rats were treated with 1,2-dimethylhydrazine to induce colorectal tumours. Generally, the tumour incidence increased with increasing fat content in the diet. Despite marked faeces bulking, dietary cellulose either had no effect or an enhancing effect on the formation of colorectal tumours in general, although the development of carcinomas was decreased. GOS appeared to be highly protective against the development of colorectal tumours, as was demonstrated by an inhibitory effect on tumour incidence, multiplicity and size, regardless of the fat content of the diet. Neither fibre source influenced the bromodeoxyuridine labelling index determined in colon crypts or tumours. In animals fed high-GOS diets, the caecal content was significantly increased in weight and significantly decreased in pH. It was concluded that tumorigenesis was enhanced by increased fat content of the diet, and that the diets containing fermentable GOS conferred a greater protection against colorectal cancer than did the diets containing non-fermentable cellulose.

Introduction

Epidemiological data indicate that diet is probably the most important factor in the etiology of human cancer, especially cancer of the alimentary tract. Fat and fibre are thought to be major elements in the concept of dietary factors associated with increased or decreased cancer risk. This is supported by a vast amount of (meta)epidemiological and experimental data, indicating that fat has a promoting effect and fibre an inhibitory effect on carcinogenesis (Alabaster *et al.*, 1996; Burkitt, 1971; Giovannucci *et al.*, 1992; Howe *et al.*, 1992; Ma *et al.*, 1996; Potter, 1995; Reddy and Maeura, 1984; Trock *et al.*, 1990; Weisburger *et al.*, 1982). Diets rich in fruits and vegetables have been associated with reduced rates of cancer. In the classical definition, dietary fibre is the sum of lignin and polysaccharides originating from plants that are not digested by the enzymes of the small intestines, but may be degraded by microbial fermentation in the large intestines. Some fibres, such as cellulose, are (practically) not fermented and leave the body unaltered. They result in faeces bulking and consequent dilution of luminal carcinogens and promoters. Furthermore, by bulking of the stool, its transit is accelerated, reducing the exposure time to irritants and (co-)carcinogens. Galacto-oligosaccharides (GOS) belong, together with fructo-oligosaccharides (FOS) and inulin, to the group of non-digestible carbohydrates (NDO). NDO may be regarded as soluble dietary fibres as regards their compliance with a generally accepted definition of dietary fibre including both biochemical and nutritional/physiological criteria. This definition, in which the non-digestibility of foodstuffs is particularly stressed, has been proposed and is promoted by the Food Industry *ad hoc* working group on dietary fibre, an association of European food manufacturers in which also dietary fibre producers participate (Food Industry *ad hoc* working group on dietary fibre, 1994). GOS, along with other soluble fibres, is a fermentable substrate for anaerobic bacteria in the large intestines. The main fermentation products are the short chain fatty acids acetate, propionate, and butyrate. Short chain fatty acids reduce the colonic pH, which results in precipitation of bile acids and inhibition of the formation of secondary bile acids. High concentrations of secondary bile acids have been associated with an increased colon cancer risk.

The present paper describes experiments investigating the effects of dietary cellulose and GOS on the development of dimethylhydrazine-induced colorectal cancer in rats fed low-, medium- or high-fat diets. The expected protective effects

of the two types of dietary fibre used in this experiment are based on different mechanisms. The objective of this study is to find out which type of fibre confers the most effective protection against colorectal cancer and whether the protective effect is influenced by the level of dietary fat.

Materials and methods

Animals and diets

Four-hundred-and-sixty-eight male specific pathogen-free Wistar WU rats (Crl:(WI)WU BR, Charles River Wiga, Sulzfeld, Germany), 8 weeks old, were divided into 12 groups of 39 animals each. The different groups were fed an AIN[76]-based diet containing low (4.51-5.19 wt%) or high (22.55-24.51 wt%) cellulose (LC or HC), or low (8.30-9.54 wt%) or high (26.34-28.63 wt%) GOS (LGOS or HGOS; kindly provided by Borculo Domo Ingredients, Borculo, The Netherlands), and low (3.45-4.51 wt%), medium (6.90-9.02 wt%) or high (14.27-16.24 wt%) fat (LF, MF or HF). The composition of the experimental diets is summarised in Table 2.1. The diets contained about equal amounts of vitamins and minerals per unit of energy, and were prepared freshly every 2 months and stored at -20 °C until use. The percentage of linoleic acid (from safflower oil, Adams Vegetable Oils, Rotterdam, The Netherlands) was kept at a constant level in the different diets. The fat content in the MF and HF diets was increased by adding a high-oleic sunflower oil (Trisun; Contined, Bennekom, The Netherlands) to avoid a possible effect of different levels of linoleic acid on the tumorigenesis. The appearance of GOS was a syrup containing 75 wt% dry substance. The composition of the dry substance was (w/w) 58.8% galacto-oligosaccharide, 21.3% lactose, 19.3% glucose and 1.1% galactose. The syrup was mixed with water to yield a GOS syrup containing 65 wt% dry substance.

Treatment and housing

All animals were treated with 10 weekly subcutaneous injections with 50 mg/kg body wt 1,2-dimethylhydrazine (DMH; Sigma, Brussels, Belgium). The first injection was given 3 weeks after the start of the experiment. The animals were housed in macrolon cages with bedding, three animals per cage, from the start of

Table 2.1. Percentage compositions of the AIN[76]-based diets.

Dietary components (wt%)	LF/LC	LF/HC	MF/LC	MF/HC	HF/LC	HF/HC	LF/LGOS	LF/HGOS	MF/LGOS	MF/HGOS	HF/LGOS	HF/HGOS
Casein	18.04	14.43	19.30	15.43	20.75	16.60	17.24	13.80	18.45	14.74	19.83	15.86
DL-Methionine	0.27	0.22	0.29	0.23	0.32	0.25	0.26	0.21	0.26	0.22	0.27	0.24
Wheat starch	57.28	44.02	50.79	39.96	41.27	28.77	57.99	50.49	51.83	46.40	42.81	36.07
Cellulose	4.51	23.45	4.83	22.55	5.19	24.51	-	-	-	-	-	-
GOS[a]	-	-	-	-	-	-	8.30	27.40	8.88	26.34	9.54	28.63
Choline bitartrate	0.18	0.14	0.19	0.15	0.21	0.16	0.17	0.14	0.17	0.15	0.18	0.16
AIN[76]-minerals	3.16	2.53	3.38	2.71	3.64	2.90	2.70	2.38	2.92	2.58	3.12	2.78
AIN[76]-vitamins	0.90	0.72	0.97	0.78	1.04	0.83	0.78	0.69	0.83	0.75	0.90	0.79
$CaHPO_4$	1.35	1.08	1.45	1.17	1.56	1.24	1.29	1.03	1.29	1.12	1.30	1.19
Safflower oil[b]	4.51	3.61	5.66	4.53	4.79	4.41	4.31	3.45	5.41	4.33	4.58	4.21
Trisun oil[c]	0	0	3.36	2.69	11.45	10.53	0	0	3.22	2.57	10.94	10.06
Water	9.79	9.79	9.79	9.79	9.79	9.79	6.96	0.42	6.76	0.79	6.53	0
Energy content (kJ/g)	14.8	11.5	15.7	12.4	17.1	13.7	15.4	14.9	16.3	15.6	17.7	17.0

LF, MF, HF: low-, medium-, high fat. LC, HC: low-, high cellulose. LGOS, HGOS: low-, high galacto-oligosaccharide.

[a] GOS: galacto-oligosaccharide syrup (dry substance: 65 wt%). Composition of dry substance (w/w): 58.8% galacto-oligosaccharide, 21.3% lactose, 19.3% glucose and 1.1% galactose. Water was added to correct for the water present in the GOS syrup, resulting in equal water contents in all diets.

[b] High linoleic safflower oil (approx. 75% C18:2-6).

[c] High oleic sunflower oil (approx. 80% C18:1-9).

the study up to 3 weeks after the treatment with carcinogen. Thereafter, the animals were housed in suspended stainless steel cages with wire mesh floor and front, three animals per cage. Feed and tap water were available *ad libitum*. The relative humidity was kept between 30 and 70%. The number of air changes was about 10/h. Lighting was artificial by fluorescent tubes and time switch controlled at a sequence of 12 h light, 12 h dark.

In-life measurements

Food intake and body weight of all animals were recorded on a regular basis. The faeces production of six animals of the LF/LC, LF/HC, HF/HC, LF/LGOS, LF/HGOS, and HF/HGOS groups was recorded for a 7 day period (week 30). The animals were checked for clinical signs regularly. Animals showing ill health were killed and necropsy was performed.

Necropsy, histology and histopathology

Nine months after the start of the experiment all animals were killed by exsanguination from the abdominal aorta under ether anaesthesia. A thorough necropsy was performed. The colon was removed, cut open longitudinally, rinsed with saline and examined for the presence of neoplastic changes. The number, size and distance from the anus of all colorectal tumours were recorded. The remaining parts of the colon were collected as Swiss rolls. The weight and pH of the caecum content were recorded. The collected tissues were preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde, embedded in paraffin wax, sectioned at 5 µm, and stained with haematoxylin and eosin. Serial sections were made whenever necessary to expose the stalk, if present, of a tumour. The collected tissues were examined microscopically and the type of the tumours (benign or malignant) was established and recorded. Microscopic classification of the tumours was done according to the criteria described by Whiteley *et al.* (1996).

Labelling index

Ten animals of the LF/LGOS, LF/HGOS, HF/LGOS, and HF/HGOS groups were intraperitoneally injected with bromodeoxyuridine (BrdU; Sigma), 25 mg/kg body wt, 1 h prior to killing for future determination of the BrdU labelling index (LI). Swiss rolls of the colon and colorectal adenomas from animals treated with BrdU were stained with a monoclonal antibody against BrdU (Beckton Dickinson,

Mountain View, 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) 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). In 10 randomly selected normal colonic crypts in slides of the Swiss rolls all labelled and non-labelled nuclei were counted. In each adenoma (if present) all labelled and non-labelled nuclei in ten randomly selected areas with a total area of 0.046 mm^2 were counted. The number of nuclei counted per area of 0.046 mm^2 ranged from 557 to 1105. The LI was expressed as the percentage of brown stained positive nuclei of the total number of nuclei counted.

Statistical analysis

The multiplicity, size and distance from the anus of the colorectal tumours, absolute and relative caecum weights, pH (for statistical analysis expressed as H^+ concentration) of the caecum content, and BrdU LI were analysed using two-way analysis of variance (ANOVA) with factors fat and fibre. Levene's test was used to test whether variances among the groups were homogeneous. If Levene's test indicated homogeneous variances, the groups were compared by a one-way ANOVA for equal variances, followed, if significant, by pooled variance *t*-tests. If Levene's test indicated heterogeneous variances, the groups were compared by a one-way ANOVA for unequal variances, followed, if significant, by separate variance *t*-tests. Tumour incidences were analysed using Pearson's χ^2 test. All statistical tests were performed using BMDP Statistical Software (Brown *et al.*, 1992). A probability value of $P < 0.05$ (two-tailed) was used as the critical level of significance.

Results

Food consumption/energy intake/terminal body weight

The experimental diets had different energy contents. Consequently, the food consumption slightly varied among the groups, since rats eat in accordance with

their caloric needs (Appel *et al.*, 1997). Although the food consumption, as anticipated, was inversely related to the energy content of the different diets, the calculated energy intake still showed marginal differences between the groups, about 10% or less (Table 2.2). As a result, the terminal body weights were also slightly different and in accordance with the calorie consumption (Table 2.2).

Table 2.2. Mean food consumption, energy intake and terminal body weight.

Group	Food consumption (g/rat/day)	Energy intake (kJ/rat/day)	Body weight (g)
LF/LC	17.8	263.4	536
LF/HC	21.0	241.5	507
MF/LC	16.7	262.2	543
MF/HC	20.0	248.0	515
HF/LC	16.0	273.6	569
HF/HC	18.2	249.3	507
LF/LGOS	16.7	257.2	575
LF/HGOS	16.1	239.9	519
MF/LGOS	16.0	260.8	543
MF/HGOS	15.4	240.2	542
HF/LGOS	15.4	272.6	568
HF/HGOS	14.6	248.2	502

LF, MF, HF: low-, medium-, high fat. LC, HC: low-, high cellulose. LGOS, HGOS: low-, high galacto-oligosaccharide. The energy content of GOS is assumed to be 12.6 kJ/g (13). The calculated energy intake showed marginal differences between the groups, about 10% or less.

Faeces production

The mean faeces production per animal was markedly increased in the animals fed HC (Table 2.3). The lowest production was measured in the HF/HGOS group, whereas the other groups produced intermediate amounts of faeces. The faeces resulting from the cellulose diets was light coloured, whereas the faeces of the

animals fed GOS was dark. It has previously been reported that feeding oligosaccharides or non-starch polysaccharides may lead to diarrhoea (Challa *et al.*, 1997). In the present study, the consistency of the faeces was slightly softer in the HGOS groups and normal in all other groups. Diarrhoea did not occur.

Table 2.3. Mean daily faeces production per rat, measured in 6 animals in week 30 of the experiment.

Diet group	Mean daily faeces production per animal (g)
LF/LC	0.87
LF/HC	3.69
HF/HC	2.94
LF/LGOS	0.75
LF/HGOS	0.76
HF/HGOS	0.50

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LGOS, HGOS: low-, high galacto-oligosaccharide. The faeces production of animals fed a HC diet was markedly increased.

Caecum weight and pH

The caecum weights and pH are summarised in Table 2.4. The absolute and relative weights of the caecum content were inversely related to the fat content of the diets ($P < 0.01$). HC- and HGOS-fed animals showed a markedly enlarged caecum in comparison with LC- and LGOS-fed animals, respectively ($P < 0.01$). The fat content of the diets did not influence the pH of the caecum content. The pH in animals fed the cellulose-diets varied from 6.4 to 6.6. The LGOS-diets resulted in a slightly lower pH, whereas the caecum pH in animals fed the HGOS diets was statistically significantly decreased ($P < 0.01$).

Incidence of colorectal tumours

The tumour yield was high with a mean incidence of 89%. The incidence of tumours per diet group is presented in Table 2.5. The incidence of adenomas is the percentage of adenoma-bearing animals with or without carcinoma. The incidence

Table 2.4. Absolute and relative weights (g ± SEM) and pH of the caecum content.

	Diet groups					
	LF/LC	LF/HC	MF/LC	MF/HC	HF/LC	HF/HC
Abs. caecum content weight (g)	2.8 ± 0.2	3.4 ± 0.1^{b}	2.9 ± 0.2	3.2 ± 0.1^{b}	2.0 ± 0.1^{a}	2.5 ± 0.1^{ab}
Rel. caecum content weight (g/kg bw)	5.2 ± 0.3	6.7 ± 0.3^{b}	5.6 ± 0.6	6.4 ± 0.3^{b}	3.6 ± 0.2^{a}	5.1 ± 0.4^{ab}
pH	6.5	6.5	6.6	6.4	6.5	6.4
	LF/LGOS	LF/HGOS	MF/LGOS	MF/HGOS	HF/LGOS	HF/HGOS
Abs. caecum content weight (g)	2.9 ± 0.1	5.5 ± 0.2^{b}	2.8 ± 0.1	5.0 ± 0.3^{b}	2.2 ± 0.2^{a}	4.6 ± 0.4^{ab}
Rel. caecum content weight (g/kg bw)	5.3 ± 0.3	10.4 ± 0.4^{b}	5.2 ± 0.4	9.2 ± 0.4^{b}	4.1 ± 0.3^{a}	9.0 ± 0.6^{ab}
pH	6.3	5.8^{c}	6.2	5.8^{c}	6.2	5.8^{c}

LF, MF, HF: low-, medium-, high fat. LC, HC: low-, high cellulose. LGOS, HGOS: low-, high galacto-oligosaccharide. The absolute and relative weight of the caecum content were inversely related to the fat content of the diets ($^{a}P < 0.01$). HC- and HGOS-fed animals showed a markedly enlarged caecum in comparison with LC- and LGOS-fed animals, respectively ($^{b}P < 0.01$). The fat content of the diets did not influence the pH of the caecum content. The caecum pH in animals fed the HGOS diets was decreased ($^{c}P < 0.01$). Two-way analysis of variance (ANOVA) with factors fat and fibre.

Table 2.5. Incidence (% tumour-bearing animals) of colorectal tumours after 9 months.

	Cellulose-diets					
	LF/LC	LF/HC	MF/LC	MF/HC	HF/LC	HF/HC
Adenomas	49	68[c]	67	71[c]	79[a]	92[ac]
Carcinomas	60	46[c]	69	39[c]	79	52[c]
Total tumours	77	86	92	79	97[a]	94[a]
	GOS-diets					
	LF/LGOS	LF/HGOS	MF/LGOS	MF/HGOS	HF/LGOS	HF/HGOS
Adenomas	71	66	61	67	79	67
Carcinomas	74	76	71	61	87[b]	77
Total tumours	95	87	90	83	100	92

LF, MF, HF: low-, medium-, high fat. LC, HC: low-, high cellulose. LGOS, HGOS: low-, high galacto-oligosaccharide. The incidence of adenomas is the percentage of adenoma-bearing animals with or without carcinoma. The incidence of carcinomas is the percentage of carcinoma-bearing animals with or without adenoma. The incidence of total tumours is the percentage of animals bearing adenoma or carcinoma or both. In the cellulose groups the incidences of adenomas and total tumours were increased with an increasing fat content of the diet ($^aP < 0.05$). In the GOS groups a HF diet resulted in an increased incidence of carcinomas ($^bP < 0.05$). A HC diet increased the incidence of adenomas and resulted in a decrease of the carcinoma incidence ($^cP < 0.05$), whereas no effect on total tumour incidence occurred. The incidence of tumours was generally decreased in the HGOS-groups when compared with the LGOS-groups, although the differences were not statistically significant. Pearson's χ^2 test.

of carcinomas is the percentage of carcinoma-bearing animals with or without adenoma. The incidence of total tumours is the percentage of animals bearing adenoma or carcinoma or both.

In the cellulose groups the incidences of adenomas and total tumours were significantly increased with an increasing fat content of the diet ($P < 0.05$). The carcinoma incidence was also elevated, but the increase did not reach the level of statistical significance. An HC diet increased the incidence of adenomas and resulted in a significant decrease of the carcinoma incidence ($P < 0.05$), whereas no effect on total tumour incidence occurred. In the GOS groups an HF diet resulted in an increased incidence of carcinomas ($P < 0.05$), but not of adenomas or total tumours. The incidence of tumours was generally decreased in the HGOS groups when compared with the LGOS groups, although the differences were not statistically significant.

Multiplicity of colorectal tumours

The multiplicity of the tumours, expressed as the mean number of tumours per tumour-bearing animal, is presented in Fig.2.1. When compared with the LF diets, the HF diets resulted in increased multiplicity of adenomas ($P < 0.01$) and total tumours ($P < 0.01$) in the cellulose-fed animals. The HC diets resulted in a significant increase of the multiplicity of adenomas ($P < 0.01$) and total tumours ($P < 0.01$), and in a significant decrease of the carcinoma multiplicity ($P < 0.01$). In the HGOS-fed animals the multiplicity of adenomas, carcinomas ($P < 0.01$) and total tumours ($P < 0.01$) was significantly decreased.

Size of colorectal tumours

The mean size of adenomas, carcinomas and total tumours are presented in Table 2.6. The amount of cellulose or GOS in the diets did not influence the mean size of the colorectal adenomas found at necropsy. However, the mean size of carcinomas and total tumours was decreased in the HC- ($P < 0.05$) and HGOS-(trend) fed animals when compared to the LC- and LGOS-fed animals, respectively. The amount of dietary fat did not affect the size of the tumours.

Location of colorectal tumours

Most tumours were found in the distal two-thirds of the colon. The location of carcinomas was slightly more cranial than the location of the adenomas in all

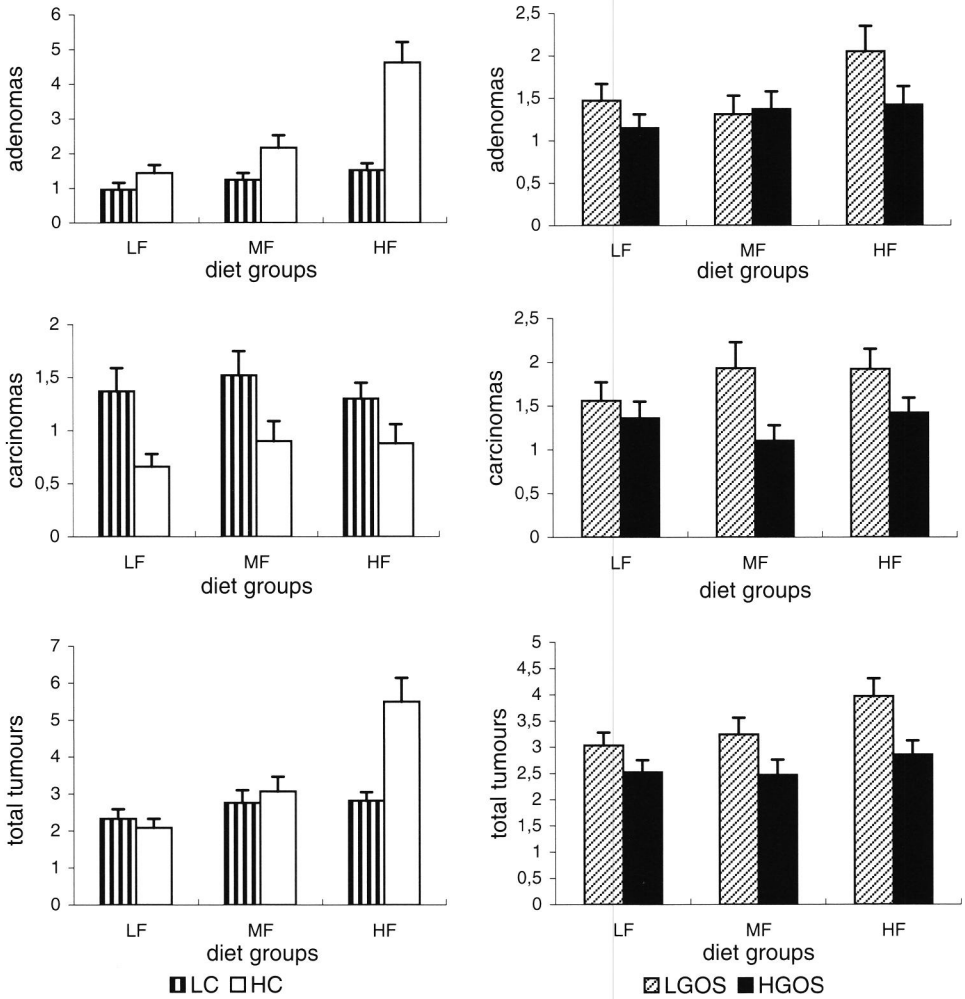
Table 2.6. Size (mm ± SEM) of colorectal tumours.

	Cellulose-diets					
	LF/LC	LF/HC	MF/LC	MF/HC	HF/LC	HF/HC
Adenomas	2.8 ± 1.0	3.3 ± 1.6	2.6 ± 0.2	2.3 ± 0.3	2.8 ± 0.3	2.0 ± 0.2
Carcinomas	7.6 ± 1.4	5.4 ± 0.6*	8.2 ± 1.9	5.4 ± 0.8*	9.6 ± 1.6	7.4 ± 1.3*
Total tumours	4.9 ± 0.9	4.4 ± 1.3*	6.0 ± 1.4	3.1 ± 0.4*	5.7 ± 0.8	3.0 ± 0.4*
	GOS-diets[1]					
	LF/LGOS	LF/HGOS	MF/LGOS	MF/HGOS	HF/LGOS	HF/HGOS
Adenomas	2.8 ± 0.3	2.2 ± 0.3	2.1 ± 0.3	2.8 ± 0.4	2.4 ± 0.3	3.0 ± 0.5
Carcinomas	9.6 ± 2.2	9.1 ± 1.2	9.3 ± 1.1	7.8 ± 1.0	9.1 ± 1.1	7.3 ± 0.9
Total tumours	6.1 ± 1.1	5.9 ± 0.8	6.1 ± 0.7	5.5 ± 0.9	6.4 ± 0.9	5.3 ± 0.7

LF, MF, HF: low-, medium-, high fat. LC, HC: low-, high cellulose. LGOS, HGOS: low-, high galacto-oligosaccharide. The mean size of carcinomas and total tumours was decreased in the HC-fed animals when compared to the LC-fed animals (*$P < 0.05$). Two-way analysis of variance (ANOVA) with factors fat and fibre.

[1] In the original article published in Carcinogenesis, by mistake, for the tumour size in the GOS groups the data from the cellulose groups were used. The data in this table are correct.

Figure 2.1. The multiplicity of the tumours, expressed as the mean number (± SEM) of tumours per tumour-bearing animal. LF, MF and HF: low, medium and high fat; LC, HC: low, high cellulose; LGOS, HGOS: low, high galacto-oligosaccharide. The HC diets resulted in an increase of the multiplicity of adenomas ($P < 0.01$) and total tumours ($P < 0.01$), but in a decrease of the carcinoma multiplicity ($P < 0.01$). In the HGOS-fed animals the multiplicity of adenomas, carcinomas ($P < 0.01$) and total tumours ($P < 0.01$) was significantly decreased. Analysis of variance with factors fat and fibre.



groups. In general, the location of the tumours was neither influenced by the amount of dietary fat, nor by the type or amount of dietary fibre.

Labelling index (LI)

The LI measured in normal colonic crypts and in adenomas are presented in Table 2.7. The LI in the adenomas was considerably higher than in the normal colonic crypts. The LI in normal colon crypts was slightly higher in animals fed an HGOS diet compared with animals fed an LGOS diet, but the difference was not statistically significant. An HF diet resulted in a statistically significantly increased LI in colon crypts when compared with an LF diet. In the adenomas the LI in the HF groups was higher than in the LF groups, and in the HGOS groups lower than in the LGOS groups, but these differences were not statistically significant.

Table 2.7. BrdU labelling index in normal colonic crypts and adenomas in animals fed GOS diets.

Groups	Labelling index (± SEM)	
	Normal crypts	Adenomas
LF/LGOS	13.88 ± 1.30^{a}	25.11 ± 2.56
LF/HGOS	14.17 ± 1.64^{a}	19.95 ± 3.21
HF/LGOS	16.47 ± 1.23^{b}	31.05 ± 5.10
HF/HGOS	21.70 ± 3.37^{b}	26.14 ± 3.35

LF, HF: low-, high fat. LGOS, HGOS: low-, high galacto-oligosaccharide.
The labelling index in normal crypts and in adenomas was increased in animals fed a HF diet.
^{a,b} Values with different superscripts are statistically significantly different ($P < 0.05$). Two-way analysis of variance (ANOVA) with factors fat and fibre.

Discussion

This paper describes the effects of dietary cellulose and GOS on the development of chemically induced colorectal cancer in rats fed diets with different

levels of fat. The most important finding was that GOS was highly protective against the development of colorectal tumours, as was demonstrated by an inhibitory effect on tumour incidence, multiplicity and size, irrespective of the fat content of the diet.

GOS is readily fermented in the caecum. The main fermentation products are short chain fatty acids (SCFA), such as acetate, propionate and butyrate, which are responsible for the observed decreased caecal pH. At a low pH the formation of secondary bile acids, which are cytotoxic and are thought to enhance carcinogenesis, is inhibited and their solubility is decreased (Cheah, 1990; Nagengast *et al.*, 1995; Van Munster and Nagengast, 1993). Butyrate has been shown to suppress colorectal tumour formation (McIntyre *et al.*, 1993), to protect the colonic epithelium from dysplastic change, and to stimulate cell differentiation, to induce apoptosis, and to decrease proliferation of colonic cells *in vivo* and *in vitro* (Awad *et al.*, 1991; Cummings and Bingham, 1987; Gamet *et al.*, 1992; Gibson *et al.*, 1992; Hague and Paraskeva, 1995; Hague *et al.*, 1996). The BrdU LI in normal colonic crypts was slightly increased in animals fed an HGOS diet. However, in adenomas from HGOS-fed animals the LI was lower when compared with those from LGOS-fed animals, which points to an inhibitory effect of GOS on proliferation of tumour cells, most probably via the formation of short chain fatty acids. Gibson *et al.* (1992) also have found that normal colonic cells may react differently to butyrate than colonic tumour cells. Other investigators have reported that oligosaccharides may promote the growth of beneficial gut microflora, such as bifidobacteria and lactobacillus (Howard *et al.*, 1995; Van Haastrecht, 1995). This phenomenon may also, at least in part, be responsible for the protective effect of GOS. In this study GOS has been shown to behave like a fibre and perfectly fits in the broader definition of dietary fibre.

The faeces production was markedly increased in the animals fed HC diets, when compared with the other groups. This can be explained by the fact that cellulose leaves the body practically unaltered. Moreover, since cellulose contributes hardly to the energy content of the diet, the animals have to eat more to meet their caloric requirements. Therefore, diets with an HC content result in enlargement of the caecum and faeces bulking. This is clearly demonstrated by comparison of the mean food consumption and faeces production in the LF/LC group with those of the LF/HC group (Tables 2.2 and 2.3). In the LF/HC group both the mean daily food consumption and the daily faeces production were three

grams higher than in the LF/LC group. Faeces bulking is one of the mechanisms thought to have a protecting effect in colorectal carcinogenesis. The protecting effect is ascribed to binding and dilution of carcinogens, and by shortening of the faecal transit time (Munakata *et al.*, 1995). Although faeces bulking was a convincing result in the present study, it did not seem to have a protecting effect on the development of colorectal tumours. This was rather surprising since previous studies have shown an inhibitory effect of cellulose on the development of colorectal cancer (Heitman *et al.*, 1989; Madar *et al.*, 1996; Sakamoto *et al.*, 1996). The multiplicity data, however, indicate that the development of carcinomas was inhibited in the HC-fed rats. Some investigators state that most carcinomas arise *de novo* from the mucosa (Maskens and Dujardin-Loits, 1981), the work of others support the hypothesis of an adenoma-carcinoma sequence (Kirkham *et al.*, 1983). Carcinomas probably develop in both ways. Nevertheless, the fact that in the present study the multiplicity of adenomas in the HC fed animals was increased suggests that the decreased multiplicity of carcinomas may have been due to an inhibited formation of carcinomas from adenomas. The decreased size of carcinomas and total tumours in HC-fed animals is also indicative for an inhibitory effect on the growth of these tumours.

Unlike in HC-fed animals, the enlargement of the caecum in HGOS-fed animals most probably occurs mainly because of water-binding properties of GOS. Resorption of the water in the colon results in normal amounts of faeces in these rats.

The promotional effect of dietary fat on carcinogenesis has been widely recognized and was confirmed in the present study. This effect is partly ascribed to the high caloric density of fat, because animal experiments have clearly shown a significant reduction of tumour formation (Klurfeld *et al.*, 1987; Kritchevsky *et al.*, 1986) and development of preneoplastic colonic aberrant crypt foci (Lasko and Bird, 1995) in animals kept on a calorie restricted diet. Other mechanisms may also play a role, for instance, dietary fat may influence the activation of protein kinase C (Birt, 1990) or elevate the levels of secondary bile acids (Reddy, 1992). Although the differences in calorie intake between the experimental groups were quite small, they cannot completely be discounted as factors responsible for the lower cancer rate in the HGOS fed animals. However, the animals fed a HC diet had the same reduced calorie intake, but in these animals the cancer rate was unaffected or even

slightly enhanced. Therefore, the reduced cancer rate in the HGOS fed animals cannot be solely ascribed to a reduced calorie intake.

From the results of the present study it may be concluded that dietary cellulose, despite marked faeces bulking, either had no effect or an enhancing effect on the formation of colorectal tumours, although the development of carcinomas was decreased, possibly through an inhibited formation of carcinomas from adenomas. Furthermore, it can be concluded that GOS is highly protective against the development of colorectal tumours, as was demonstrated by an inhibitory effect on tumour incidence, multiplicity and size, regardless of the fat content of the diet. GOS may be a valuable functional food with a high potency to decrease the risk to develop colorectal cancer.

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Chapter 3

Effect of dietary galacto-oligosaccharides on azoxymethane-induced aberrant crypt foci and colorectal cancer in Fischer 344 rats

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Abstract

The aim of the present study was to investigate the effects of galacto-oligosaccharides (GOS, Elix'or) on the development of aberrant crypt foci (ACF) and colorectal tumours in rats treated with azoxymethane (AOM). Two groups of 102 male Fischer 344 rats were injected twice with AOM to induce colorectal tumours, and fed diets containing either a low [5% (w/w); LGOS] or a high [20% (w/w); HGOS] concentration of GOS. Four weeks after the last AOM injection, 18 animals from each group were killed and their colon was removed for scoring ACF. Half of the animals in the LGOS group were switched to an HGOS diet (L/HGOS) and half of those in the HGOS group to an LGOS diet (H/LGOS). Six weeks after the change in diet, nine animals per group were killed for scoring ACF. Ten months after the start of the study the remaining animals were killed for scoring colorectal tumours. The aberrant crypt multiplicity scored after 13 weeks and the colorectal tumour incidence in rats fed an HGOS diet were significantly lower than in rats fed an LGOS diet. However, the induction of ACF by AOM, the proliferation rate and apoptotic index of the adenomas, and the size and multiplicity of colorectal tumours were not influenced by the amount of GOS in the diet. The aberrant crypt multiplicity, scored after 13 weeks, was predictive for the tumour outcome at the end of the study. It was concluded that an HGOS diet has a protective effect against the development of colorectal tumours in rats and that this protective effect is exerted during the promotion phase rather than the initiation phase of carcinogenesis.

Introduction

Over the last decades, dietary fibre has been recognized as an important food constituent. There is evidence from many epidemiological studies and animal models that it has protective properties against cancer, especially cancer of the alimentary tract, in humans and animals (Alabaster *et al.*, 1996; Burkitt, 1971; Giovannucci *et al.*, 1992; Howe *et al.*, 1992; Ma *et al.*, 1996; Potter, 1995; Trock *et al.*, 1990; Weisburger *et al.*, 1982). Dietary fibre is, by definition, not digestible by the enzymes of the small intestines, but fermentable dietary fibres are degraded by microbial fermentation in the large intestines. Galacto-oligosaccharides (GOS) belong to the group of non-digestible carbohydrates that may be regarded as soluble dietary fibres, because they fit the generally accepted definition of dietary fibre including both biochemical and nutritional/physiological criteria (Food Industry *ad hoc* Working Group on Dietary Fibre, 1994). The main constituent of the GOS (Elix'or) syrup is a mixture of galacto-oligosaccharides, produced by treatment of lactose with β-galactosidase. The composition of the GOS is: $(galactose)_n glucose$, where n is 1-7.

A previous experiment (Wijnands *et al.*, 1999) showed that GOS were highly protective against the development of colorectal tumours in Wistar rats, treated with 10 weekly subcutaneous injections of 1,2-dimethylhydrazine (DMH) at 50 mg/kg body weight, as was demonstrated by an inhibitory effect on tumour multiplicity (mean number of tumours per tumour-bearing animal), tumour incidence (percentage of tumour-bearing animals) and tumour size. The multiplicity of colorectal tumours in animals fed a high-GOS (HGOS) diet was statistically significantly lower than that in animals fed a low-GOS (LGOS) diet. Both the incidence and size of colorectal tumours were lower in animals fed an HGOS diet than in those fed an LGOS diet, although the differences were not statistically significant.

The aim of the present study was to investigate the effects of GOS on the development of aberrant crypt foci (ACF) and tumours in the colon and rectum of rats treated with azoxymethane (AOM). AOM, a metabolite of DMH, is a potent carcinogen. Two injections of AOM, 1 week apart, are sufficient to induce colorectal cancer in rats, resulting in a much shorter initiation phase than in the DMH model. This makes the AOM model more suitable for studying events taking place during either the initiation or promotion phase of carcinogenesis. It is

generally accepted that carcinogenesis is a multihit/multistep process in which a tumour can develop after exposure of cells to an initiator, followed by exposure to a promoter. During the initiation phase a carcinogen causes changes in a target tissue, such that the altered cells become susceptible to the promotional effects of a promoter. During the promotion phase the initiated tissue may develop focal proliferative lesions, some of which may undergo further changes leading to the development of a malignant tumour (Slauson and Cooper, 1990).

ACF are considered putative preneoplastic lesions, which may develop into colorectal tumours. ACF do not occur in untreated rats, but appear in the colon and rectum within a few weeks (during the post-initiation phase) after treatment with carcinogens such as DMH or AOM.

In order to investigate whether the expected effect of GOS on tumorigenesis occurs during the initiation phase or the promotion phase, the LGOS diets were replaced by an HGOS diet, and vice versa, 7 weeks after the start of the study. Ten months after the start of the experiment the animals were killed and examined for the presence of colorectal tumours. The number, size and distance from the anus of all colorectal tumours were recorded. The growth rate of adenomas was studied by counting the relative number of proliferating and apoptotic adenoma cells after making the typical nuclear changes visible. The growth of a tumour is largely determined by the balance between increase in cell numbers (proliferation) and loss of cells by apoptosis. Influencing either of these processes may play a role in enhancement or inhibition of carcinogenesis.

Materials and methods

Animals, diets and time schedule

Two hundred and four male specific-pathogen free Fischer 344 rats (Harlan Sprague Dawley, Indianapolis, IN, USA), 3 weeks old, were divided into two groups of 102 animals each. Rats were fed an AIN^{93}-based diet containing a low [5% (w/w); LGOS] or high [20% (w/w); HGOS] concentration of GOS (Elix'or; Borculo Domo Ingredients, Borculo, The Netherlands). The diets contained approximately equal amounts of vitamins and minerals per unit of energy; they were prepared freshly every 2 months and stored at -20 °C until use. GOS was a

syrup containing 75% (w/w) dry substance. The composition of the dry substance was (by weight): 58.8% galacto-oligosaccharides, 21.3% lactose, 19.3% glucose and 1.1% galactose. The syrup was mixed with water to yield a GOS syrup containing 65% (w/w) dry substance. The GOS syrup was added to the diets in place of some of the wheat starch and water. The composition of the experimental diets is summarised in Table 3.1.

Table 3.1. Composition of the experimental diets.

Dietary component	% by weight in diet	
	LGOS diet	HGOS diet
Casein	20.00	20.00
L-Cystine	0.30	0.30
Wheat starch	49.70	39.95
Cellulose	5.00	5.00
GOS	5.00	20.00
Choline bitartrate	0.25	0.25
AIN^{93} - minerals	3.50	3.50
AIN^{93} - vitamins	1.00	1.00
Soya oil	10.00	10.00
Water[a]	5.25	0.00
Total	100.00	100.00
Energy content (kJ/g)	16.3	15.9

GOS syrup (Elix'or) contained 65 % (w/w) dry substance. Composition of dry substance (w/w): 58.8% galacto-oligosaccharides, 21.3% lactose, 19.3% glucose and 1.1% galactose.
[a] Water was added to correct for the water present in the GOS syrup, resulting in equal water contents in all diets.

Seven weeks after the start of the study (i.e. 4 weeks after the last AOM treatment), 18 animals from the LGOS and HGOS groups were killed and their

colon removed for scoring ACF. Half of the animals in each group where then switched to the other diet. Thus, two additional experimental groups were formed: L/HGOS (which received the LGOS diet for the first 7 weeks of the study and the HGOS diet thereafter) and H/LGOS (which received the HGOS diet for the first 7 weeks of the study and the LGOS diet thereafter). Six weeks after the change in diet, 9 animals per group were killed for scoring ACF again. Ten months after the start of the study the remaining animals were killed. The time schedule is summarised in Fig.3.1.

Figure 3.1. Design of the study. AOM, treatment with azoxymethane; ACF, scoring aberrant crypt foci; LGOS and HGOS, low- and high galacto-oligosaccharides. L/HGOS, this group received the LGOS diet for the first 7 weeks of the study and the HGOS diet thereafter. H/LGOS, this group received the HGOS diet for the first 7 weeks of the study and the LGOS diet thereafter.

week 0	week 2	week 3	week 7	week 13----------/ /----------10 months	
Start	AOM I	AOM II			
			Interim kill I	Interim kill II	Final necropsy
			ACF ACF		Tumours
			n = 18 (LGOS) n = 18 (HGOS)	n = 9 (LGOS) n = 9 (L/HGOS) n = 9 (HGOS) n = 9 (H/LGOS)	
			↑ Diet change		
Animals in study					
					All remaining rats:
n = 102 (LGOS) n = 102 (HGOS)			n = 42 (LGOS) n = 42 (L/HGOS) n = 42 (HGOS) n = 42 (H/LGOS)	n = 33 (LGOS) n = 33 (L/HGOS) n = 33 (HGOS) n = 33 (H/LGOS)	n = 33 (LGOS) n = 30 (L/HGOS) n = 31 (HGOS) n = 29 (H/LGOS)

Treatment and housing

All animals were treated with two subcutaneous injections with AOM (Sigma, Brussels, Belgium), 15 mg/kg body wt, in the second and third week after the start of the experiment, respectively. The animals were housed in macrolon cages with

bedding (three animals per cage) from the start of the study up to 3 weeks after the treatment with carcinogen. Thereafter, the animals were housed in suspended stainless steel cages (three animals per cage) with wire mesh floor and front. Feed and tap water were available *ad libitum*. The relative humidity was kept between 30 and 70%. The number of air changes was ~10 per hour. Artificial light was supplied from fluorescent tubes in a 12 h light - 12 h dark cycle. The food intake, body weight and clinical signs of all animals were recorded regularly. Moribund animals were killed and necropsy was performed.

Aberrant crypt foci

The colons of the animals killed at the interim necropsies were removed, opened longitudinally, rinsed with saline, fixed flat between filtration paper, and preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde. The ACF were made visible by staining the colons with 0.1% methylene blue in saline for about 7 minutes. ACF can easily be identified using a light microscope at 40 x magnification. Aberrant crypts have large, usually elongated openings. The lining epithelial cells are larger and more intensely stained with methylene blue than the surrounding normal epithelial cells. The results of previous experiments of other investigators have indicated that especially the development of larger ACF (four or more crypts/focus) might have particularly good predictive value for tumour yield at the end of the experiment (Pretlow *et al.*, 1992; Davies *et al.*, 1999). Therefore, the number of aberrant crypts (AC) in each focus was recorded to determine the AC multiplicity.

Necropsy, histology and histopathology

Ten months after the start of the experiment all remaining animals were killed by exsanguination from the abdominal aorta under ether anaesthesia. A thorough necropsy was performed. The colon was removed, cut open longitudinally, rinsed with saline, and examined for the presence of neoplastic changes. The number, size and distance from the anus of all colorectal tumours were recorded. The remaining parts of the colon were collected as 'Swiss rolls'. Collected tissues were preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde, embedded in paraffin wax, sectioned at 5 µm, and stained with haematoxylin and eosin. Serial sections were made when necessary to expose the stalk, if present, of a tumour. The collected tissues were examined microscopically and the type of the tumours

(benign or malignant) was established and recorded. Microscopic classification of the tumours was done according to the criteria described by Whiteley *et al.* (1996). The tumour incidence (percentage of tumour-bearing animals) and multiplicity (mean number of tumours per tumour-bearing animal) were determined for adenomas, carcinomas, and total tumours (adenomas and carcinomas). Data on properties of tumours groups maintained on the same diet during the last phase of the study (from week 13 until necropsy at about 10 months) were combined.

Proliferation labelling index

Sections of all colorectal adenomas found were stained with a monoclonal antibody against Ki-67 and examined by light microscopy. The Ki-67 staining protocol included the following: after deparaffination, the slides were incubated for 10 min in 3% hydrogen peroxide in methanol to block endogenous peroxidase. Slides were rinsed in distilled water, immersed in 10 mMol citric acid pH 6.0 and boiled for 15 min for antigen retrieval. After cooling at room temperature for about 30 min, the slides were rinsed in phosphate-buffered saline (PBS) and covered with 25% goat serum for 15 min. They were then incubated with primary antibody (Ki-67, Novocastra) for 1 h. Slides were rinsed in PBS and then incubated in secondary antibody (biotinylated rabbit anti-mouse; Dako, Denmark) for 30 min. After rinsing the slides in PBS, horseradish peroxidase-conjugated streptavidin (Dako, Denmark) was applied for 30 min. Finally, peroxidase activity was developed for 10 min with 5 mg diaminobenzidine (Sigma) in 10 ml PBS and 5 µl hydrogen peroxidase. To increase the staining intensity, 100 µl $CoCl_2$ was added to the staining solution. The slides were counterstained with nuclear Fast Red. In the adenomas, all labelled and unlabelled nuclei in 10 randomly selected areas with a total area of 0.046 mm^2 were counted. The labelling index was expressed as the percentage of nuclei counted that stained brown.

Apoptosis

Apoptotic cells in colorectal adenomas were visualised using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) method. After deparaffination, the endogenous peroxidase was blocked by immersing the slides in 0.3% hydrogen peroxide in methanol for 30 min. After rinsing three times in PBS, the slides were incubated in proteinase K (15 µg/ml in PBS pH 7.4; Sigma) for 15 min at 37 °C. Slides were rinsed twice in PBS and dried

around the sample; TUNEL reaction mixture (Boehringer, Germany) was added. The sections were covered by a coverslip to avoid evaporation and incubated at 37°C for 60 min. Slides were rinsed twice in PBS for 10 min and then dried around the sample; a converter-POD was added and the sections were covered by a coverslip and incubated at 37°C for 30 min. Slides were rinsed twice in PBS for 10 min, diaminobenzidine was added for 10 min and then slides were rinsed in running water and counterstained with haematoxylin. In the adenomas all labelled and unlabelled nuclei in 10 randomly selected areas with a total area of 0.046 mm^2 were counted. The apoptotic index was expressed as the percentage of nuclei that stained positively.

Statistical analysis

The multiplicity of AC and ACF, the multiplicity and size of the colorectal tumours, the Ki-67 labelling index and the apoptotic index were analysed using one-way analysis of variance (ANOVA). Levene's test was used to test whether variances among the groups were homogeneous. If Levene's test indicated homogeneous variances, the groups were compared by one-way ANOVA for equal variances, followed, if significant, by pooled variance *t*-tests. If Levene's test indicated heterogeneous variances, the groups were compared by one-way ANOVA for unequal variances, followed, if significant, by separate variance *t*-tests. Tumour incidences were analysed using Pearson's χ^2 test. A P value of < 0.05 (two-tailed) was considered significant.

Results

Food consumption, energy intake and terminal body weight

The LGOS and HGOS diets had a similar caloric content (Table 3.1). The food consumption and (calculated) energy intake of animals fed the LGOS diet was slightly higher than that of animals fed the HGOS diet. This may explain the slightly higher final body weights of the animals maintained on the LGOS diet (Table 3.2). However, the differences were only marginal (about 7% or less).

Table 3.2. Mean food consumption, GOS consumption, energy intake and final body weight.

	Food consumption (g/rat/day)		GOS consumption (g/rat/day)		Eenergy intake (kJ/rat/day)		Final body weight (g)
LGOS		17.5		0.9		285.3	497
L/HGOS	16.0[a]	16.8[b]	0.8[a]	3.4[b]	260.8[a]	267.1[b]	470
HGOS		16.7		3.3		265.5	459
H/LGOS	16.3[a]	16.9[b]	3.3[a]	0.8[b]	259.2[a]	275.5[b]	477

[a] Before diet change.
[b] After diet change.
There were marginal differences in calculated energy intake between the groups.

Aberrant crypt foci

ACF were present in all animals. After the first 7 weeks the total number of ACF in animals fed an HGOS diet was lower than that in animals fed an LGOS diet (Table 3.3). The number of large ACF (four or more crypts) and the AC multiplicity were highest in the HGOS-fed animals ($P < 0.05$ for large ACF). From week 7 to week 13 (after the diet change), the number of total as well as large ACF increased in all animals as expected. However, the increase was less in rats that had been switched from an LGOS to an HGOS diet than in rats that had been switched from an HGOS to an LGOS diet. At 13 weeks, the AC multiplicity in the H/LGOS group was statistically significantly higher ($P < 0.05$) than that in all other groups. The number of total as well as large ACF was also highest in this group, but this was not statistically significant. To investigate the effect of the different diets during weeks 7-13 of the experiment, the results of the LGOS and H/LGOS groups were combined, as were those of the HGOS and L/HGOS groups. The number of total as well as large ACF was lower in the combined HGOS group than in the combined LGOS group (not statistically significant). The AC multiplicity was statistically significantly ($P < 0.05$) lower in the combined HGOS group.

Table 3.3. Mean number of ACF (± SD) in the colon and rectum of animals killed 7 or 13 weeks after the start of the study.

Group	Week of sacrifice	No. of rats	ACF/rat		AC multiplicity (no. of crypts/focus)
			Total no.	No. with ≥ 4 crypts	
LGOS	7	18	85.3 ± 29.9	10.0 ± 5.8	2.29 ± 0.16
HGOS	7	18	69.6 ± 29.2	15.2 ± 7.9[b]	2.65 ± 0.28
LGOS	13	9	100.8 ± 33.9	32.7 ± 13.4	3.01 ± 0.24
L/HGOS	13	9	89.0 ± 49.5	31.0 ± 24.9	3.06 ± 0.32
HGOS	13	9	96.4 ± 48.9	34.8 ± 22.6	3.09 ± 0.32
H/LGOS	13	9	106.2 ± 39.0	54.9 ± 30.1	3.80 ± 0.62[c]
Combined groups after 13 weeks[a]					
LGOS	13	18	103.5 ± 35.5	43.8 ± 25.3	3.40 ± 0.61
HGOS	13	18	92.7 ± 47.9	32.9 ± 23.1	3.07 ± 0.31[d]

[a] Combined: LGOS = LGOS + H/LGOS, HGOS = HGOS + L/HGOS, week 13.
[b] $P < 0.05$, HGOS vs LGOS, week 7.
[c] $P < 0.05$, H/LGOS vs LGOS, L/HGOS, HGOS, week 13.
[d] $P < 0.05$, HGOS combined vs LGOS combined.

Properties of colorectal tumours

The overall mean incidence of colorectal tumours was 76.4%. The incidence of tumours in each diet group is presented in Table 3.4. The incidence of adenomas is the percentage of adenoma-bearing animals with or without carcinoma. The incidence of carcinomas is the percentage of carcinoma-bearing animals with or without adenoma. The incidence of total tumours is the percentage of animals bearing adenoma or carcinoma or both. An HGOS diet resulted in a decreased incidence of colorectal adenomas and carcinomas. The decrease in incidence of all tumours combined (adenomas + carcinomas) was significant ($P = 0.05$). The multiplicity of the tumours, expressed as the mean number of tumours per tumour-bearing animal, is presented in Table 3.4. In general, the multiplicity was quite low in all animals. The experimental groups did not show statistically significant

differences with respect to the multiplicity of adenomas, carcinomas or all tumour types combined. The experimental groups did not show statistically significant differences with respect to the mean size of adenomas, carcinomas or all tumour types combined (Table 3.4). Most tumours were found in the distal two-thirds of the colon. The location of carcinomas was slightly more cranial (mean distance from the anus: 64 mm) than the location of the adenomas (mean distance from the anus: 44 mm) in all groups. In general, the location of the tumours was not influenced by the diet.

Table 3.4. Incidence, multiplicity and size of colorectal tumours.

	LGOS and H/LGOS combined	HGOS and L/HGOS combined
Incidence (% tumour-bearing animals)		
Adenomas	47.8	43.1
Carcinomas	58.2	52.3
All tumours	83.6	69.2*
Multiplicity (mean ± SD)		
Adenomas	1.0 ± 1.2	0.9 ± 0.9
Carcinomas	1.0 ± 0.9	1.0 ± 0.8
All tumours	2.0 ± 1.0	1.9 ± 1.1
Size (mm ± SD)		
Adenomas	2.4 ± 1.2	2.2 ± 0.9
Carcinomas	5.8 ± 3.7	6.1 ± 3.6
All tumours	4.4 ± 3.3	4.5 ± 3.4

The incidence of adenomas is the percentage of adenoma-bearing animals with or without carcinoma. The incidence of carcinomas is the percentage of carcinoma-bearing animals with or without adenoma. The incidence of total tumours is the percentage of animals bearing adenoma or carcinoma or both.

* $P = 0.05$ (Pearson's χ^2 test).

Labelling index and apoptotic index

To determine the labelling index in adenomas, using Ki-67 as a marker, a total number of 24205 nuclei was counted. The labelling index in the adenomas of LGOS-fed animals was 42.84 ± 7.22 and that in HGOS-fed animals was 40.72 ± 13.22. The difference was not statistically significant.

To determine the apoptotic index in adenomas a total number of 14764 nuclei was counted. The apoptotic index in the adenomas of LGOS-fed animals was 0.76 ± 0.39 and in HGOS-fed animals 0.90 ± 0.64. The difference was not statistically significant.

Discussion

In the present study the incidence of colorectal tumours in rats fed an HGOS diet was lower than that in rats fed an LGOS diet. The results also demonstrate that an HGOS diet decreased the aberrant crypt multiplicity scored after 13 weeks but failed to affect the total number of ACF induced in rat colon by AOM. Although the effect of the different diets on the development of ACF was rather inconsistent, the aberrant crypt multiplicity scored after 13 weeks appeared to be predictive of the tumour outcome at the end of the study.

Several animal models are available for investigating the influence of dietary factors on the development of colorectal tumours. In the DMH model, rats are subjected to multiple (up to 10) high doses of carcinogen. This injection protocol leads to the development of a rather high number of colorectal tumours in almost all animals. The results of previously performed studies, conducted at our Institute, have shown that the incidence of colorectal tumours is hardly affected using this animal model. In most studies an effect is found on the multiplicity rather than on the incidence of colorectal tumours. An undesired effect of the DMH model is the frequent development of tumours at other sites, such as the duodenum and the Zymbal gland, causing health problems and death before the end of the experiment. AOM induces these unwanted effects at a much lower incidence than DMH. Another important advantage of the AOM model is the short injection protocol, which allows studying the effects of compounds during the initiation or the promotion phase of the carcinogenic process, separately.

In the present study, the effects of GOS during the initiation phase were determined by monitoring the development of putative preneoplastic ACF, induced in rats by two consecutive injections with AOM. A protective effect of an HGOS diet during the initiation phase of carcinogenesis should, theoretically, be reflected by a lower number and/or smaller size of ACF in animals fed an HGOS diet in comparison with animals fed an LGOS diet. The results of the present study, however, pointed to some inconsistent effects of GOS during the initiation phase, although the differences among the groups reached the level of statistical significance in one group only. Animals maintained on an HGOS diet for 7 weeks developed significantly more large (four or more crypts) ACF, but fewer total ACF, than animals maintained on an LGOS diet. After 13 weeks the aberrant crypt multiplicity (number of aberrant crypts per focus) was significantly higher in animals maintained on an HGOS diet during the first 7 weeks and on an LGOS diet from week 7 onwards. After combining the animals maintained on the same diets from week 7 to week 13 (LGOS + H/LGOS, and HGOS + L/HGOS), the number of total as well as large ACF was lower (not statistically significant) in the combined HGOS group than in the combined LGOS group. The AC multiplicity was statistically significantly ($P < 0.05$) lower in the combined HGOS group.

The results of studies into effects of different compounds on colon carcinogenesis in rats by other investigators have indicated that the predictive value of ACF for the tumour outcome is inconsistent. Some investigators (Alabaster *et al.*, 1995; Pretlow *et al.*, 1992) have found a clear correlation between number and size of ACF, and the tumour outcome in studies with sodium phytate, a chemopreventive agent or with β-carotene and wheat bran fibre. In another study with wheat bran, Young *et al.* (1996) found a correlation between the number but not the size of ACF and tumour outcome. Magnuson *et al.* (1993) found just the opposite in rats fed cholic acid: not the number but the size of ACF correlated with tumour outcome. A plausible explanation was not always given. It has been demonstrated that ACF observed in the colon of humans and laboratory animals are histologically heterogeneous lesions (Di Gregorio *et al.*, 1997; Fenoglio-Preiser and Noffsinger, 1999; Bouzourene *et al.*, 1999) and it has been speculated that only a selective group of ACF is susceptible to the effects of chemicals and dietary components. Thorup *et al.* (1994), who failed to find a correlation between ACF parameters and tumour outcome in animals fed a high fibre diet, even questioned the hypothesis that ACF are really preneoplastic lesions. These investigators have

postulated that ACF and colorectal tumours possibly represent two parallel, independent consequences of cancer initiation. Nevertheless, the results from most previous studies indicate that the AC multiplicity is a more consistent predictor of tumour outcome than the number of ACF.

In the present study the ACF score and AC multiplicity after 13 weeks contrast with those after 7 weeks. Knowing the protective effect of GOS against the development of colorectal tumours, it can be concluded that, in this study, the AC multiplicity after 13 weeks but not that after 7 weeks has a predictive value for ultimate tumour outcome. This means that the beneficial effect of an HGOS diet is mainly exerted during the post-initiation phase. These data support the hypothesis that some ACF are transient lesions that are either eliminated or are remodelled to produce normal colonic crypts. Other ACF, the so-called persistent ACF, may develop into colorectal tumours. The occurrence of persistent nodules induced in the liver with various carcinogens is a comparable phenomenon (Bannasch and Zerban, 1994). Based on the tumour size, proliferation index and apoptosis data found in the present study, it was concluded that the growth rate of colorectal adenomas was not significantly influenced by the different diets.

In a previous study (Wijnands *et al.*, 1999) we showed that GOS have a significant inhibitory effect on the development of colorectal tumours. In that study, the exposure of rats to the carcinogen DMH (10 weekly injections of 50 mg/kg) was very high, resulting in a high tumour incidence (almost 100%) and a high tumour multiplicity. In that study, GOS demonstrated a significant inhibitory effect on the multiplicity of colorectal tumours, but only a slight inhibitory effect on the incidence. Interestingly, the results of the present study with AOM showed a significant inhibitory effect of GOS on the incidence but not on the multiplicity of colorectal tumours. This may be due to the low number of tumours per animal, because an animal without a colorectal tumour will be omitted in the multiplicity score.

Although the present results are less pronounced than those observed in the DMH study, the results of both studies together strongly support the conclusion that a diet high in GOS inhibits the development of colorectal tumours in carcinogen-treated rats. This beneficial effect of an HGOS diet may be explained by the general properties of GOS. GOS escapes enzymatic digestion in the small intestines but is readily fermented in the caecum. The main fermentation products are short-chain fatty acids (SCFA), such as acetate, propionate and butyrate. We

have analysed the stored caecum content of several animals from the DMH study for SCFA using gas chromatography. The results are presented in Table 3.5. The total amount of SCFA in the caecum of animals fed an HGOS diet was considerably higher than that in animals fed an LGOS diet. After correction for the differences in weight of the caecal content between the groups, the amounts of acetate, propionate and butyrate were significantly increased in the HGOS-fed animals.

Table 3.5. Absolute amount (mg) of SCFAs in the caecum (mean ± SEM)

	*n*1	*n*2	Acetate	Propionate	Butyrate	Total SCFA
LF/LGOS	9	8	13.7 ± 3.6^{ac}	2.7 ± 0.5^{c}	4.0 ± 1.5^{c}	20.4 ± 5.5^{ac}
LF/HGOS	9	9	52.6 ± 7.5^{ad}	7.7 ± 0.9^{d}	5.4 ± 0.7^{d}	65.6 ± 8.2^{ad}
HF/LGOS	8	5	9.4 ± 1.9^{bc}	2.2 ± 0.2^{c}	1.4 ± 0.4^{c}	13.0 ± 2.4^{bc}
HF/HGOS	10	8	28.1 ± 4.3^{bd}	6.6 ± 1.1^{d}	6.4 ± 1.7^{d}	41.2 ± 5.6^{bd}

LF, HF: low (4% w/w) and high (15% w/w) fat; LGOS, HGOS: low (9% w/w) and high (28% w/w) GOS; *n*1: number of animals from which samples were taken; *n*2: number of samples analysed after pooling; total SCFA: sum of acetate, propionate and butyrate.
[ab]Effect of fat: [b] significantly lower than [a] ($P < 0.05$).
[cd]Effect of GOS: [d] significantly higher than [c] ($P < 0.001$; for butyrate, $P < 0.05$) (two-way ANOVA).

SCFA production decreases the pH in the large intestines (in the DMH study, the caecum pH in animals fed diets with cellulose as fibre source ranged from 6.4 to 6.6; the caecum pH in LGOS-fed animals was 6.2 and in HGOS-fed animals was 5.8). At a low pH the formation of secondary bile acids, which are cytotoxic and are thought to enhance carcinogenesis (Narisawa *et al.*, 1974; Reddy *et al.*, 1977), is inhibited and their solubility decreased (Cheah, 1990; Nagengast *et al.*, 1995; Van Munster and Nagengast, 1993). Butyrate suppresses colorectal tumour formation (McIntyre *et al.*, 1993) and both butyrate and propionate have been shown to inhibit proliferation and enhance differentiation of colon cancer cell lines (Gamet *et al.*, 1992; Scheppach *et al.*, 1995). In the present study, however, the proliferation index was only marginally lower in HGOS-fed animals. Other investigators have reported that oligosaccharides may promote the growth of

beneficial gut microflora, such as bifidobacteria and lactobacilli (Howard *et al.*, 1995; Van Haastrecht, 1995). This phenomenon may also have contributed to the protective effect of GOS on the development of colorectal tumours.

Taking into account the inhibitory effect of an HGOS diet on colorectal tumour multiplicity in rats, previously demonstrated in an experiment using the DMH model, it seems justifiable to conclude that an HGOS diet has a protective effect against the development of colorectal tumours in rats. The protective effect of GOS on colorectal carcinogenesis is exerted during the promotion phase rather than during the initiation phase of the carcinogenic process. SCFA, the fermentation products of GOS, probably play a key role in the mechanism of protection.

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Chapter 4

Effects of cellulose, Novelose 330 and fat on induced aberrant crypt foci (ACF) and colorectal cancer in azoxymethane-treated rats: no predictive value of ACF for the development of colorectal cancer

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Abstract

Male F344 rats, treated with azoxymethane (AOM), were fed diets either low (5% w/w) or high (25% w/w) in cellulose (LC, HC) or low (5% w/w) or high (25% w/w) in Novelose 330 [containing resistant starch (RS) as main component: LRS, HRS], combined with low (5-7% w/w) or high (13-16% w/w) fat (LF, HF). ACF were scored at 11 weeks. Ten months after the study start colorectal tumours were scored and caecal weight and pH, caecal short chain fatty acids (SCFA) and faecal bile acids were measured. HC- and HF-fed animals developed more and larger ACF than LC- and LF-fed animals. RS had no effect on development of ACF. In general, the development of colorectal tumours was enhanced in rats of the high-cellulose groups, but was inhibited in high-RS-fed animals. A high-fat diet resulted in an increased incidence of colorectal tumours, but had no effect on tumour multiplicity. Caecal weight and the concentration of SCFA were increased in animals fed the high-cellulose diets as well as in those fed the high-RS diets. The caecal pH was decreased in high-RS-fed animals and in low-fat/high-cellulose-fed animals. It was concluded that colorectal carcinogenesis was enhanced by dietary cellulose and fat, and inhibited by dietary RS. The ACF score was predictive for the tumour outcome in the cellulose groups but not in the RS groups. Clearly, ACF are not suitable as biomarker for colorectal cancer.

Introduction

Animal models are widely used to study the effects of dietary factors on the development of colorectal tumours. Rats are treated with carcinogens such as 1,2-dimethylhydrazine (DMH), azoxymethane (AOM), or N-methyl-N-nitro-N-nitrosoguanidine (MNNG), that are known to induce colorectal tumours (Rogers and Nauss, 1985). Disadvantages of these models are that rather large numbers of animals are needed, because not all animals develop colorectal tumours, and the animals that do may have discomfort or die because of the induced tumours. Moreover, the carcinogen may also induce tumours at other sites than the colon, such as the Zymbal gland or the duodenum, which may cause severe clinical symptoms leading to early withdrawal of animals from the experiment.

A few weeks after injection of the carcinogen aberrant crypt foci (ACF) develop in the colorectal mucosa. ACF are single or groups of several hyperproliferative and/or dysplastic crypts. They are readily recognisable at low magnification after staining the mucosa with methylene blue. The incidence of ACF in carcinogen-treated animals is 100%, but they do not occur in untreated animals (Kawamori *et al.*, 1995; Tudek *et al.*, 1989). ACF are considered putative preneoplastic lesions that may develop into colorectal tumours (McLellan and Bird, 1988a; Pretlow *et al.*, 1991).

The main goal of the present experiment was to investigate whether various types of diet would influence the development of ACF and colorectal tumours, and whether the occurrence of ACF correlated with the colorectal tumour response. The dietary variables examined were cellulose, Novelose 330 (the main component of which is resistant starch, which has been proposed to be of importance as fermentable substrate with protective properties against colorectal cancer) and fat.

If ACF could be used as biomarker for colorectal cancer, one would have a method to screen food components or other substances for their potential role in colorectal carcinogenesis in a relatively short period of time. Moreover, from an ethical point of view, less suffering of animals (not having to get tumours), and the use of smaller numbers of animals per group would be achieved.

Materials and methods

Animals and diets

Three hundred and thirty six male specific-pathogen free Fischer 344 rats (Charles River Deutschland, Sulzfeld, Germany), three weeks old, were divided into 8 groups of 42 animals each. The different groups were fed an AIN[93]-based diet low (5% w/w) or high (25% w/w) in cellulose (LC, HC) or low (5% w/w) or high (25% w/w) in Novelose 330 (containing resistant starch (RS) as main component: LRS, HRS, National Starch & Chemical, Neustadt a.d. W., Germany), combined with low (5-7% w/w) or high (13-16% w/w) fat (LF, HF). The diets contained about equal amounts of vitamins and minerals per unit of energy and were prepared freshly every two months and stored at -20 °C until use. Novelose 330 consists almost entirely of carbohydrates (~ 50% resistant starch, ~ 35% total dietary fibre, ~ 5% simple sugars). The resistant starch in Novelose 330 is retrograded or crystalline non-granular starch, a type similar to that present in for example cooked and cooled potato, bread crust, cornflakes and retrograded high amylose starch. The composition of the experimental diets is summarised in Table 4.1.

Treatment and housing

All animals were treated with two weekly subcutaneous injections with azoxymethane (AOM, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands), 15 mg/kg body weight. The first injection was given two weeks after the start of the experiment. The animals were housed in macrolon cages with bedding, three animals per cage, from the start of the study up to three weeks after the treatment with carcinogen. Thereafter, the animals were housed in suspended stainless steel cages with wire mesh floor and front, three animals per cage. Feed and tap water were available *ad libitum*. The relative humidity was kept between 30 and 70%. The number of air changes was about 10 per hour. Lighting was artificial by fluorescent tubes and time switch controlled at a sequence of 12 hours light, 12 hours dark.

Table 4.1. Percentage compositions of the AIN^{93}-based diets.

Dietary components (wt%)	LF/LC	LF/HC	HF/LC	HF/HC	LF/LRS	LF/HRS	HF/LRS	HF/HRS
Casein	18.00	15.00	20.00	17.60	18.00	18.00	20.00	20.00
L-Cystine	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Wheat starch	66.95	48.85	55.45	37.45	66.95	45.95	56.35	34.45
Cellulose	5.00	25.00	5.00	25.00	-	-	-	-
Novelose 330 (RS)[a]	-	-	-	-	5.00	25.00	5.00	25.00
Choline bitartrate	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
AIN^{93}-minerals	3.50	2.80	3.90	3.10	3.50	3.50	4.00	3.90
AIN^{93}-vitamins	1.00	0.80	1.10	0.90	1.00	1.00	1.10	1.10
Soya oil	5.00	7.00	14.00	16.00	5.00	6.00	13.00	15.00
Energy content (kJ/g)	16.70	13.80	18.50	15.60	17.30	16.40	18.90	18.30

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LRS, HRS: low-, high Novelose 330.
[a] Novelose 330: main component resistent starch, energy content 11.75 kJ/g.

In-life measurements

Food intake and body weight of all animals were recorded on a regular basis. The animals were checked for clinical signs regularly. Animals showing ill health were killed and necropsy was performed.

Necropsy, histology and histopathology

Eight weeks after the last AOM injection, nine animals per group were killed for counting ACF. From these animals the colon was removed, cut open longitudinally, rinsed with saline, and fixed flat between filtration paper in a neutral aqueous phosphate-buffered 4% solution of formaldehyde. Ten months after the start of the experiment the remaining animals were killed by exsanguination from the abdominal aorta under ether anaesthesia. A thorough necropsy was performed. The colon was removed, cut open longitudinally, rinsed with saline, and examined for the presence of neoplastic changes. The number, size, and distance from the anus of all colorectal tumours were recorded. The remaining parts of the colon were collected as Swiss rolls. The weight and pH (Sentron 2001 pH System, Europhysics, Erkelenz, Germany) of the caecum content were recorded. The caecum content was frozen for analysis of short chain fatty acids (SCFA). The content of the colon was frozen for analysis of faecal bile acids. The collected tissues were preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde, embedded in paraffin wax, sectioned at 5 µm, and stained with haematoxylin and eosin. Serial sections were made whenever necessary to expose the stalk, if present, of a tumour. The collected tissues were examined microscopically and the type of the tumours was established and recorded.

Aberrant Crypt Assay

The flat-fixed colons of the animals sacrificed eight weeks after the last AOM injection were stained with a 0.1% solution of methylene blue for nine minutes. They were examined at low magnification and the number of ACF was recorded. Furthermore, the multiplicity of aberrant crypts was recorded by counting the number of crypts per ACF.

SCFA analysis

SCFA analysis was performed in the caecum content of 12 randomly selected animals of all groups. After thawing, the samples were homogenised. SCFA (acetate, butyrate, propionate, valeriate, isobutyrate and isovaleriate) were extracted by 115 mg 2-ethylbutyrate/7.15 ml formic acid, made up to 1 ltr. with methanol. The samples were thoroughly mixed and then centrifuged (4000 rpm for 10 minutes). The supernatant was analysed using gaschromatography with flame ionisation detector. The concentrations of the SCFA were calculated by comparison with the internal standard (2- ethylbutyrate) and the standard solutions.

Faecal bile acids analysis

Faeces from 8 randomly chosen rats from each of the different diet groups were freeze-dried and ground in a homogeniser. Faecal bile acids (cholic acid, lithocholic acid, deoxycholic acid, α-muricholic, β-muricholic, ω-muricholic, hyodeoxycholic and ursodeoxycholic acid) were analysed quantitatively by gas chromatography.

Statistical analysis

The multiplicity, size and distance from the anus of the colorectal tumours, number of ACF, multiplicity of aberrant crypts, absolute and relative caecum weights, pH (for statistical analysis expressed as H^+- concentration) of the caecum content, the concentration and amount of SCFA and secondary bile acids were analysed using two-way analysis of variance (ANOVA) with factors fat and fibre. Levene's test was used to test whether variances among the groups were homogeneous. If Levene's test indicated homogeneous variances, the groups were compared by a one way ANOVA for equal variances, followed if significant by pooled variance t-tests. If Levene's test indicated heterogeneous variances, the groups were compared by a one way ANOVA for unequal variances, followed if significant by separate variance t-tests. Tumour incidences were analysed using Pearson's χ^2 test. A probability value of $P < 0.05$ (two-tailed) was used as the critical level of significance.

Results

Food consumption/energy intake/terminal body weight

The experimental diets had different energy contents. Consequently, the food consumption slightly varied among the groups, since rats eat in accordance with their caloric needs. Although the food consumption, as anticipated, was inversely related to the energy content of the different diets, the calculated energy intake still showed marginal differences between the groups, at most 8.7% or 5.2% in the cellulose or RS groups, respectively (Table 4.2). As a result, the terminal body weights were also slightly different and in accordance with the calorie consumption (Table 4.2).

Table 4.2. Mean food consumption, energy intake and terminal body weight.

Diet groups	Food consumption (g/rat/day)	Energy intake (kJ/rat/day)	Body weight (g)	Energy intake (kJ/kg body weight/day)
LF/LC	14.4	240	402	598
LF/HC	16.6	229	394	581
HF/LC	13.3	246	439	561
HF/HC	16.3	254	417	610
LF/LRS	14.5	251	421	596
LF/HRS	14.4	236	389	607
HF/LRS	13.5	255	431	592
HF/HRS	13.0	238	412	577

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LRS, HRS: low-, high Novelose 330. The calculated energy intake showed marginal differences between the groups, at most 8.7% in the cellulose groups, at most 5.2% in the RS groups.

Aberrant crypt foci

The number and size of ACF are presented in Table 4.3. The number of ACF in HC groups was statistically significantly higher than in the LC groups ($P < 0.05$).

In the cellulose groups, a high fat diet resulted in more ACF than a low fat diet ($P < 0.05$). This held true for the total number of ACF but also when the different groups were compared with regard to ACF of increasing size. This was done because it has been suggested by several investigators that only large ACF may develop into colorectal tumours and that, therefore, the number of large ACF (for example 4 or more aberrant crypts per focus) may be more predictive than the total number of ACF for the development of colorectal tumours (Lafave *et al.*, 1994; Shirtliff and Bird, 1996). The number of crypts per focus in the HC groups was statistically significantly increased when compared with the LC groups ($P < 0.001$), but fat had no effect. In the Novelose 330 groups, fat or RS had no significant effect on the number or size of ACF.

Caecum weight and pH

The caecum weights and pH are summarised in Table 4.4. The absolute and relative weights of the caecum content were inversely related to the fat content of the diets ($P < 0.05$). HC- and HRS-fed animals showed a markedly enlarged caecum in comparison with LC- and LRS-fed animals, respectively ($P < 0.001$). The caecum content in the HRS groups was watery, most probably due to the water holding capacity of Novelose 330 (2.2 g H_2O/g). The fat content of the diets did not influence the pH of the caecum content. In the LF/HC group the caecum pH was significantly lower than in the LF/LC group ($P < 0.05$). The caecum pH in animals fed the HRS diets was markedly decreased when compared with the LRS groups ($P < 0.001$).

Incidence of colorectal tumours

The incidence of tumours in the different diet groups is presented in Table 4.5. The incidence of adenomas is the percentage of adenoma-bearing animals with or without carcinoma. The incidence of carcinomas is the percentage of carcinoma-bearing animals with or without adenoma. The incidence of total tumours is the percentage of animals bearing adenoma or carcinoma or both. The adenoma incidence was statistically significantly increased in the LF/HC group when compared with the LF/LC group ($P < 0.05$). The carcinoma incidence was statistically significantly increased in the HF/HC group when compared with the HF/LC group ($P < 0.01$) and the LF/HC group ($P < 0.05$). The incidence of total

Table 4.3. Number of ACF of various sizes, and of crypts per focus (mean ± SEM) eight weeks after the last injection of AOM in nine animals per group.

	Diet groups							
	LF/LC	LF/HC	HF/LC	HF/HC	LF/LRS	LF/HRS	HF/LRS	HF/HRS
Total no. of ACF	112 ± 15	168 ± 15[a]	167 ± 12[b]	195 ± 23[ab]	122 ± 11	97 ± 7	114 ± 14	107 ± 13
No. with ≥ 2 crypts	95 ± 14	146 ± 13[a]	139 ± 9[b]	174 ± 20[ab]	103 ± 9	84 ± 22	96 ±12	88 ± 10
No. with ≥ 3 crypts	57 ±10	95 ± 9[a]	75 ± 7[b]	122 ± 13[ab]	55 ± 6	49 ± 5	55 ± 7	50 ± 5
No. with ≥ 4 crypts	26 ± 5	54 ± 6[a]	33 ± 4[b]	76 ± 8[ab]	25 ± 4	21 ± 3	25 ± 5	23 ± 2
No. with ≥ 5 crypts	8 ± 2	27 ± 3[a]	11 ± 2[b]	42 ± 4[ab]	10 ± 2	9 ± 2	9 ± 2	8 ± 1
No. of crypts/focus	2.64 ± 0.08	3.05 ± 0.06[c]	2.59 ± 0.07	3.33 ± 0.04[c]	2.63 ± 0.11	2.71 ± 0.09	2.67 ± 0.07	2.69 ± 0.08

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LRS, HRS: low-, high Novelose 330. ACF: aberrant crypt focus. Cellulose groups: HC gives more ACF than LC ($^{a}P < 0.05$), HF gives more ACF than LF ($^{b}P < 0.05$). Number of crypts per focus: HC gives larger ACF than LC ($^{c}P < 0.001$), no effect of fat. Novelose 330 groups: no effects of fat or RS on size or number of ACF. Two-way analysis of variance (ANOVA) with factors fat and carbohydrate.

Table 4.4. Absolute and relative weights (g ± SEM) and pH of the caecum content

	Diet groups							
	LF/LC	LF/HC	HF/LC	HF/HC	LF/LRS	LF/HRS	HF/LRS	HF/HRS
Abs. caecum content weight (g)	2.3 ± 0.1	3.9 ± 0.2[b]	2.2 ± 0.1[a]	3.2 ± 0.1[ab]	2.3 ± 0.1	7.6 ± 0.7[b]	2.2 ± 0.1[a]	5.9 ± 0.3[ab]
Rel. caecum content weight (g/kg bw)	5.7 ± 0.3	9.8 ± 0.4[b]	5.0 ± 0.2[a]	7.7 ± 0.3[ab]	5.5 ± 0.2	19.7 ± 1.7[b]	5.2 ± 0.3[a]	14.5 ± 0.9[ab]
PH	6.2	6.0[c]	6.2	6.1	6.2	5.6[d]	6.2	5.7[d]

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LRS, HRS: low-, high Novelose 330. The absolute and relative weight of the caecum content were inversely related to the fat content of the diets ([a]$P < 0.05$). HC- and HRS-fed animals showed a markedly enlarged caecum in comparison with LC- and LRS-fed animals, respectively ([b]$P < 0.001$). The fat content of the diets did not influence the pH of the caecum content. In the LF/HC group the caecum pH was lower than in the LF/LC group ([c]$P < 0.05$). The caecum pH in animals fed the HRS diets was markedly decreased ([d]$P < 0.001$). Two-way analysis of variance (ANOVA) with factors fat and carbohydrate.

Table 4.5. Incidence (% tumour-bearing animals) of colorectal tumours after 10 months.

	Diet groups							
	LF/LC	LF/HC	HF/LC	HF/HC	LF/LRS	LF/HRS	HF/LRS	HF/HRS
Adenomas	33	61[a]	42	58	42	23	45	33
Carcinomas	42	55	45	79[b]	48	35	42	52
Total tumours	70	79	58	91[e]	73	48[c]	70	76[d]

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LRS, HRS: low-, high Novelose 330. The incidence of adenomas is the percentage of adenoma-bearing animals with or without carcinoma. The incidence of carcinomas is the percentage of carcinoma-bearing animals with or without adenoma. The incidence of total tumours is the percentage of animals bearing adenoma or carcinoma or both. The incidence of adenomas was increased in the LF/HC group when compared with the LF/LC group ($^{a}P < 0.05$). The incidence of carcinomas was increased in the HF/HC group when compared with the HF/LC group ($^{b}P < 0.01$) and the LF/HC group ($^{b}P < 0.05$). The incidence of total tumours was decreased in the LF/HRS group when compared with the LF/LRS group ($^{c}P < 0.05$), increased in the HF/HRS group when compared with the LF/HRS group ($^{d}P < 0.05$) and increased in the HF/HC group when compared with the HF/LC group ($^{e}P < 0.05$). Pearson's χ^2 test.

tumours was statistically significantly decreased in the LF/HRS group when compared with the LF/LRS group ($P < 0.05$), increased in the HF/HRS group when compared with the LF/HRS group ($P < 0.05$), and increased in the HF/HC group when compared with the HF/LC group ($P < 0.05$).

Multiplicity of colorectal tumours

The multiplicity of the colorectal tumours, expressed as the mean number of tumours per tumour-bearing animal, is presented in Table 4.6. The multiplicity of adenomas and total tumours was statistically significantly increased in the HC groups when compared with the LC groups ($P < 0.05$). In the animals fed the HF/HRS diet the multiplicity of total tumours was statistically significantly lower than in the HF/LRS-fed animals ($P < 0.05$). An inhibiting effect of HRS was not observed in the low fat groups. There were no significant effects of fat on the multiplicity of tumours.

Size and position of colorectal tumours

The mean size of adenomas, carcinomas and total tumours are presented in Table 4.7. In the cellulose groups, the HF diets resulted in a statistically significantly decreased size of adenomas, carcinomas and total tumours when compared with the LF diets ($P < 0.05$). In the Novelose 330 groups no effect of fat or carbohydrate on tumour size was observed. In all groups, the mean distance of carcinomas from the anus was greater than that of adenomas. The grand mean distance from the anus was 52 mm for adenomas and 75 mm for carcinomas.

SCFA

Results of the SCFA analyses are presented in Tables 4.8a,b. Valeriate, isobutyrate and isovaleriate are summarised as 'other SCFA'. Carbohydrates that escape enzymatic digestion in the small intestines, such as resistant starch, can be degraded by microbial fermentation in the caecum. The main fermentation products are the SCFA acetate, propionate and butyrate. The concentration of SCFA in the large intestines will gradually increase when the content of the caecum passes the colon, because of the resorption of water in the colon. At the same time part of the SCFA will be used as energy source by the colonocytes. The water content of the caecum differed considerably among the various diet groups. In general, the caecum content of the cellulose-fed animals was relatively dry,

Table 4.6. Multiplicity of colorectal tumours, expressed as the mean number (± SEM) of tumours per tumour-bearing animal.

	Diet groups							
	LF/LC	LF/HC	HF/LC	HF/HC	LF/LRS	LF/HRS	HF/LRS	HF/HRS
Adenomas	0.6 ± 0.1	1.5 ± 0.3[a]	0.9 ± 0.2	1.4 ± 0.3[a]	0.9 ± 0.2	0.7 ± 0.2	1.0 ± 0.2	0.5 ± 0.1
Carcinomas	1.0 ± 0.2	1.0 ± 0.2	1.2 ± 0.2	1.4 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.9 ± 0.2	0.7 ± 0.1
Total tumours	1.6 ± 0.1	2.6 ± 0.2[a]	2.1 ± 0.3	2.8 ± 0.4[a]	1.8 ± 0.2	1.7 ± 0.2	1.9 ± 0.3	1.2 ± 0.1[b]

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LRS, HRS: low-, high Novelose 330. The multiplicity of adenomas and total tumours was increased in the HC groups when compared with the LC groups ($^aP < 0.05$). Two-way analysis of variance (ANOVA) with factors fat and carbohydrate. In the animals fed the HF/HRS diet the multiplicity of total tumours was lower than in the HF/LRS fed animals ($^bP < 0.05$, Student's *t*-test).

Table 4.7. Size (mm ± SEM) of colorectal tumours.

	Diet groups							
	LF/LC	LF/HC	HF/LC	HF/HC	LF/LRS	LF/HRS	HF/LRS	HF/HRS
Adenomas	2.2 ± 0.3	2.4 ± 0.3	1.6 ± 0.2[a]	1.9 ± 0.2[a]	1.9 ± 0.2	1.6 ± 0.4	1.9 ± 0.2	2.4 ± 0.4
Carcinomas	5.7 ± 0.9	5.4 ± 0.4	4.8 ± 0.8[a]	3.9 ± 0.3[a]	4.6 ± 0.6	5.5 ± 1.3	12.7 ± 7.2	3.6 ± 0.7
Total tumours	4.3 ± 0.7	3.7 ± 0.4	3.2 ± 0.5[a]	3.0 ± 0.2[a]	3.3 ± 0.4	4.3 ± 1.1	7.5 ± 4.2	2.9 ± 0.3

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LRS, HRS: low-, high Novelose 330. In the cellulose goups, the HF diets resulted in decreased size of adenomas, carcinomas and total tumours when compared with the LF diets ($^aP < 0.05$). In the Novelose 330 groups no effect of fat or carbohydrate on tumour size was observed. Two-way analysis of variance (ANOVA) with factors fat and carbohydrate.

Table 4.8a. Concentration of SCFA (mg/100 g ± SEM) in the caecum content of twelve randomly selected animals of all groups.

	Diet groups							
	LF/LC	LF/HC	HF/LC	HF/HC	LF/LRS	LF/HRS	HF/LRS	HF/HRS
Acetate	596 ± 114	396 ± 55	389 ± 17	382 ± 25	437 ± 55	695 ± 163	433 ± 20	453 ± 40
Propionate	73 ± 10	67 ± 6	54 ± 3[a]	59 ± 3[a]	54 ± 6	50 ± 5	51 ± 2	47 ± 3
Butyrate	171 ± 19	168 ± 21	139 ± 5	184 ± 10[b]	159 ± 15	251 ± 41[b]	145 ± 5	260 ± 25[b]
Other SCFA	78 ± 10	60 ± 8[c]	69 ± 4	59 ± 4[c]	69 ± 9	63 ± 9	64 ± 5	45 ± 4
Total SCFA	919 ± 148	691 ± 76	650 ± 23	684 ± 31	718 ± 80	1059 ± 197	693 ± 25	806 ± 50

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LRS, HRS: low-, high Novelose 330. SCFA: short chain fatty acids. Propionate in HF/LC and HF/HC groups was lower than in LF/LC and LF/HC groups ($^{a}P < 0.05$). Butyrate in the HF/HC, LF/HRS and HF/HRS groups was higher than in the HF/LC, LF/LRS and HF/LRS groups, respectively ($^{b}P < 0.05$). Other SCFA were lower in HC than in LC groups ($^{c}P < 0.05$). Two-way analysis of variance (ANOVA) with factors fat and carbohydrate.

Table 4.8b. Absolute amounts of SCFA (mg ± SEM) in the caecum content of twelve randomly selected animals of all groups.

	Diet groups							
	LF/LC	LF/HC	HF/LC	HF/HC	LF/LRS	LF/HRS	HF/LRS	HF/HRS
Acetate	14.7 ± 3.9	17.1 ± 3.2	8.5 ± 0.7^{a}	11.4 ± 1.0^{ab}	9.9 ± 1.6	44.3 ± 9.6^{b}	9.3 ± 0.8	26.4 ± 2.7^{b}
Propionate	1.7 ± 0.3	2.8 ± 0.3^{b}	1.1 ± 0.1^{a}	1.7 ± 0.1^{ab}	1.2 ± 0.2	3.4 ± 0.3^{b}	1.1 ± 0.1	2.9 ± 0.3^{b}
Butyrate	4.1 ± 0.8	7.6 ± 1.5^{b}	3.0 ± 0.2	5.5 ± 0.4^{b}	3.6 ± 0.5	17.0 ± 2.8^{b}	3.1 ± 0.2	1 5.4 ± 1.6^{ab}
Other SCFA	1.8 ± 0.3	2.6 ± 0.4^{b}	1.5 ± 0.1^{a}	1.7 ± 0.1^{ab}	1.6 ± 0.2	4.6 ± 0.8^{b}	1.4 ± 0.1	2.7 ± 0.3^{b}
Total SCFA	22.3 ± 5.2	30.1 ± 5.0	14.2 ± 1.1^{a}	20.4 ± 1.3^{ab}	16.3 ± 2.4	69.3 ± 11.5^{b}	14.8 ± 1.1	47.4 ± 3.9^{b}

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LRS, HRS: low-, high Novelose 330. The absolute amounts of all SCFA were lower in HF diets than in LF diets ($^{a}P < 0.05$). SCFA were increased in animals fed either HC or HRS when compared to LC or LRS ($^{b}P < 0.05$). Two-way analysis of variance (ANOVA) with factors fat and carbohydrate.

whereas the Novelose-fed animals had quite watery caecum content. In order to make a more accurate comparison between the groups with respect to the amount of SCFA formed, the SCFA are expressed as concentration (mg/100g caecum content), and also as absolute amount (mg) by taking into account the wet weight of the caecum content.

In the cellulose groups, an HF diet resulted in lower propionate concentration than an LF diet ($P < 0.05$). The butyrate concentration in the HF/HC group was significantly higher than in the HF/LC group ($P < 0.05$). The concentrations of other SCFA were lower in HC- than in LC-fed animals ($P < 0.05$). The absolute amounts of all SCFA were lower in HF diets than in LF diets ($P < 0.05$, in most cases). Propionate, butyrate and others were higher in HC- than in LC-fed rats ($P < 0.05$).

In the Novelose 330 groups, HRS-fed rats had a higher concentration of butyrate as well as higher absolute amounts of all SCFA than LRS-fed rats ($P < 0.05$).

Faecal bile acids

The results of the analyses of faecal bile acids are presented in Table 4.9. In the cellulose groups no cholic acid was detected in LF-fed animals. Lithocholic acid, deoxycholic acid, others (the sum of α-muricholic, β-muricholic, ω-muricholic, hyodeoxycholic and ursodeoxycholic acid) and total bile acids were significantly lower in HC-fed animals than in LC-fed animals ($P < 0.05$), whereas the concentration of these bile acids was not influenced by the fat content of the diet. Novelose 330 groups: cholic acid was significantly higher in HRS-fed animals than in LRS-fed animals. The amount of RS or fat in the diet had no statistically significant effect on the concentration of lithocholic acid, deoxycholic acid, others or total bile acids, although in general the concentration of most of these bile acids showed a tendency to decrease in the HRS groups.

Table 4.9. Faecal bile acids of eight randomly selected animals per group (μmol/g ± SEM) at study termination.

	Diet groups							
	LF/LC	LF/HC	HF/LC	HF/HC	LF/LRS	LF/HRS	HF/LRS	HF/HRS
Cholic acid	0	0	0.12 ± 0.06	0.10 ± 0.07	0.06 ± 0.06	0.25 ± 0.12[b]	0.02 ± 0.02	0.24 ± 0.10[b]
Lithocholic acid	0.48 ± 0.05	0.15 ± 0.03[a]	0.48 ± 0.03	0.21 ± 0.01[a]	0.52 ± 0.06	0.51 ± 0.08	0.67 ± 0.05	0.45 ± 0.05
Deoxycholic acid	2.68 ± 0.49	1.00 ± 0.30[a]	2.72 ± 0.41	1.26 ± 0.06[a]	4.17 ± 0.51	3.28 ± 0.36	4.50 ± 0.75	4.50 ± 0.59
Other bile acids	5.68 ± 0.46	1.43 ± 0.34[a]	4.79 ± 0.48	1.51 ± 0.17[a]	4.87 ± 0.46	5.09 ± 0.75	5.47 ± 0.82	3.68 ± 0.40
Total bile acids	8.84 ± 0.97	2.58 ± 0.67[a]	8.13 ± 0.93	3.20 ± 0.19[a]	9.62 ± 0.71	9.00 ± 0.79	10.54 ± 1.55	8.84 ± 0.86

LF, HF: low-, high fat. LC, HC: low-, high cellulose. LRS, HRS: low-, high Novelose 330. Other bile acids: α-muricholic, β-muricholic, ω-muricholic, hyodeoxycholic and ursodeoxycholic acid. Bile acids were lower in HC-fed animals than in LC-fed animals ($^aP < 0.05$). Cholic acid was higher in HRS-fed animals than in LRS-fed animals ($^bP < 0.05$). Most secondary bile acids tended to be lower in HRS groups. Two-way analysis of variance (ANOVA) with factors fat and carbohydrate.

Discussion

The main goals of the present study were to investigate whether various types of diet would influence the development of ACF and colorectal tumours, and whether the results of the ACF scores at an early stage of the experiment had predictive value for the ultimate tumour yield.

Cellulose groups

The HC groups developed significantly more and larger ACF than the LC groups. In general, the incidence of tumours at the end of the study was higher in HC groups than in LC groups. The differences were statistically significant for adenomas in the LF diet groups, and for carcinomas and total tumours in the HF diet groups. The multiplicity of adenomas and total tumours in LF/HC- and HF/HC-fed rats was significantly increased when compared with LC-fed rats. The multiplicity of carcinomas was about comparable. The cellulose content of the diets did not influence the size of the tumours. Based on the above findings it was concluded that eight weeks after the last AOM injection the ACF score in the cellulose groups was predictive for the incidence and multiplicity, but not for the size of the tumours at the end of the study. The enhancing effect of HC diets on colorectal carcinogenesis was a surprising finding, because of the general contention that diets rich in fibre have protecting properties. Specifically, cellulose has been found to protect against colorectal cancer in animal studies as well as in human epidemiological studies (Madar *et al.*, 1996; Negri *et al.*, 1998; Sakamoto *et al.*, 1996), although a lack of protection also has been described (Jacobs and Lupton, 1986; Wilpart and Roberfroid, 1987).

Novelose 330 (RS) groups

The RS diet groups had slightly less ACF than the cellulose diet groups. No significant differences in the number or size of the ACF could be detected between the groups fed either LRS or HRS, combined with either LF or HF. However, the incidence of total tumours (adenomas + carcinomas) was significantly decreased in the LF/HRS group, when compared with both the HF/HRS group and the LF/LRS group. The multiplicity of total tumours in HF/HRS-fed rats was significantly lower than in HF/LRS-fed rats. These observations justify the conclusion that the

ACF scores in the Novelose 330 groups did not offer a reliable prediction for the tumour outcome at the end of the study.

Effects of dietary fat

In the cellulose groups, an HF diet resulted in more ACF than an LF diet. The differences were statistically significant for each of the size groups of the ACF. The incidence of carcinomas in the HF/HC group was higher than in the LF/HC group. The multiplicity of tumours tended to be slightly higher in HF-fed animals than in LF-fed animals but the differences did not reach the level of statistical significance. The size of benign as well as malignant tumours was inversely related to the fat content of the diets. In the Novelose 330 groups the amount of dietary fat did not influence the development of ACF. However, at the end of the study an HF-diet resulted in an increased incidence of total tumours. The multiplicity and size of the tumours did not show differences related to the dietary fat content. Based on the above data it was concluded that the enhancing effect of dietary fat on the development of ACF in the cellulose groups was predictive for the incidence of carcinomas but not of adenomas. In the Novelose 330 groups, however, there was no correlation between the effects of fat on the ACF score and the tumour outcome. Results from epidemiological as well as animal model studies have provided convincing evidence that high fat diets have promotional effects on colorectal carcinogenesis (Reddy, 1992; Reddy, 1995; Zhao *et al.*, 1991). Absence of an enhancing effect of the HF diet in the Novelose 330 groups as observed in the present study may be explained by the counteracting inhibitory effects of RS.

Animal experiments addressing the predictive value of ACF as early biomarker of colorectal cancer are numerous. Their results are conflicting. Some investigators found a correlation between the number and/or size of ACF and tumour development (Pretlow *et al.*, 1992; Alabaster *et al.*, 1993; Kawamori *et al.*, 1995; Young *et al.*, 1996), others did not (Takahashi *et al.*, 1991; Thorup *et al.*, 1994; Cameron *et al.*, 1996; Zheng *et al.*, 1999). Pereira *et al.*, (1994), Young *et al.*, (1996) and Matsukawa *et al.*, (1997) studied, respectively, chemicals, carbohydrates, quercetin and restraint stress for their effects on the formation of ACF and extended their conclusions to presumable effects on carcinogenesis without performing a long-term carcinogenesis study. The present study confirms the idea of other investigators (Hardman *et al.*, 1991; Kristiansen *et al.*, 1995;

Moen *et al.*, 1996), that ACF can not be used as biomarker for colorectal cancer. Obviously, it would be very useful to have a sensitive model which would make it possible and manageable to screen compounds for their ability to influence colorectal carcinogenesis. The number and multiplicity of ACF have proven to be unreliable predictors. In other words, conclusions about products being promising inhibitors of colorectal carcinogenesis based on ACF assessment in short-term studies only are questionable and should be approached with caution. In order to learn more about ACF, their heterogeneous morphology has been studied to find out if a class of 'true' preneoplastic ACF could be identified (Bouzourene *et al.*, 1999; Fenoglio-Preiser and Noffsinger, 1999; Di Gregorio *et al.*, 1997). It has been proposed that only dysplastic ACF progress to tumours (Jen *et al.*, 1994). Extensive genotyping and phenotyping of unchanged colon epithelium, ACF and tumours using modern techniques such as genomics, transcriptomics and proteomics possibly will provide additional tools to get better understanding of the processes taking place in tissues evolving from normal to malignant.

The incidence and multiplicity of total tumours were statistically significantly decreased in the HRS-fed rats when compared with LRS-fed rats. The HRS-fed rats had a higher concentration of butyrate as well as higher absolute amounts of all SCFA than LRS-fed rats. This is consistent with the conception of SCFA being protective against colorectal carcinogenesis. Only few animal studies with RS have been published. Young *et al* (1996) found enhancement of carcinogenesis in RS-fed dimethylhydrazine-treated rats. However, epidemiological data suggest a strong inverse association between RS consumption and colorectal cancer (Cassidy *et al.*, 1994).

The increase of SCFA in the caecum content of animals fed the HC diets was a surprising finding, since cellulose is known as a non-fermentable fibre. Although a small fraction of cellulose may be susceptible to microbial enzymes it was not expected that this would result in a significant increase of SCFA in the caecum. This finding can possibly be explained by the fact that other, fermentable, substances, entrapped in the cellulose, might have reached the caecum and were then fermented. Despite the increased amounts of butyrate and propionate the HC-fed animals exhibited a higher tumour yield than the LC-fed animals, which was unexpected because of the well known inhibiting effect of especially butyrate against colorectal tumour development (McIntyre *et al.*, 1993; Hague *et al.*, 1997; Medina *et al.*, 1998; Van Munster and Nagengast, 1993). On the other hand,

cellulose has a marked effect on bulking of the colonic content and it shortens its transit time (Harris and Ferguson, 1993; Munakata *et al.*, 1995). Therefore, it may be possible that butyrate and propionate have not had the chance to exert their protective effect because they were diluted and possibly adsorbed to the cellulose in the colon; moreover, they were present for a short period of time only.

At lower pH of the contents of the large intestines the conversion from primary into secondary bile acids, which are cytotoxic and are thought to promote carcinogenesis, is inhibited (Nagengast *et al.*, 1995; Narisawa *et al.*, 1974; Reddy *et al.*, 1977; Reddy and Watanabe, 1979). This may explain the increased amount of primary bile acids (cholic acid) and the decreased amount, although the latter only a tendency, of secondary bile acids (deoxycholic and lithocholic acid) in the HRS groups, which had a significantly lower caecal pH than the LRS groups. The significantly decreased concentration of secondary bile acids in the HC groups cannot be explained by this mechanism, because the decrease of the caecal pH in the HC groups was only slight when compared to the HRS groups. The main reason for the decreased concentration of bile acids is most probably dilution by the bulking effect of the HC diet. In a previous study we demonstrated a three- to fourfold increase of faeces production in animals fed diets containing 25% cellulose as opposed to a diet with 5% cellulose (Wijnands *et al.*, 1999).

From the results of the present experiment it was concluded that colorectal carcinogenesis was enhanced by dietary cellulose, but inhibited by dietary RS. A high fat diet had either no effect or an enhancing effect on carcinogenesis. The ACF score was predictive for the tumour outcome in the cellulose groups but not in the Novelose 330 groups. Therefore, ACF are considered unsuitable as biomarker for colorectal cancer.

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Chapter 5

Effects of dietary fish oil on azoxymethane-induced DNA adduct formation and colorectal carcinogenesis in F344 rats

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Abstract

Azoxymethane (AOM)-treated male F344 rats were fed a diet containing low fat (LF, 5% safflower/sunflower oil), high fat (HF, 25% sunflower oil) or high fish oil (HFO, 5% safflower/sunflower oil + 20% fish oil). The HFO diet was fed during the initiation phase of carcinogenesis; during post-initiation these rats were fed the HF diet (HFO/HF group). One day after the last AOM treatment DNA adducts were measured in liver and colon. Aberrant crypt foci (ACF) were counted in the colon 8 weeks after the diet switch. Colorectal tumours were scored 9 months after the study start. The HFO-fed animals demonstrated inhibited DNA adduct formation in the liver and enhanced development of ACF. There was no correlation between development of ACF and the occurrence of colorectal tumours. There were no statistically significant differences between the various groups with respect to incidence, multiplicity or size of colorectal tumours. The lack of an enhanced development of colorectal tumours in the HF group, as compared with the LF group, was an unexpected result. Since the HF group was included to serve as a positive control group, it was not possible to draw conclusions with respect to a potential effect on colorectal carcinogenesis of dietary fish oil fed during the initiation phase.

Introduction

It has been estimated that approximately one third of all cancers in the Western world is diet related. Results from epidemiological and animal model studies provided evidence that a diet high in fat has an enhancing effect on cancer of the colon and other organ systems (Potter and McMichael, 1986; Reddy, 1992; Reddy, 1995; Zhao *et al.*, 1991). However, the effect of dietary fat depends on the type of fat. Sakaguchi *et al.* (1984) demonstrated a significantly higher incidence of colorectal tumours in rats fed a 5% linoleic acid diet than in those fed a 4.7% stearic acid plus 0.3% linoleic acid diet. Polyunsaturated fatty acids (PUFA) from the ω-3 family, which are abundant in fish oil, have been shown to inhibit the carcinogenic process in several organs; the formation of colorectal tumours was inhibited in carcinogen-treated rats (Reddy and Maruyama, 1986; Minoura *et al.*, 1988; Nelson *et al.*, 1988; Reddy and Sugie, 1988). Populations such as Eskimos, consuming large amounts of fat derived from marine oil containing ω-3 PUFA, specifically, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have low incidences of cancer (Blot *et al.*, 1975). A case-control study in Italy conducted between 1992 and 1996 revealed an inverse association with the consumption of fish and colorectal cancer (Franceschi *et al.*, 1998). Protective associations for fish were also observed by Neugut *et al.* (1993) in a case-control study in New York City and by Willett *et al.* (1990) in a prospective study in women.

There are only few reports of animal model studies that address the question whether the effect of dietary fish oil on colorectal carcinogenesis takes place primarily during the initiation or the post-initiation phase. The present study was designed to investigate whether the effect, if any, of dietary fish oil on colorectal carcinogenesis takes place during the initiation phase. Furthermore, the possible effect of dietary fish oil on the formation of AOM-induced DNA adducts was studied.

Materials and methods

Animals and diets

One hundred and fifty male specific-pathogen free Fischer 344 rats (Charles River Deutschland, Sulzfeld, Germany), four weeks old, were divided into 3 groups of 50 animals each. The different groups were fed a diet containing low fat (LF, 5% (w/w) safflower/sunflower oil), high fat (HF, 25% (w/w) sunflower oil) or high fish oil (HFO, 5% (w/w) safflower/sunflower oil + 20% (w/w) fish oil). The fat sources used for the diets were high-linoleic (ca 76% C18:2) safflower oil (Unilever, Vlaardingen, The Netherlands), high oleic (ca 80% C18:1) sunflower oil (Trisun™; Contined, Bennekom, The Netherlands) and fish oil (MaxEPA; Seven Seas, Hull, United Kingdom). Vitamin E consisting of 50% DL-tocopherolacetate was added to all diets as extra antioxidant. The diets were prepared monthly and stored at -20°C until use. All diets were AIN[76]-based. The composition of the experimental diets is summarised in Table 5.1.

Table 5.1. Percentage compositions of the AIN[76]-based diets.

Dietary components (wt%)	LF	HF	HFO
Casein	20.00	25.00	25.00
DL-methionine	0.30	0.70	0.70
Wheat starch	63.50	35.79	35.79
Cellulose	5.00	6.18	6.18
Choline bitartrate	0.20	0.25	0.25
AIN[76]-minerals	3.50	4.32	4.32
AIN[76]-vitamins	1.00	1.24	1.24
$CaHPO_4$	1.50	1.85	1.85
Safflower oil	3.05	-	1.76
Trisun oil	1.94	24.98	3.24
MaxEPA	-	-	20.00
Vitamin E[1]	0.10	0.10	0.01
Energy (kJ/g)	16	20	20

LF: low fat; HF: high fat; HFO: high fish oil.
[1] Vit E: 50% DL-tocopherolacetate.

The HFO diet was fed only during the first 5 weeks (initiation phase). During this period the animals received fresh feed daily to minimize oxidation of the fish oil. This procedure warrants peroxide values to stay within acceptable values as was shown in previous work performed at our Institute (Appel and Woutersen, 1994). Thereafter, the HFO rats were switched to the HF diet (HFO/HF).

Treatment and housing

All animals received 2 subcutaneous injections, 1 week apart, with AOM (Sigma-Aldrich, Zwijndrecht, The Netherlands), 15 mg/kg body weight, the first in week 4 and the second in week 5. The animals were housed in macrolon cages with bedding, three animals per cage. Feed and tap water were available *ad libitum*. The relative humidity was kept between 30 and 70%. The number of air changes was about 10 per hour. Lighting was artificial by fluorescent tubes and time switch controlled at a sequence of 12 hours light, 12 hours dark.

In-life measurements

Food consumption was recorded daily in week 2, weekly from week 6 to week 13 and monthly thereafter. Body weight of all animals was recorded weekly during the first 4 months of the study and monthly thereafter. The animals were checked for clinical signs regularly.

Necropsy, histology and histopathology

One day after the last AOM treatment 9 rats/group were killed. Liver and colon were collected for measuring DNA adducts in these organs. At necropsy 8 weeks after the last AOM injection the colon of all animals was removed, cut open longitudinally, rinsed with phosphate-buffered saline and fixed flat between filter paper in a neutral aqueous phosphate-buffered 4% solution of formaldehyde. After fixation the tissue samples were stained with 0.1% methylene blue for 9 minutes, according to the method described by Bird (1987). Then, the colons were examined microscopically at low magnification for the presence of ACF. The total number of ACF and the number of aberrant crypts (AC) per ACF were recorded (AC/ACF). In addition, the number of ACF with 4 or more AC was recorded, because it has been proposed that larger ACF may have better predictive value for colorectal cancer than the total number of ACF (Shirtliff and Bird, 1996).

Tumours were histologically processed, sectioned at 5 μm, and stained with haematoxylin and eosin. Serial sections were made whenever necessary to expose the stalk, if present. They were examined microscopically to establish whether they were benign or malignant. Classification of the tumours was done according to the criteria described by Whiteley *et al.* (1996).

Measurement of DNA adducts

The analysis of methyl-DNA adducts in DNA from liver and colon was performed as previously described (Van Delft *et al.*, 1994). The procedure for DNA isolation involved first isolating the nuclei, followed by treatment with proteinase K, sodium dodecyl sulphate and RNAse A, salt precipitation of proteins and precipitation with iso-propanol. For the analysis of the N^7-methylguanine levels the DNA was sonicated and treated with alkali in order to form the imidazole ring-open derivative. These derivatives were detected by use of the monoclonal antibody N^7E-026 in an immunoslot-blot assay. The detection limit of this assay is one N^7-methylguanine adduct per 10^6 nucleotides.

Statistical analysis

The multiplicity and size of ACF and colorectal tumours were analysed using analysis of variance (ANOVA) followed by Student's *t*-test. Tumour incidences were analysed using Pearson's χ^2 test. A probability value of $P < 0.05$ (two-tailed) was used as the critical level of significance.

Results

Survival, food consumption, energy intake and body weight gain

Survival of the animals was 100% in all groups. The mean food consumption during the experiment was 14.4, 11.8 and 12.3 g/animal/day for the LF, HF and HFO/HF group, respectively. The LF diet had the lowest energy content. The animals fed that diet had the highest food consumption, which was anticipated since rats tend to eat according to their caloric need. This resulted in a comparable energy intake for all groups throughout the study.

The animals fed the HFO/HF diet grew slightly faster than the other groups during the first weeks of the study. After the diet switch they kept their higher body

weights and their body weight gain became similar to that of the other groups (Fig.5.1).

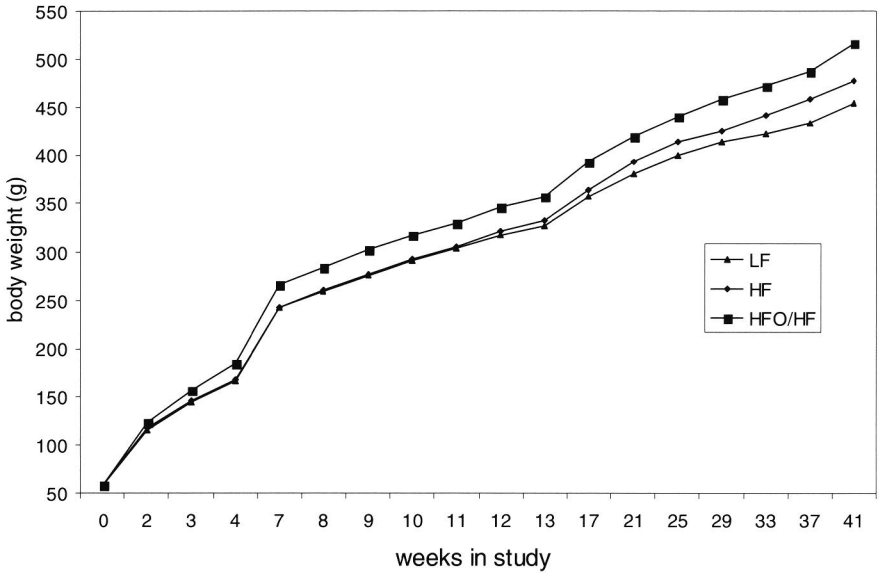

Figure 5.1. Body weights.

DNA adducts

The number of N^7-adducts in the liver ($P < 0.05$) and colon (not statistically significant) of the HFO/HF rats was lower than in the LF and HF groups and comparable in the LF and HF groups (Table 5.2).

Table 5.2. DNA adducts in liver and colon (mean ± SEM).

	LF	HF	HFO
$N^7/10^6$ nucl. in liver	1049 ± 60	1055 ± 47	845 ± 31[a]
$N^7/10^6$ nucl. in colon	11.9 ± 4.3	10.4 ± 2.8	4.9 ± 0.8

LF: low fat; HF: high fat; HFO: high fish oil. [a]$P < 0.05$: HFO vs LF, HF (Student's *t*-test).

ACF

As was expected, all animals developed ACF. The total number of ACF and the number of large ACF (4 or more AC/ACF) were highest in the HFO/HF group ($P < 0.05$). This group had also the largest ACF, expressed as mean number of aberrant crypts per ACF ($P < 0.05$). The total numbers of ACF, large ACF and crypts/ACF in the LF group were comparable with those in the HF group (Table 5.3).

Colorectal tumours

The incidence, multiplicity and size of colorectal tumours are presented in Table 5.3.

Table 5.3. Multiplicity of all ACF, ACF with 4 or more aberrant crypts and tumours per animal (mean ± SEM), number of aberrant crypts per ACF (mean ± SD), tumour incidence (%) and tumour size (mm ± SD).

	LF	HF	HFO/HF
	ACF after 8 weeks		
No. of animals	9	9	9
ACF multiplicity	135 ± 8	129 ± 17	182 ± 12[a]
ACF ≥ 4 AC	23 ± 2	20 ± 3	39 ± 5[a]
No. of AC / ACF	2.46 ± 0.07	2.42 ± 0.15	2.59 ± 0.17[b]
	Colorectal tumours after 8 months		
No. of animals	32	32	32
Adenoma incidence	38	38	28
Carcinoma incidence	53	41	34
Total tumour incidence	66	53	50
Adenoma multiplicity	0.62 ± 0.13	0.88 ± 0.19	0.75 ± 0.21
Carcinoma multiplicity	1.38 ± 0.21	1.24 ± 0.25	0.94 ± 0.19
Total tumour multiplicity	2.00 ± 0.20	2.12 ± 0.24	1.69 ± 0.18
Adenoma size	1.7 ± 0.6	2.1 ± 0.9	1.9 ± 0.5
Carcinoma size	5.0 ± 2.3	4.5 ± 2.1	5.1 ± 2.6
Total tumour size	4.2 ± 2.5	3.5 ± 2.2	3.9 ± 2.2

LF: low fat; HF: high fat; HFO/HF: first high fish oil, high fat thereafter.
[a]$P < 0.05$: HFO/HF vs LF, HF. [b]$P < 0.05$: HFO/HF vs HF (Student's *t*-test).

The incidence of adenomas is the percentage of adenoma-bearing animals with or without carcinoma. The incidence of carcinomas is the percentage of carcinoma-bearing animals with or without adenoma. The incidence of total tumours is the percentage of animals bearing adenoma or carcinoma or both. The multiplicity is the number of colorectal tumours per tumour-bearing animal.

The various experimental groups did not show statistically significant differences in incidence, multiplicity or size of colorectal tumours.

Discussion

In the present study, it was investigated whether the effect, if any, of dietary fish oil on colorectal carcinogenesis takes place during the initiation phase. Furthermore, the effect of the different diets on the formation of AOM-induced DNA adducts was studied.

In a previous study (Wijnands *et al.*, 1999) we observed that a high-sunflower diet had a promoting effect on chemically induced colorectal cancer in rats. Therefore, it was anticipated that in the present study a high-sunflower diet would have a similar effect and could be used as positive control. However, the HF diet failed to show a promoting effect on the development of colorectal tumours, which was an unexpected finding. The HFO/HF group demonstrated a non-significant trend towards a decreased incidence and multiplicity of colorectal tumours. But, because of the lack of a proper positive control group in the present study, we feel that any conclusion with respect to potential effects of fish oil fed during the initiation phase on colorectal carcinogenesis is unjustifiable.

The HFO diet, fed during initiation, inhibited the formation of DNA adducts in the liver ($P < 0.05$). In the colon of the HFO-fed animals, DNA adduct formation was markedly lower than in the colon of the other animals, but the difference did not reach the level of statistical significance. This may be due to the fact that the data showed large variation in the various groups. Lower AOM-induced DNA adduct formation has been observed by Hong *et al.* (2000), who fed AOM-treated SD rats with either 15% corn oil or 15% fish oil. They measured DNA adducts 3, 6, 9 or 12 h after AOM injection. Compared with the animals fed corn oil the fish oil-fed animals had a reduced formation of O^6-methylguanine adducts in colonic crypts. The inhibition of DNA adduct formation at an early stage in carcinogenesis

may contribute to a protective effect of fish oil, but in the present study this was not reflected by a statistically significant inhibition of tumour development at the end of the study.

The enhanced development of ACF ($P < 0.05$) in the HFO/HF group in comparison with the other groups did not correlate with the ultimate tumour yield, which confirms our view that the proposed correlation between these phenomena (Pretlow *et al.*, 1992; Alabaster *et al.*, 1995; Kawamori *et al.*, 1995; Young *et al.*, 1996) is questionable.

Reddy *et al.* (1991) found in AOM-treated F344 rats, that dietary menhaden oil rich in ω-3 fatty acids fed during initiation prevented the enhancing effect of a high-corn oil diet fed during postinitiation. The best protection, however, occurred in the animals fed menhaden oil during the initiation as well as the postinitiation phase. This finding showed that the protective effect of fish oil was exerted during the early as well as late events of carcinogenesis. It is difficult to determine which phase of the carcinogenic process is more important in terms of susceptibility for the effects of fish oil or other dietary factors. It seems logical to assume that there is a greater chance to influence the postinitiation phase since this period lasts for the rest of an individual's life, as opposed to the initiation which comprises in fact only the period of exposure to a carcinogen. On the other hand, in real life the exposure to carcinogens from the environment, cigarette smoke, (overheated) food, etc., etc. is a continuous process.

There are indications, however, that fish oil is more effective during the postinitiation phase. Caygill and Hill (1995) correlated mortality data for colorectal cancer in 24 European countries with fish and fish oil consumption. They found that in males there was an inverse correlation between colorectal cancer mortality and current intake of fish, a weaker correlation with fish consumption 10 years earlier, and none with consumption 23 years earlier. They concluded that fish consumption is associated with protection against the later promotional stages of colorectal carcinogenesis, but not with the early initiation stages. Minoura *et al.* (1988) observed a lower incidence and multiplicity of colorectal tumours in EPA-fed rats. EPA suppressed the tumour content of prostaglandin E_2, suggesting that fish oil exerts its inhibitory effect on colorectal carcinogenesis by influencing the lipid metabolism and by inhibiting prostaglandin E_2 synthesis in tumour cells, hence during postinitiation. Good *et al.* (1998) maintained AOM-treated F344 rats on diets with different fat sources during postinitiation. They found that rats fed a

high-fat fish oil diet had a lower tumour incidence and multiplicity than those fed a high-fat corn oil diet. Singh *et al.* (1997) reported that AOM-treated F344 rats fed dietary fish oil, as opposed to animals fed corn oil, demonstrated inhibited carcinogenesis and a significant reduction of cyclooxygenase-2 expression, which is considered to be an early event in the postinitiation phase. Anti *et al.* (1994) found that fish oil supplementation had normalizing effects on the abnormal rectal proliferation patterns in patients with increased colon cancer risk.

A striking result of the present study was that the HF diet failed to have an enhancing effect on colorectal cancer when compared with the LF diet. The lack of a promoting effect of a high fat diet is an uncommon finding but has been observed occasionally by other investigators (Bird *et al.*, 1996; Nicholson *et al.*, 1990). This kind of unexpected observations is difficult to explain. It does not necessarily mean that mistakes have been made, or that the experiment involved is completely invaluable. It is merely an example of a pitfall which may occur when experimental models are used to study complex phenomena. Evidently, it can never be excluded that other, unknown, factors play an important role in the determination of the course of events. Therefore, it is important to repeat experiments in order to verify obtained results.

From the results of the present study, it was concluded that dietary fish oil inhibited AOM-induced DNA adduct formation and that there was no correlation between the development of ACF and the occurrence of colorectal tumours. Because of the unexpected lack of promoting effect on colorectal cancer of the HF diet, it was considered unjustifiable to draw conclusions with respect to a potential effect on colorectal carcinogenesis of dietary fish oil fed during the initiation phase.

Chapter 6

Do aberrant crypt foci have predictive value for the occurrence of colorectal tumours? Potential of gene expression profiling in tumours

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Abstract

The effects of different dietary compounds on the formation of aberrant crypt foci (ACF) and colorectal tumours and on the expression of a selection of genes were studied in rats. Azoxymethane-treated male F344 rats were fed either a control diet or a diet containing 10% wheat bran (WB), 0.2% curcumin (CUR), 4% rutin (RUT) or 0.04% benzyl isothiocyanate (BIT) for 8 months. ACF were counted after 7, 15 and 26 weeks. Tumours were scored after 26 weeks and 8 months. We found that the WB and CUR diets inhibited the development of colorectal tumours. In contrast, the RUT and BIT diets enhanced colorectal carcinogenesis. In addition, the various compounds caused different effects on the development of ACF. In most cases the number or size of ACF was not predictive for the ultimate tumour yield. The expression of some tumour-related genes was significantly different in tumours from the control group as compared to tumours from the treated groups. It was concluded that WB and CUR, as opposed to RUT and BIT, protects against colorectal cancer and that ACF are unsuitable as biomarker for colorectal cancer. Expression of some tumour-related genes in colon tissue might be more predictive, for example metalloproteinase 1 (TIMP-1).

Introduction

Colorectal cancer is one of the most common causes of death from cancer in the western world. It has been generally accepted that food plays an important role in the risk of this disease. In the past decades, a vast amount of data has been obtained from laboratory animal models in which effects of food components on carcinogenesis were studied in carcinogen-treated rats. The merits of these widely used animal models have been proven. Nevertheless, there are some disadvantages: they are time consuming and take a considerable amount of animals and costs. Faster methods have been searched to study the effects of food components or chemicals on colorectal carcinogenesis. An example of such a method is the aberrant crypt assay. Aberrant crypt foci (ACF) are putative preneoplastic lesions that develop in the colon and rectum of carcinogen-treated rats. However, the predictive value of ACF for the development of colorectal tumours has proven to be inconsistent (Whiteley, 1999).

Colorectal cancer is the result of an accumulation of changes in the expression of certain genes. Investigation of these changes may provide valuable information which can add to the understanding of carcinogenesis.

In the present experiment groups of azoxymethane (AOM)-treated rats were fed a control diet or diets containing either wheat bran (WB), curcumin (CUR), rutin (RUT) or benzyl isothiocyanate (BIT). These four compounds were chosen because they have been related to an inhibitory effect on the development of colorectal cancer (Alabaster *et al.*, 1995; Rao *et al.*, 1995; Deschner *et al.*, 1991; Sugi *et al.*, 1994). WB is the grind husk of wheat and belongs to the group of dietary fibres. By definition, dietary fibre is not susceptible to digestive enzymes in the small intestines and arrives unchanged in the large intestines where it may or may not be fermented by intestinal microflora (Englyst *et al*, 1987). CUR is a phenolic compound naturally occurring in plants, that is used as a spicing and colouring agent. It is a natural non-steroidal anti-inflammatory drug (NSAID) (Satoskar *et al.*, 1986; Srimal and Dhawan, 1973) and acts as an antioxidant (Sharma, 1976; Toda *et al.*, 1985). RUT is a glycoside form of quercetin. In the large intestine the rutinol is split off by microbial enzymes, releasing quercetin (Goldin *et al.*, 1988). Quercetin is a flavonoid, occurs naturally in plants and has antioxidative properties (Robak and Gryglewski 1988). BIT is one of the

isothiocyanates originating from glucosinolates in cruciferous vegetables. It is an antioxidant and has cytostatic properties (Zhang, 2001).

For expression analysis the following genes were chosen, based on reports that their expression is up- or down-regulated in colorectal cancer: carbonic anhydrase II (CA2), cyclin-dependent kinase 4 (CDK4), cyclooxygenase 2 (COX-2), cytochrome P 450 1A1 (CYP1A1), K-ras, p53, p27 and tissue inhibitor of metalloproteinase 1 (TIMP-1). Expression of CA2 was found to be down-regulated in colon tumours (Kitahara *et al.*, 2001; Kivela *et al.*, 2001). CDK4 is involved in cell cycle regulation and is a catalytic subunit of cyclin D1. COX-2 expression is up-regulated in colon tumours and metastases. Down-regulation of COX-2 could be important in protection against development and progression of colorectal cancer (Zhang *et al.*, 2002; Kawai *et al.*, 2002). CYP1A1, involved in metabolism of xenobiotics, is more expressed in colon adenomas and carcinomas than in normal mucosa (McKay *et al.*, 1993). The oncogene K-ras is activated by mutation, which can lead to an increase in cell proliferation (Bos, 1989). P53 is a transcription factor that has a role in cell cycle control (Levine, 1997). Overexpression of p53 is found in colon tumours and is associated with lower disease-free survival (Diez *et al.*, 2000). The genes K-ras and p53 are often mutated during development of colorectal cancer, however, mutations in both genes are rare (Smith *et al.*, 2002). p27 is a cyclin-dependent kinase inhibitor, which can regulate cyclin D-cdk4/6 activity (Obaya *et al.*, 2002). In rat colorectal tumours p27 levels were found to be decreased compared to normal mucosa (Tao *et al.*, 2002). TIMP-1 levels were higher in plasma from patients with colon cancer (Holten-Andersen *et al.*, 2002) and gene expression of TIMP-1 was higher in colon tumours and liver metastases compared to corresponding normal tissue (Zeng *et al.*, 1995). Additionally, TIMP-1 gene expression was higher in colon tissue from patients with metastases than from patients without metastases (Zeng *et al.*, 1995), suggesting that TIMP-1 could be involved in tumour invasion.

The aim of the present study was to investigate whether the effects of a number of dietary compounds on the development of AOM-induced ACF and colorectal tumours correlated. In addition, the effects of these compounds on the expression of some tumour-related genes in colorectal tumours were studied.

Materials and methods

Animals and diets

Two hundred and fifty male specific-pathogen free Fischer 344 rats (Charles River Deutschland, Sulzfeld, Germany), four weeks old, were divided into 5 groups of 50 animals each. The control group was fed an AIN^{93}-based diet. The other four groups were fed similar diets supplemented with either 10% (w/w) WB (Meneba Feed Ingredients, Rotterdam, The Netherlands), 0.2% (w/w) CUR (Fisher Scientific BV, 's-Hertogenbosch, The Netherlands), 4% (w/w) RUT (Sigma-Aldrich, Zwijndrecht, The Netherlands) or 0.04% (w/w) BIT (Sigma Aldrich, Zwijndrecht, The Netherlands). 4% RUT is approximately equimolecular to 2% quercetin (Deschner *et al.*, 1991). The composition of the experimental diets is summarised in Table 6.1.

Table 6.1. Percentage compositions of the AIN^{93}-based diets.

Dietary components (wt%)	Control	WB	CUR	RUT	BIT
Casein	20.00	19.00	20.00	20.00	20.00
L-Cystine	0.30	0.30	0.30	0.30	0.30
Wheat starch	62.95	53.95	62.75	58.95	62.91
Cellulose	5.00	5.00	5.00	5.00	5.00
Choline bitartrate	0.25	0.25	0.25	0.25	0.25
AIN^{93}-minerals	3.50	3.50	3.50	3.50	3.50
AIN^{93}-vitamins	1.00	1.00	1.00	1.00	1.00
Soya oil	7.00	7.00	7.00	7.00	7.00
Wheat bran	-	10.00	-	-	-
Curcumin	-	-	0.20	-	-
Rutin	-	-	-	4.00	-
Benzyl isothiocyanate	-	-	-	-	0.04
Energy (kJ/g)	17	17	17	17	17

WB: wheat bran, CUR: curcumin, RUT: rutin, BIT: benzyl isothiocyanate.

Treatment and housing

All animals were treated with 3 weekly subcutaneous injections with AOM (Sigma-Aldrich, Zwijndrecht, The Netherlands), 15 mg/kg body weight. The first

injection was given one week after the start of the experiment. The animals were housed in macrolon cages with bedding, three animals per cage. Feed and tap water were available *ad libitum*. The relative humidity was kept between 30 and 70%. The number of air changes was about 10 per hour. Lighting was artificial by fluorescent tubes and time switch controlled at a sequence of 12 hours light, 12 hours dark.

In-life measurements

Food intake and body weight of all animals were recorded weekly during the first 3 months of the study and monthly thereafter. The animals were checked for clinical signs regularly. Animals showing a poor health condition were killed by decapitation under O_2/CO_2 anaesthesia. Necropsy was performed on these animals and on those that were found dead.

Necropsy, histology and histopathology

Seven weeks after the start of the experiment 6 animals per group were killed by decapitation under O_2/CO_2 anaesthesia. From 5 animals the colon was removed. Halfway, a small ring (~ 3 mm) was cut out, covered with Tissue-Tek (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands), snap-frozen in liquid nitrogen and stored at –80 °C. The two remaining parts of the colon were cut open longitudinally and fixed flat between filtration paper in 70% ethanol. From the 6^{th} animal of each group the colon was removed and rinsed with chilled 70% ethanol. Next, the colon was cut in 4 equal parts; each part was stored as Swiss Roll covered with Tissue-Tek at –80 °C.

Fifteen weeks after the start of the experiment another 6 animals per group were killed and the same procedure as described above was followed.

Twenty six weeks after the start of the experiment 9 animals per group were sacrificed. Again, the same procedures were followed, but the group of 5 animals was extended to 8. In addition, colon tumours were collected if present. Eight months after the start of the experiment the remaining animals were killed. The colon was removed, rinsed with 70% ethanol and examined for the presence of neoplastic changes. The number, size, and location (distance from the anus) of all colorectal tumours were recorded. Large tumours ($\geq$ 5 mm) were cut in half; the part (with the stalk, if present) attached to the colon was covered with Tissue-Tek, snap-frozen in liquid nitrogen and stored at –80 °C. The other half was covered

with RNA-Later (Ambion, Austin, Texas, USA) and stored at –80 °C. RNA-Later was used to prevent breakdown of RNA. Small tumours were sampled ~50/50, covered with either Tissue-Tek or RNA-Later and stored at –80 °C. Remaining parts of the colon were stored as Swiss Rolls covered with Tissue-Tek, snap-frozen in liquid nitrogen and stored at –80 °C.

The flat-fixed colons of the animals sacrificed in week 7, 15 and 26 were stained with a 0.1% solution of methylene blue for 7 minutes to make ACF visible. They were examined at low magnification. The number of ACF was recorded and their size was determined by counting the number of crypts per ACF (AC/ACF).

From each group 7 large (≥ 5 mm) tumours were selected. From the selected tumours the half part attached to the colon was histologically processed, sectioned at 5 µm, and stained with haematoxylin and eosin. Serial sections were made whenever necessary to expose the stalk, if present. They were examined microscopically and the type of the tumours was established and recorded. Microscopic classification of the tumours was done according to the criteria described by Whiteley *et al.* (1996). The other halfs of the large tumours mentioned above were used for isolation of RNA and real-time reverse transcription polymerase chain reaction (real-time RT-PCR). In the groups fed the diets with WB and CUR only few tumours larger than 5 mm had developed (2 and 3 respectively). Therefore, some smaller tumours were also analysed to attain the number of 7. Consequently, those additional small tumours were not available for microscopical classification.

RNA isolation

Colorectal tumours were disrupted using sonication and subsequent centrifugation over a QIAshredder spin column (QIAGEN, Westburg, Leusden, The Netherlands) in a buffer containing β-mercapthoethanol and guanidine thiocyanate (QIAGEN, Westburg, Leusden, The Netherlands). RNA was isolated from the supernatants using RNA-binding silica-gel membrane containing spin columns (QIAGEN, Westburg, Leusden, The Netherlands). In addition, RNA solutions were treated with RNase-free DNase I (QIAGEN, Westburg, Leusden, The Netherlands) to remove trace amounts of co-isolated DNA. RNA concentrations were determined using RiboGreen® RNA Quantitation Reagent (Molecular Probes, Leiden, The Netherlands).

cDNA synthesis and quantitative real-time RT-PCR

Total RNA (150 ng) was reversely transcribed using oligo(dT) VN_{15} primers (Promega, Madison, WI, USA) with avian myeloblastosis virus (AMV) reverse transcriptase (Promega, Madison, WI, USA). Quantitative real-time RT-PCR were performed using TaqMan® probes (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) or QuantiTect™ SYBR® Green (QIAGEN, Westburg, Leusden, The Netherlands). The TaqMan® assays were performed in a total volume of 25 µl 1x TaqMan Universal Mastermix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) in the iCycler iQ™ Real-Time PCR Detection System (Biorad, Veenendaal, The Netherlands). An initial denaturation step of 10 min at 95 °C was followed by 40-50 cycles of 95 °C for 15 s and 60 °C for 1 min. QuantiTect™ SYBR® Green PCR reactions were performed in a total volume of 20 µl 1x QuantiTect SYBR Green Master Mix (QIAGEN, Westburg, Leusden, The Netherlands) in the iCycler iQ™ Real-Time PCR Detection System. An initial denaturation step of 10 min at 95 °C was followed by 40-50 cycles of 95 °C for 15 s, 49 °C for 30 s and 72 °C for 20 s. Subsequently, a melting curve was generated by decreasing the setpoint temperature from 95 °C to 55 °C and measuring the fluorescence. Absolute amount of copies of the gene of interest in the experimental complementary DNA (cDNA) samples were calculated from the linear regression of a standard curve. The expression of the measured genes in each tumour was normalized for β-actin expression. Per tumour the expression of each gene was measured in duplo. The sequences of the primers and the TaqMan® probes used for quantitative real-time RT-PCR were as follows: β-actin forward: 5'-TTC AAC ACC CCA GCC ATG T-3', reverse: 5'-GTG GTA CGA CCA GAG GCA TAC A-3', probe: 5'-CGT AGC CAT CCA GGC TGT GTT GTC C-3'. CA2 forward: 5'-AGG ACT TTG CAG TGC TGA AAG A-3', reverse: 5'-GCC CTG GCC ATC AGA TGA-3', probe: 5'-CCC TCA GTG GCT CCT ACA GAT TGA TCC A-3'. CDK4 forward: 5'-AAG GAT CTG ATG CGC CAG TTT-3', reverse: 5'-CAG GTC CCG GTG AAC AAT G-3', probe: 5'-CGG CCT AGA TTT CCT TCA TGC A-3'. K-ras forward: 5'-AGG AAA CAA GTA GTA ATT GAT GGA GAA A-3', reverse: 5'-GTA CTG GTC CCT CAT TGC ACT GTA-3', probe: 5'-TCT CTT GGA TAT TCT CGA CAC AGC AGG TCA-3'. P53 forward: 5'-CCA TCA TCA CGC TGG AAG ACT-3', reverse: 5'-CCC AGG ACA GGC ACA AAC AC-3', probe: 5'-AAC CTC AAA GCT GTC CCG TCC CAG A-3'. TIMP-1 forward: 5'-

GGG CTA CCA GAG CGA TCA CTT-3', reverse: 5'-AAG GTA TTG CCA GGT GCA CAA-3', probe: 5'-CCT GCC TGC CAC GGA ATC CAG A-3'. COX-2 forward: 5'-TCC CTT CGC CTC TTT CAA TG-3', reverse: 5'-GGA GGC ACT TGC GTT GAT G-3', probe: 5'-AAG ACC CGC AGG CTA CCA AGA CAG C-3'. CYP1A1 forward: 5'-ACA GAC CTC AGC TGC CCT ATC GT-3', reverse: 5'-TGA ATG GGA CAA AGG ATG AAT G-3', probe: 5'-AGG CCT TCA TCC TGG AGA CCT TCC G-3'. p27 forward: 5'-GCG ACC TGC GGC AGA A-3', reverse: 5'-GGG AAC CGT CTG AAA CAT TTT C-3' (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

Statistical analysis

The multiplicity and size of ACF and colorectal tumours were analysed using analysis of variance (ANOVA) followed by Student's *t*-test. Gene expression results were analysed using a *t*-test. Tumour incidences were analysed using Pearson's χ^2 test. A probability value of $P < 0.05$ (two-tailed) was used as the critical level of significance.

Results

Survival of the animals

Survival of the animals was 90% in the control group, 100% in the WB group, 94% in the CUR group, 88% in the RUT group and 94% in the BIT group. Nine animals were found dead, 8 were euthanised because of poor health condition. The main cause of death and clinical problems was a tumour in the small intestines. One animal showed invagination of the colon due to a colon tumour. Other animals had haemorrhagic gastroenteritis, pleuropneumonia or had died of unknown cause. These animals were excluded from the study to ensure proper comparison of the different groups.

Food consumption, energy intake and terminal body weight

The different experimental groups had comparable food consumption: 13.9, 14.4, 13.5, 14.2 and 13.0 g/animal/day for the control, WB, CUR, RUT and BIT group, respectively. Since the different foods had similar energy content the overall mean energy intake of the animals was also comparable.

Compared with the controls, the WB group had the best growth performance, while the BIT group showed slight growth retardation (Fig.6.1). This may be explained by the fact that, in contrast to some animals of other groups, none of the WB animals had showed health problems during the experiment.

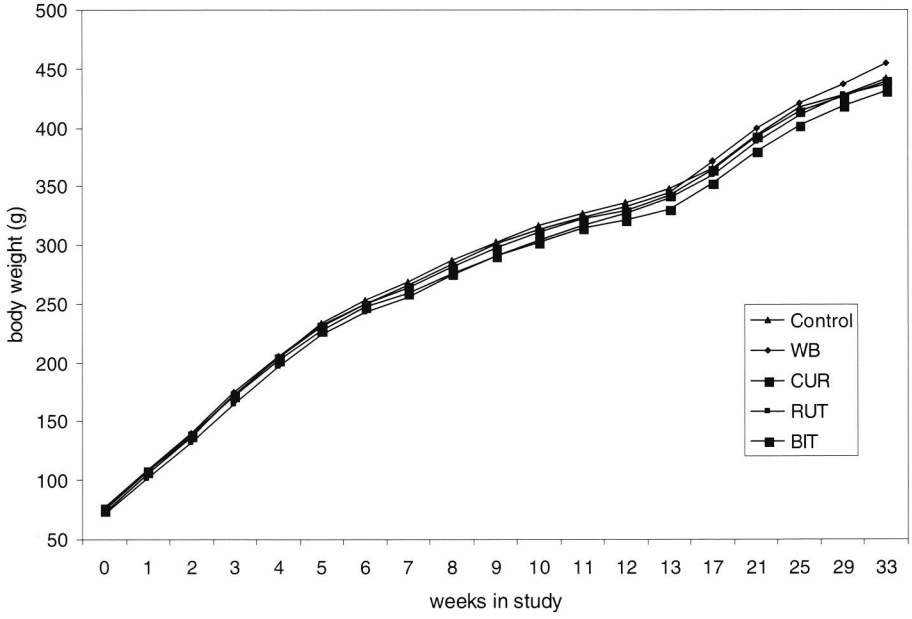

Figure 6.1. Body weights.

ACF

All animals developed ACF. The ACF scores are presented in Table 6.2. In week 7, the highest numbers of ACF were present in the WB and BIT groups. The lowest numbers were counted in the CUR and RUT groups ($P < 0.05$, compared to the control group). The highest numbers of large ACF (4 or more AC/ACF) were present in the control, WB and BIT groups.

Compared with week 7, the number of ACF was markedly decreased in week 15 in the control, WB and BIT groups, but only slightly different in the CUR and RUT groups. In general, in week 15, the numbers of ACF in all experimental

groups were comparable, but the number of AC/ACF was higher in the WB group than in the control group ($P < 0.05$). In week 26, the lowest number of ACF was found in the WB group. The number of AC/ACF was highest in the RUT group ($P < 0.05$, compared to the control group). The other groups were comparable with the control group. At this time point the number of ACF was markedly decreased compared with week 7 in the control group (- 27%), the WB group (- 70%) and the BIT group (- 56%), whereas it was increased in the CUR group (+ 17%) and RUT group (+ 89%). The mean number of AC/ACF increased from week 7 to week 26 in all groups.

Colorectal tumours

The incidence, multiplicity and size of colorectal tumours are presented in Table 6.2. The incidence is the percentage of animals bearing one or more colorectal tumours. The multiplicity is the number of colorectal tumours per tumour-bearing animal. The design of the study did not allow microscopical examination of all tumours to establish whether they were benign or malignant. Therefore, the table refers to the total number of macroscopically observed tumours. In the animals killed in week 7 no tumours were found. In week 15 again no tumours were found except for one small polyp in a RUT-fed animal. At necropsy in week 26, most animals had developed one or more colorectal tumours. The incidence was 86% in the control group, 89% in the WB group, 89% in the CUR group, 100% in the RUT group and 100% in the BIT group. The WB group had the lowest multiplicity and the BIT group had the highest multiplicity, but the differences were not statistically significant compared to the control group. The CUR and RUT groups had the largest mean tumour size ($P < 0.05$, compared to the control group).

Upon final necropsy at 8 months, the highest incidence of colorectal tumours was found in CUR-fed animals (100%); the WB group showed the lowest incidence (76%). In the WB and CUR groups, the multiplicity of total tumours was statistically significantly lower than in the control group ($P <0.05$). The RUT and BIT groups showed the highest tumour multiplicity, but the difference was not statistically significant compared to the control group. The mean tumour size in all groups was not statistically significantly different from that in the control group. In all groups, the distribution of the tumours over the colon was comparable. Deep intramural processes, representing the typical macroscopic picture of a carcinoma, were generally slightly closer to the caecum than polypoid tumours.

Table 6.2. Multiplicity of all ACF, ACF with 4 or more aberrant crypts and tumours per animal (mean ± SEM), number of aberrant crypts per ACF (mean ± SD), tumour incidence (%), tumour size (mm ± SD) and tumour types used for RT-PCR.

	Experimental groups				
	Control	WB	CUR	RUT	BIT
	ACF after 7 weeks				
ACF multiplicity	46 ± 7	55 ± 3	29 ± 5*	19 ± 3*	57 ± 4
ACF ≥ 4 AC	21 ± 5	20 ± 1	11 ± 2	8 ± 2	21 ± 2
No. of AC / ACF	3.40 ± 0.53	3.14 ± 0.19	3.22 ± 0.38	3.16 ± 0.61	3.08 ± 0.08
	ACF after 15 weeks				
ACF multiplicity	20 ± 3	26 ± 6	24 ± 3	21 ± 6	28 ± 5
ACF ≥ 4 AC	13 ± 4	17 ± 4	16 ± 2	15 ± 5	18 ± 5
No. of AC / ACF	4.34 ± 0.26	4.76 ± 0.30*	4.94 ± 1.62	4.78 ± 0.73	4.26 ± 1.07
	ACF and colorectal tumours after 26 weeks				
ACF multiplicity	33 ± 8	17 ± 3	34 ± 8	36 ± 7	24 ± 6
ACF ≥ 4 AC	25 ± 5	13 ± 2	27 ± 6	31 ± 5	18 ± 5
No. of AC / ACF	5.57 ± 0.73	4.97 ± 0.76	5.92 ± 0.90	6.83 ± 0.56*	6.18 ± 0.92
Tumour incidence	86	89	89	100	100
Tumour multiplicity	2.00 ± 0.36	1.12 ± 0.12	1.62 ± 0.50	1.86 ± 0.34	2.50 ± 0.54
Tumour size	2.3 ± 1.2	3.7 ± 2.6	3.8 ± 1.4*	4.1 ± 2.9*	3.2 ± 2.0
	Colorectal tumours after 8 months				
No. of animals	26	29	26	25	27
Tumour incidence	92	76	100	88	96
Tumour multiplicity	3.29 ± 0.42	1.86 ± 0.23*	2.27 ± 0.22*	4.41 ± 0.44	3.85 ± 0.33
Tumour size	3.84 ± 0.44	2.95 ± 0.38	2.94 ± 0.26	4.31 ± 0.27	4.13 ± 0.30
Total no. of tumours	79	41	59	97	100
	Tumour types used for RT-PCR				
Adenomas	2		1	1	
Carcinomas	5	2	2	6	7
Unknown[1]		5	4		
Total	7	7	7	7	7

WB: wheat bran; CUR: curcumin; RUT: rutin; BIT: benzyl isothiocyanate; AC: aberrant crypt; ACF: aberrant crypt focus.

* $P < 0.05$, vs control group (Student's *t*-test).

[1] No histologic diagnosis because completely used for RT-PCR.

Quantitative real-time RT-PCR

We investigated whether the effects of the different dietary compounds on the development of colorectal tumours was reflected by the expression of a subset of genes. Therefore, 7 tumours per group were analysed for differential expression of 8 genes by quantitative real-time RT-PCR. Microscopic evaluation of these tumours showed that most tumours in the control, RUT and BIT group were carcinomas (Table 6.2). In the WB and CUR group 5 and 4 tumours, respectively, could not be microscopically classified, because of the small size of these tumours. From the selected tumours total RNA was isolated of which cDNA was synthesized. For one tumour from the RUT group and two tumours from the BIT group no cDNA could be synthesized. Subsequently, expression of the following genes was analysed: CA2, CDK4, COX-2, CYP1A1, K-ras, p27, p53 and TIMP-1. β-actin expression was measured in each tumour and used to normalize the expression of the genes of interest. Gene expression is presented in arbitrary units.

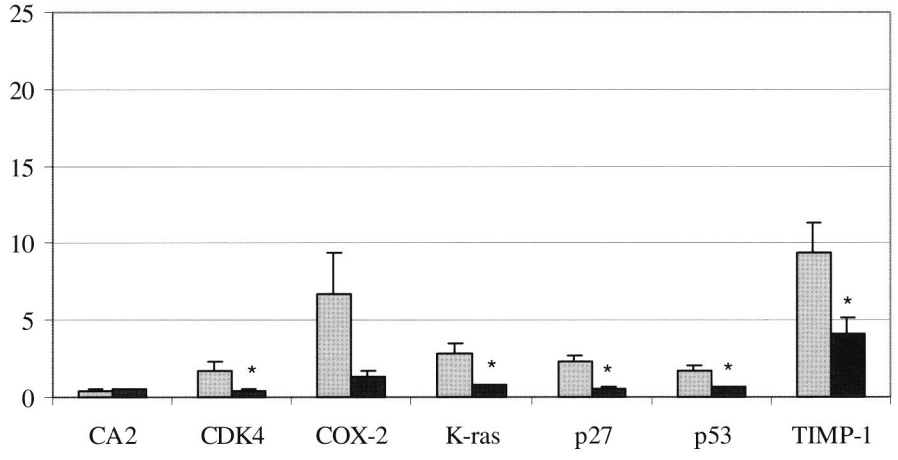

Figure 6.2. Expression of CA2, CDK4, COX-2, K-ras, p27, p53 and TIMP-1, corrected for β-actin expression, in control group (grey bars) and WB group (black bars), ± SEM. $*P < 0.05$, compared to control group.

As can be seen in Fig.6.2, quantitative real-time RT-PCR revealed that tumours from the WB group had a significantly lower expression of CDK4, K-ras, p27, p53 and TIMP-1 as compared to tumours from the control group. Tumours from the

CUR group had significantly lower expression of COX-2 and K-ras as compared to tumours from the control group (Fig.6.3).

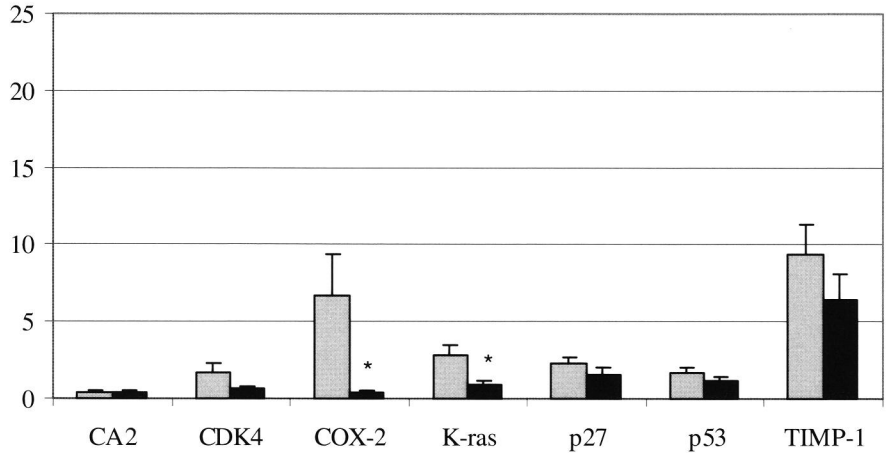

Figure 6.3. Expression of CA2, CDK4, COX-2, K-ras, p27, p53 and TIMP-1, corrected for β-actin expression, in control group (grey bars) and CUR group (black bars), ± SEM. *$P < 0.05$, compared to control group.

A significantly lower expression of COX-2 and a significantly higher expression of TIMP-1 were observed in tumours from the RUT group in comparison with tumours from the control group (Fig.6.4).

In most tumours, CYP1A1 was not expressed. Gene expression in tumours from the BIT group was not different from that in tumours from the control group. However, all tumours from the BIT group were carcinomas. When they were compared with the carcinomas only from the control group, gene expression of K-ras was significantly lower (1.19 ± 0.08 vs 3.61 ± 0.79 in control group; $P < 0.05$).

Putting together the gene expression and the tumour data demonstrates that the effects of the different dietary compounds on TIMP-1 expression correlated well with the effects of the dietary compounds on the ultimate tumour yield: TIMP-1 expression was lower in the WB group than in the control group and it was higher in the RUT group than in the control group. Therefore, TIMP-1 might be a biomarker for the development of colorectal cancer.

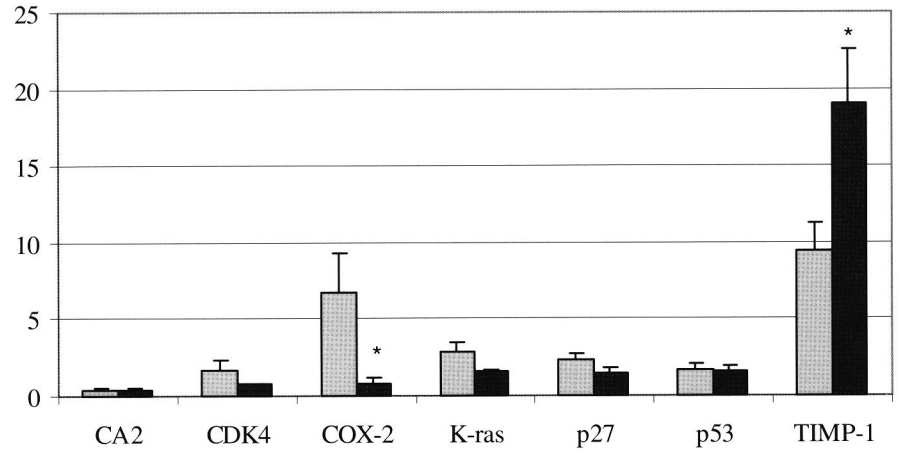

Figure 6.4. Expression of CA2, CDK4, COX-2, K-ras, p27, p53 and TIMP-1, corrected for β-actin expression, in control group (grey bars) and RUT group (black bars), ± SEM. $*P < 0.05$, compared to control group.

Discussion

In the present study we investigated whether the effects of dietary compounds on the development of AOM-induced ACF and colorectal tumours correlated. In addition, the effects of these compounds on the expression of tumour-related genes were studied.

We assessed ACF by recording their total number and the number with 4 or more aberrant crypts, since it has been postulated that larger ACF may be more predictive than the total number of ACF (Davies *et al.*, 1999; Shirtliff and Bird, 1996), and we assessed the size, expressed as number of AC/ACF. If the effect of a food compound on the formation of ACF at an early stage of carcinogenesis would have a reliable predictive value for either an increased or decreased risk of colorectal cancer, then at least one of the above mentioned features of ACF should consistently correlate with the development of colorectal tumours. It was concluded that this was not the case in the present study because of the following observations. In the WB group, at 7 and 15 weeks, the ACF scores tended to be higher than in the control group and at 15 weeks the number of AC/ACF was

higher ($P < 0.05$) than in the control group. However, at 8 months, the WB group had a lower tumour multiplicity than the control group ($P < 0.05$). In comparison with the controls, the ACF multiplicity in the RUT group was lower at 7 weeks ($P < 0.05$), but at 26 weeks the number of AC/ACF and the tumour size were higher ($P < 0.05$), while at 8 months the tumour multiplicity and size were highest of all groups. Another notable finding was that although the ACF figures in the WB and BIT groups were very similar at 7 and 15 weeks, the numbers of tumours found in the BIT group at 26 weeks and at 8 months were more than twice as high as those found in the WB group. In the CUR group both the ACF multiplicity at 7 weeks and the tumour multiplicity at 8 months were lower than those in the control group ($P < 0.05$). Summarizing, a good prediction of ACF for colorectal tumours was only observed for the CUR group at 7 weeks. In all other groups, ACF had no predictive value.

Failure of ACF to predict the development of colorectal tumours is consistent with results of previous studies (Cameron *et al.*, 1996; Takahashi *et al.*, 1991; Thorup *et al.*, 1994; Wijnands *et al.*, 2001, 2003; Zheng *et al.*, 1999). In contrast, on some occasions there was a correlation between occurrence of ACF and colorectal tumours (Alabaster *et al.*, 1995; Kawamori *et al.*, 1995; Pretlow *et al.*, 1992; Shivapurkar *et al.*, 1992; Young *et al.*, 1996). These conflicting results indicate that recording number and size of ACF cannot be used as a reliable screening assay. Nevertheless, ACF are putative preneoplastic lesions and studying them may yield relevant knowledge about the carcinogenic process. It is interesting to note that the experimental diets apparently had a different effect on their development. In rats and mice ACF appear in the colon and rectum about 2 weeks after treatment with a carcinogen. In the following months their number increases and they become larger. Next, some ACF may disappear because they regress, most will stay present, probably as innocent bystander lesions, while one or more of these persistent ACF may develop into a tumour (Bird, 1995). This general pattern is applicable to the WB and BIT groups in the present study. In the CUR and RUT groups, however, the number of ACF continued to increase, especially the larger ones. The controls showed an increased number after an initial decrease. This means that a dietary compound may influence not only the number of ACF, but it also may determine i) the moment they appear, ii) the moment they start to regress or become dysplastic and iii) their growth rate. Since it is unpredictable how a potential chemoprotective agent may influence these factors, the ACF score

established at an arbitrary time point is unreliable as predictive factor for colorectal cancer.

In the present study, WB effectively inhibited colorectal carcinogenesis, which confirmed the observations of numerous other investigators (Alabaster *et al.*, 1995; Barnes *et al.*, 1983; McIntyre *et al.*, 1993; Reddy and Mori, 1981; Reddy *et al.*, 1981; Sinkeldam *et al.*, 1986; Sinkeldam *et al.*, 1990; Watanabe *et al.*, 1979; Young *et al.*, 1996). Furthermore, Alberts *et al.* (1990) found that a dietary supplement of 13.5 g WB/day inhibited DNA synthesis and rectal mucosa cell proliferation in high-risk patients. Although this was only a pilot study, the results suggested that relatively small amounts of dietary fibre may already have a preventive effect. The protective effect of WB against colorectal cancer is associated with stool bulking, a shorter intestinal transit time and the formation of short chain fatty acids, of which butyrate is probably the most important one (Munakata *et al.*, 1995; McIntyre *et al.*, 1993).

Seven out of 41 tumours in the WB group differed significantly from an equal number of tumours in the control group in expression of CDK4, K-ras, p27, p53 and TIMP-1. Down-regulation of protein levels of p53 was also found in invasive colonocytes treated with short chain fatty acids (Emenaker *et al.*, 2001). In epidermal cells co-expression of ras and CDK4 promoted cell growth and stimulated development of invasive neoplasia (Lazarov *et al.*, 2002). Butyrate down-regulated gene expression of N-ras in colon carcinoma cells in vitro (Mariadason *et al.*, 2000). Reddy *et al.* (2000) demonstrated that specifically the lipid fraction of WB could down-regulate COX-2 protein expression. In this study, however, gene expression of COX-2 in colorectal tumours was not significantly down-regulated by WB. Protein levels of p27 are low in colorectal cancers, and high expression of p27 in tumours is reported to be correlated to survival rate (Palmqvist *et al.*, 1999). Tumours in the WB group had a lower expression of p27 than tumours in the control group. In contrast to the consistent effect of WB on gene expression of CDK4, K-ras, p53 and TIMP-1, the effect of WB on gene expression of p27 does not seem to correspond with the inhibitory effect of WB on colorectal cancer.

Although in the present study after 8 months dietary CUR had a protective effect on tumour multiplicity only, it has been shown to inhibit both the incidence and multiplicity of colorectal tumours in animal models (Huang *et al.*, 1994; Pereira *et al.*, 1996; Rao *et al.*, 1995). The protective effect of CUR has been

related to its antioxidant and antimutagenic effects (Nagablushan *et al.*, 1987), to its influence on arachidonic acid metabolism (Rao *et al.*, 1995), and to its ability to inhibit prostaglandin synthesis (Huang *et al.*, 1991) and to enhance apoptosis (Samaha *et al.*, 1997). Tumours of the CUR group showed down-regulated gene expression of COX-2 and K-ras in comparison with tumours of the control group. These results were in accordance with published studies, in which tumours were induced by other carcinogens. Rao *et al.* (1995) reported lower COX-2 activity in tumours induced by AOM from rats that were fed a diet with 2000 ppm CUR. In mice CUR decreased expression of ras oncogenes in carcinogen-induced skin tumours (Limtrakul *et al.*, 2001). Further, CUR decreased expression of ras oncogenes induced by diethylnitrosamine in rat liver (Chuang *et al.*, 2000) and lowered COX-2 protein and mRNA levels in human colon cancer cells *in vitro* (Goel *et al.*, 2001).

At 26 weeks, the tumour incidence and tumour size of the RUT group was enhanced compared to the control group. After 8 months, the total number of tumours was higher compared to the control group, but the incidence was lower and no significant differences with respect to tumour multiplicity and size were observed in the RUT group. Several studies did not reveal a carcinogenic or tumour-promoting effect of rutin/quercetin (Deschner *et al.*, 1991; Dunnick and Hailey, 1992; Hirono *et al.*, 1981; Ito *et al.*, 1989; National Toxicology Program, 1992). Furthermore, quercetin has been shown to exert chemoprotective properties such as antioxidant activity (Robak and Gryglewski 1988) and the ability to inhibit arachidonic acid metabolism (Welton *et al.*, 1988). On the other hand, Pereira *et al.* (1996) found enhancement of colorectal tumorigenesis in AOM-treated rats. The lack of protection against carcinogenesis in this study could not be explained.

Even though RUT did not protect against the development of colorectal cancer, gene expression of COX-2 was decreased in tumours of the RUT group, compared to tumours of the control group. In AOM-treated rats fed RUT for 7 days, p27 levels in colorectal tumours did not change (Tao *et al.*, 2002). In this study rats were fed RUT for a longer period and also no effect on expression of p27 in colorectal tumours was found. In the RUT group the expression of TIMP-1 in colorectal tumours was increased compared to the control group. In contrast, in humans supplemented with quercetin for 14 days plasma levels of TIMP-1, mRNA and protein were decreased (Morrow *et al.*, 2001). It is known that high TIMP-1 expression is correlated with tumour progression and liver metastasis (Hewitt *et al.*

2000; Zeng *et al.*, 1995). TIMP-1 may exert its effect on colorectal carcinogenesis through stimulation of cell growth, which may be influenced by interaction with ras (Hayakawa *et al.*, 1992; Wang *et al.*, 2002). Interestingly, in this study TIMP-1 gene expression was down-regulated in tumours of rats fed a WB diet, which protected against development of colorectal tumours and up-regulated in tumours of rats fed a RUT diet, which showed a trend to enhance colorectal tumour development. Thus, increased and decreased TIMP-1 gene expressions in colorectal tumours seem to correlate with promotion and inhibition of colorectal cancer, respectively.

In contrast with the results of the present study, Sugi *et al.* (1994) found that rats fed a diet containing 0.04% BIT showed decreased development of colorectal tumours. This may be explained by the difference in study protocol. Sugi *et al.* used methylazoxymethanol-treated female ACI/N rats and the incidence and multiplicity of colorectal tumours was reduced if BIT was given during initiation, but not if BIT was given during promotion. Although BIT did not protect against development of colorectal cancer in this study, carcinomas from the BIT group had a lower expression of K-ras than carcinomas from the control group.

In summary, it can be concluded that WB and CUR had a protective effect on the development of colorectal tumours. Despite their assumed protective properties, the RUT and BIT diets showed an enhancing rather than inhibitory effect on colorectal carcinogenesis. The ACF score was not considered to be suitable as biomarker for colorectal cancer. Since the effects of the different dietary compounds on the expression of TIMP-1 correlated well with the effects of the different compounds on the ultimate tumour yield, it can be hypothesized that TIMP-1 expression in colorectal tissue is a good predictor for the development of colorectal tumours. The reliability of TIMP-1 as a biomarker for colorectal cancer has to be verified in further studies.

By using microarrays for gene expression profiling, more new biomarkers for colorectal cancer can be found (van Ommen and Stierum, 2002). Next, we will study gene expression changes in intestinal cells of AOM-treated rats in response to different food compounds at several time points.

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Chapter 7

General discussion and conclusions

Introduction

There is a large body of data from epidemiological and animal model studies linking dietary factors with the occurrence of cancer. Dietary fibre and fat are regarded to play a major role in the etiology of colorectal cancer. Diets low in fibre and high in fat, characteristic for many Western countries are associated with a high risk of colorectal cancer, whereas diets high in fibre and low in fat, characteristic for most African and Asian countries are associated with a low risk. The relevance of the diet is emphasised by the observation that people migrating from an area where low-risk diets are common to an area where high-risk diets are common, rapidly acquire an increased risk for cancer and other diseases, typical for the new environment (Faivre, 1998; Potter, 1995; Reddy, 1995; Smigel, 1992). Obviously, diets from geographically different areas also differ with respect to many other components other than fibre and fat. Moreover, the effects of dietary fibre and fat cannot be generalised; they have been shown to depend on the type of fibre and fat (Hill, 1997; Klurfeld and Bull, 1997).

Much information has been obtained from animal model experiments, designed to study the effects of a food ingredient or other variables on the development of chemically induced colorectal cancer under controlled conditions. A disadvantage of this type of studies is that they require a considerable number of animals and much time. The discovery of ACF, that develop in the large intestines of rodents within a few weeks after carcinogen treatment (Bird, 1987), provided scientists with a tool to study early events in colorectal carcinogenesis in short-term studies using fewer animals. There is little doubt that ACF are putative preneoplastic lesions. Several investigators have proposed that ACF are suitable as biomarker and that, consequently, changes in ACF development may be extrapolated to changes in cancer risk. However, this view is subject to debate.

In this thesis five animal studies are described and discussed. In all experiments classical rat models were used. The models proved to be effective in all cases. All stages of carcinogenesis were observed, from ACF up to and including invasive adenocarcinomas. The major objectives of the studies were to investigate effects of different types of fibre and fat on the development of chemically induced ACF and colorectal tumours and to investigate whether ACF can be used as biomarker.

Dietary fibre and colorectal cancer

The experimental diets used contained various levels of different fibre types:
- Cellulose; insoluble and hardly fermentable.
- Galacto-oligosaccharides (GOS); soluble with the appearance of a thick syrup and readily fermentable.
- Resistant starch (RS); insoluble (in cold water) and readily fermentable.
- Wheat bran (WB); insoluble and (partly) fermentable, though slowly.

The results summarised in Table 7.1 demonstrate that apart from an inhibitory effect on carcinoma formation in one experiment, cellulose had generally no effect or an enhancing effect on colorectal tumour development, whereas GOS, WB and RS demonstrated inhibitory effects.

To put these findings into perspective, the general hypotheses of the protective effects of dietary fibre, summarised in the General Introduction (Chapter 1), should be considered. Investigation of changes of the composition of the gut microflora was not within the scope of this thesis. Therefore, this discussion is confined to other properties of dietary fibre, namely bulking effect and fermentability.

Cellulose

Taking into account the physico-chemical properties of cellulose, diets containing high levels of cellulose can be expected to lead to faeces bulking. Indeed, this was a very convincing effect in the experiments presented in Chapter 2 and 4 and confirmed the observations of other investigators (Harris and Ferguson, 1993; Munakata *et al.*, 1995). In addition, cellulose has been shown to suppress the DMH-induced increase in colon crypt mitotic activity (Cameron *et al.*, 1989) and to decrease the activity of faecal β-glucuronidase (Freeman, 1986), phenomena which are both associated with inhibition of carcinogenesis. Undoubtedly, the bulking effect has consistently been denoted the major reason why cellulose protects against colorectal cancer. Having reviewed epidemiological literature, also Hill (1998) stressed that stool bulking and consequent dilution of luminal carcinogens and promoters are the most important protective mechanisms of dietary fibre in general. However, despite marked faeces bulking, cellulose did not inhibit tumorigenesis in our experiments. On the contrary, it showed even an enhancing effect.

Table 7.1. Effects of high levels of various fibres on the development of colorectal tumours. -: negative association; +: positive association; 0: no statistically significant effect.

	Adenomas	Carcinomas	Total tumours
Fibre types		Incidence	
Cellulose[1]	+	-	0
Cellulose[2]	+	+	+
GOS[1]	0	0	0
GOS[3]	0	0	-
Resistant starch	0	0	-[a]
Wheat bran	0	0	0
		Multiplicity	
Cellulose[1]	+	-	+
Cellulose[2]	+	0	+
GOS[1]	-	-	-
GOS[3]	0	0	0
Resistant starch	0	0	-[b]
Wheat bran	0	0	-
		Size	
Cellulose[1]	0	-	-
Cellulose[2]	0	0	0
GOS[1]	0	0	0
GOS[3]	0	0	0
Resistant starch	0	0	0
Wheat bran	0	0	0

[1] Chapter 2
[2] Chapter 4
[3] Chapter 3
[a] only when combined with low fat
[b] only when combined with high fat

Previous data concerning dietary cellulose have been conflicting. Jacobs and Lupton (1986) fed DMH-treated rats a fibre free diet or a diet containing 10% cellulose and found no difference in tumour yield. In DMH-treated Sprague-Dawley rats fed diets with 20% cellulose, Madar *et al.* (1996) observed increased

faeces production accompanied with a decreased tumour incidence, whereas in a similar experiment Tempero *et al.* (1988) found no protecting effect. Wilpart and Roberfroid (1987) increased the dietary cellulose level from 5% during initiation to 15% during promotion in DMH-treated Wistar rats, but this did not inhibit carcinogenesis. Heitman *et al.* (1989) observed that the incidence of colorectal adenocarcinomas was significantly lower in DMH-treated Sprague-Dawley rats maintained on diets containing various levels of cellulose than in rats fed a fibre-free diet. Sakamoto *et al.* (1996) found that colorectal tumours were significantly smaller in DMH-treated Sprague-Dawley rats fed a diet with 10% cellulose, compared to rats fed a fibre-free diet, but there were no differences in tumour incidence or multiplicity. Ward *et al.* (1973) did not find differences in tumour yield between AOM-treated rats fed a low-fibre diet and those fed 20% or 40% dietary cellulose, despite markedly increased bulking resulting from the high-cellulose diets. Results from other experiments indicate that a protecting effect of dietary cellulose, if any, is weaker than that of other fibre sources (Kritchevsky and Klurfeld, 1997; Takahashi *et al.*, 1999). In a large human case-control study in Italy the relationship between various types of fibre and colorectal cancer risk was investigated (Negri *et al.*, 1998). The risk of colorectal cancer was inversely related to fibre intake. Especially fibre from vegetables and, to a lesser extent, from fruit protected against colorectal cancer. The protective effect of cellulose and soluble non-cellulose polysaccharides (NCP) was slightly stronger than that of insoluble NCP and lignin.

Faeces bulking is a consistent feature of high-cellulose diets. The inconsistent effects of cellulose on colorectal carcinogenesis justify the question: is the bulking effect alone really a protective factor? The hypothesis that bulking dilutes and adsorbs potentially harmful substances and enables the gut to get rid of them quickly by shortened transit time makes good sense, but all other substances in the intestinal content are also subjected to dilution, adsorption and rapid excretion. This means that under certain circumstances bulking may have an adverse effect because it may prevent other protective factors to exert their protective effects.

From the results of our studies it is concluded that dietary cellulose, despite marked faeces bulking, either had no effect or an enhancing effect on the formation of colorectal tumours, although the development of adenocarcinomas was decreased in one experiment, possibly through inhibition of the progression of

adenomas to carcinomas. Furthermore, it is concluded that faeces bulking as sole factor may fail to protect against colorectal cancer.

Galacto-oligosaccharides, resistant starch and wheat bran

GOS, RS and WB all had an inhibitory effect on the formation of colorectal tumours. These three fibre sources have in common that they are fermentable by the large intestinal microflora. This property is generally considered to be the main reason for the suppressive effect of these and other fermentable fibres on colorectal carcinogenesis. In short, the hypothesis is that fermentation results in the formation of short chain fatty acids (SCFA) of which butyrate is probably the most important one, a decreased pH of the intestinal content and consequently, reduced amounts of potentially harmful secondary bile acids. In addition, some fermentable fibres may also have a bulking effect.

Animals fed the high-GOS diets had a markedly enlarged caecum. Unlike the animals fed the high-cellulose diets however, they did not show stool bulking. The caecum enlargement was caused by a high fluid content, which was readily absorbed in the colon. The protective effect of GOS was most probably related to its fermentation, resulting in decreased pH and increased amounts of SCFA. It has been shown that the proliferation of colon cancer cell lines can be influenced by butyrate (Gamet *et al.*, 1992). In our studies, however, the proliferation index, determined in colorectal adenomas either by BrdU or Ki-67 labelling, was not statistically significantly different in animals fed high-GOS diets compared with animals fed low-GOS diets.

It has been reported that oligosaccharides may promote the growth of beneficial gut microflora, such as bifidobacteria and lactobacillus (Howard *et al.*, 1995; Rowland and Tanaka, 1993; Van Haastrecht, 1995). In fact, this property of GOS was essentially the reason to market this product. Yamazaki *et al.* (2000) found that Lactobacillus casei strain Shirota inhibited AOM-induced colorectal cancer in rats. Thus, the influence on gut microflora may also have contributed to the inhibitory effect of GOS on the development of colorectal tumours.

The results of the study described in Chapter 3 indicated that feeding a low or high level of GOS during the initiation stage did not result in clear differences in tumour yield. Therefore, it was concluded that the high-GOS diets exerted their protective effect during the promotion stage.

Epidemiological data suggest a strong inverse association between RS consumption and colorectal cancer (Cassidy *et al.*, 1994). A high-RS diet caused increased faecal concentration and daily excretion of butyrate, increased faecal output and decreased faecal pH in human volunteers (Phillips *et al.*, 1995). In our experiment, the high-RS-fed rats had a lower caecal pH and increased amounts of SCFA compared with the low-RS-fed rats. It is likely that these phenomena played a role in the inhibition of colorectal carcinogenesis. The low pH may have been responsible for an inhibited transformation of primary bile acids into secondary bile acids, which can be concluded from the increased amount of primary bile acids (cholic acid) and the relatively low level of secondary bile acids (deoxycholic and lithocholic acid) in the high-RS groups.

The protective effect of WB on colorectal cancer is associated with stool bulking, a shorter intestinal transit time and the formation of short chain fatty acids (Munakata *et al.*, 1995; McIntyre *et al.*, 1993). In addition, certain components of WB, such as phytic acid and phenolic components (phenolic acid, lignans and flavonoids) may have specific protective properties (Ferguson and Harris, 1999). WB is fermented slowly, allowing continuing fermentation in the more distal regions of the colon. Therefore, a low pH and a high butyrate concentration are still present in the distal colon. This may explain the effectiveness of WB against colorectal cancer (McIntyre *et al.*, 1991).

The protective role of butyrate has been questioned by Hill (1995), mainly because many data regarding the properties of butyrate have been obtained from in vitro experiments. Nonetheless, results from animal experiments are in favour of the view that butyrate does have beneficial effects (McIntyre *et al.*, 1993; Hague *et al.*, 1997; Medina *et al.*, 1998; Van Munster and Nagengast, 1993).

In conclusion, there has been considerable debate about the proposed anti-cancer effects of dietary fibre. The results described in this thesis confirm that not all types of dietary fibre have protective effects, but it seems justifiable to state that *fermentable* fibres do.

Dietary fat and colorectal cancer

In a series of animal experiments described in this thesis three different types of fat were studied for their effect on induced colorectal carcinogenesis:

- Trisun sunflower oil, which is rich in oleic acid (Chapters 2 and 5)
- Soya oil, rich in ω-6 linoleic acid (Chapter 4).
- MaxEPA fish oil, rich in ω-3 fatty acids (Chapter 5).

The effects of the different fat types are summarised in Table 7.2.

Sunflower oil and fish oil

A high level of dietary sunflower oil had a promoting effect on colorectal cancer in the experiment described in Chapter 2. This was possibly related to an increased proliferative activity observed in colonic crypts. Proliferation indices in colonic crypts have been found to be positively correlated with the risk of colorectal cancer in laboratory animals and humans (Alberts *et al.*, 1990; Barns *et al.*, 1996; Heitman *et al.*, 1989; Ochiai *et al.*, 1996), although it has also been shown that differentiation and apoptosis had greater predictive value to detect dietary effects on tumour incidence than cell proliferation had (Chang *et al.*, 1997).

In contrast to the first experiment, in the experiment described in Chapter 5, a high level (as compared to a low level) of dietary sunflower oil did not result in increased incidence, multiplicity, or size of colorectal tumours. However, methodologically both experiments were quite different. Main differences were that in the experiment described in Chapter 2, we used DMH-treated Wistar rats fed AIN^{76}–based diets, whereas in the experiment described in Chapter 5 we used AOM-treated F344 rats fed AIN^{93}–based diets. Nonetheless, it was expected that the promoting effect of a high-sunflower oil diet could be reproduced, especially since the level of sunflower oil was more than twice as high in the second study as compared with the first one. The animals receiving high-sunflower oil during both the initiation and promotion period were included to serve as a positive control group. The lack of a promoting effect of the high sunflower oil on colorectal carcinogenesis makes the results of that experiment very difficult to interpret, if not uninterpretable. Apparently, an unknown factor has interfered with the anticipated enhanced tumour formation in the intended positive control group.

Table 7.2. Effects of high fat diets on the development of colorectal tumours. -: negative association; +: positive association; 0: no statistically significant effect; ?: inconclusive.

	Adenomas	Carcinomas	Total tumours
Fat types		Incidence	
Sunflower oil (+cellulose)[1]	+	0	+
Sunflower oil (+GOS)[1]	0	+	0
Sunflower oil[2]	0	0	0
Soya oil (+cellulose)	0	+	0
Soya oil (+RS)	0	0	+
Fish oil	?	?	?
		Multiplicity	
Sunflower oil (+cellulose)[1]	+	0	+
Sunflower oil (+GOS)[1]	0	0	0
Sunflower oil[2]	0	0	0
Soya oil (+cellulose)	0	0	0
Soya oil (+RS)	0	0	0
Fish oil	?	?	?
		Size	
Sunflower oil (+cellulose)[1]	0	0	0
Sunflower oil (+GOS)[1]	0	0	0
Sunflower oil[2]	0	0	0
Soya oil (+cellulose)	-	-	-
Soya oil (+RS)	0	0	0
Fish oil	?	?	?

[1] Chapter 2
[2] Chapter 5

The fish oil diet resulted in a decreased formation of DNA adducts, indicating a possible inhibitory effect on carcinogenesis at an early stage, since high levels of DNA adducts in the human colon have been associated with colorectal cancer (Pfohl -Leszkowicz *et al.*, 1995). However, further conclusions with respect to the effect of dietary fish oil, fed during the initiation period, on the development of colorectal cancer are considered unjustifiable because of the absence of an enhancing effect of the positive control group.

Soya oil

Soya oil or soybean oil was introduced in the AIN[93] rodent diet by the American Institute of Nutrition as one of the measures to reformulate and improve the AIN[76] diet. A change in the fat source from 5% corn oil to 7% soya oil was recommended primarily to meet the requirements for an adequate amount and balance of both essential fatty acids, linoleic (ω-6) and linolenic (ω-3) acid (Reeves *et al.*, 1993).

It is shown in Chapter 4 that a high level of dietary soya oil, combined with either cellulose or RS, increased the incidence of colorectal cancer. Reports regarding effects of soya oil on colorectal cancer are scarce. Hirose *et al.* (1990) observed that the incidence and multiplicity of chemically induced colorectal tumours in DMH-treated rats fed diets with 10% soya oil were comparable with those fed diets with 10% safflower oil as fat source (in that study, 10% perilla oil had an inhibiting effect on colorectal carcinogenesis).

The mechanisms involved in the promotion of colorectal cancer by high-fat diets are still not fully understood. The energy content of the diet is generally accepted to be of great importance. A high energy intake has consistently been found to have an enhancing effect on carcinogenesis, whereas calorie restriction and physical activity are inversely related to the occurrence of cancer. There is no evidence that these factors have played a role in the experiments described in this thesis. The housing conditions, determining the possibility for physical activity, were the same for all animals. There were inevitable caloric differences between the various experimental diets. However, rats tend to eat according to their caloric needs and they had the opportunity to do so because they were fed *ad libitum*. Consequently, as anticipated, the animals fed the low-energy diets generally ate more than those fed the high-energy diets to meet their caloric requirements. As a result, the differences in energy intake between groups were limited and are not likely to be responsible for the differences in tumour yield.

For the digestion of dietary lipids bile acids are required. A small portion of the primary bile acids reaches the large intestines and can be transformed into secondary bile acids by the intestinal microflora. Secondary bile acids are cytotoxic and are thought to promote colorectal carcinogenesis. It can be assumed that for the digestion of high-fat diets more bile acids are required than for the digestion of low-fat diets. Consequently, higher amounts of bile acids may be expected in the large intestines. However, this was not the case in the experiment described in

Chapter 4. The different fat contents of the various diets did not result in significant differences in faecal bile acids. Hence, the promotional effect of the high-fat diets, at least that described in Chapter 4, must have been based on another mechanism.

High -fat diets have been shown to result in changes in activity of protein kinase C (Birt, 1986; Lafave *et al.*, 1994; Pajari *et al.*, 1997), a key enzyme in signal transduction and growth regulation (Murray *et al.*, 1999). Rao *et al.* (1996) proposed that dietary fat may influence colorectal carcinogenesis by changing the activity of colonic mucosal phospholipases A_2 and C, which play a role in the pathways for arachidonic acid release and formation of eicosanoids (prostaglandins and thromboxane B_2). Singh *et al.* (1997) reported that the expression of cyclooxygenase-2, an important enzyme in the prostaglandin biosynthesis, was up-regulated in animals fed a tumour promoting high-corn oil diet, whereas its expression was inhibited in animals fed a fish oil diet with antitumour effect. Ornithine decarboxylase (ODC) is an enzyme in the biosynthesis pathway of polyamines, which play a role in cell proliferation and differentiation. The activity of ODC is increased in the presence of high levels of secondary bile acids and can be influenced by different levels and types of fat (Kawamori *et al.*, 1995; Narisawa *et al.*, 1991; Rao and Reddy, 1993). Besides the above mentioned factors, other known as well as unknown factors may have played a role in the development of colorectal tumours in our experiments.

General conclusions with respect to the effects of fat on colorectal carcinogenesis in the experiments described in this thesis are far from straight-forward. High-soya oil diets clearly showed a promoting effect, even in the presence of tumour inhibiting RS. High-sunflower oil diets had a promoting effect as described in Chapter 2. However, this effect appeared to be not reproducible in another experiment (Chapter 4). As a direct result of the latter observation, it was not possible to interpret the effect of dietary fish oil fed during the initiation stage.

These results may seem disappointing. On the other hand, they show the vulnerability of conclusions drawn from seemingly very successful single experiments, and stress the importance of repeating experiments in order to verify results. They also indicate the importance of studies into the mechanism underlying a promoting or protective effect.

Effects of combinations of dietary fibre and fat

In the experiments described in Chapters 2 and 4 different levels of dietary fibre were combined with different levels of dietary fat. Such a design makes it possible to study whether fibre and fat influence each other's effects. It seems obvious to assume that inhibitory effects interfere with enhancing effects on colorectal cancer. In that case it could be expected, for example, that dietary fat has a promotive effect only in the absence of protecting factors, or that some types of fibre are only protective when the level of dietary fat is not too high. This view is confirmed by Slattery *et al.* (1997), who conducted a population-based case-control study of colon cancer in subjects from California, Utah and Minnesota in 1991-1994. A high-energy diet increased the risk of colorectal cancer. However, people who consumed a high-calorie diet dense in fibre and calcium appeared to be at lower risk than people with the same caloric intake who consumed smaller amounts of dietary fibre and calcium. In our experiments we also found indications of counteracting effects of fibre and fat. Diets high in cellulose, GOS and RS all resulted in markedly increased caecum weight, whereas the caecum weight was inversely related to the fat content of the diet. Enlargement of the caecum is an effect of dietary fibre, due to either increased bulking or stimulated fermenting activity or both. Dietary fat reduces the weight of the caecum, which may indicate that, apart from other mechanisms, the promoting effect of a high-fat diet is in part due to a counteracting effect on the protection by dietary fibre. Another example of a counteracting effect is that the high-GOS diets resulted in significantly higher amounts of caecal SCFA, whereas the SCFA levels were inversely related to the fat content of the diets (Chapter 3). Further, fermentable fibres can influence the effects of dietary fat in an indirect way by inhibiting the transformation from primary to secondary bile acids. A high fat diet increases the total of bile acids in the gut. Fibres with stool bulking effect dilute the concentration of bile acids, which was observed by Weisburger *et al.* (1983) and confirmed by our results presented in Chapter 4. Munakata *et al.* (1995) found that fat absorption was suppressed in rats fed diets containing 15% cellulose or 15% pectin compared with rats fed a fibre free diet. As described in Chapter 4, high dietary cellulose increased the incidence of adenomas when combined with low fat, and of carcinomas and total tumours when combined with high fat, suggesting that the promoting effects of cellulose and fat were additive.

Based on the above reasoning, it is tempting to draw general conclusions with respect to the effect of combinations of dietary fibre and fat on the development of colorectal cancer. However, our results indicate that inconsistencies can occur. In Chapter 4 it is shown that the tumour incidence was decreased only when high RS was combined with low fat, but not with high fat, whereas the tumour multiplicity was decreased when HRS was combined with high fat, but not with low fat. In that experiment the high-fat diets resulted in an increased incidence of carcinomas in the high-cellulose group despite faecal bulking and increased concentrations of caecal SCFA, which have potentially counteracting effects. Knowing that a high-fat diet sometimes may fail to promote colorectal cancer, as we demonstrated in Chapter 5, it may even be questioned whether it is possible to draw conclusions concerning the effects of a combination of fibre and fat at all. The above-mentioned inconsistencies demonstrate that unknown factors apparently can play a role in the carcinogenic process.

In conclusion, the results of our experiments and data from literature are in favour of the view that diets low in fat and high in fermentable fibre have protective properties against colorectal cancer. However, one should be cautious to generalise this view and bear in mind that unknown factors may govern the final effect of the diet on colorectal cancer.

Aberrant crypt foci

Since the first description of ACF by Bird (1987) many studies have been conducted to assess their potential preneoplastic nature, biological significance and relationship to colorectal cancer. Some investigators have questioned the hypothesis that ACF are preneoplastic lesions and suggested that ACF and colorectal tumours possibly represent two parallel independent events as a consequence of cancer initiation (Thorup *et al.* 1994). A few years later Thorup (1997) published a study indicating that ACF are preneoplastic lesions with the potential to progress to tumours. However, it was not possible to identify particular characteristics of ACF indicating specific proneness to tumour development. Genetic alterations, such as mutations of APC and K-ras, proven to be important genetic events implicated in colorectal carcinogenesis have been found in ACF

(Losi *et al.*, 1996; Smith *et al.*, 1994; Stopera and Bird, 1992; Stopera *et al.*, 1992). Mutations in p53, characteristic for the later stages of carcinogenesis are virtually absent in ACF (Shivapurkar *et al.*, 1997; Yamashita *et al.*, 1995), but if present, they are associated with large ACF (Stopera and Bird, 1993), which are considered to have better predictive value for the development of colorectal cancer compared with small ACF. At present, there is little dispute about the view that ACF are preneoplastic lesions. In numerous experiments ACF have been studied for their predictive value for the development of colorectal cancer. A reliable short-term screening assay which can be used to identify compounds with modifying effects on colorectal cancer would be very valuable. Such an assay should demonstrate a consistent positive correlation between certain features of ACF and ultimate tumour yield.

Up to now, published reports have failed to demonstrate a consistent correlation between effects on the development of ACF and colorectal tumour yield in rodents. The same holds true for humans. Dolara *et al.* (1997) searched ACF with a magnifying endoscope in human colorectal cancer patients and controls after staining the colon mucosa with methylene blue. ACF could be found in all subjects and there was no difference in density. These investigators concluded that this technique failed to provide a way to identify individuals at either low or high risk of colorectal cancer.

The first reports were focused on the total number of ACF. When it became clear that this was not a consistent predictive parameter, it was suggested that the number of large ACF (for example 4 or more aberrant crypts per focus) may be more predictive (Lafave *et al.*, 1994; Shirtliff and Bird, 1996). This parameter also proved to be inconsistent. Next, detailed microscopic examination of ACF (Bouzourene *et al.*, 1999; Di Gregorio *et al.* 1997) revealed that they have a heterogenous morphology. This observation led to the postulate that only a specific subset of ACF, demonstrating dysplasia, represents an early step in colorectal carcinogenesis. Other ACF are regarded innocent bystander lesions and irrelevant for cancer development. Though scientifically probably valid, the practical value of this observation is limited because it cannot easily be applied in a screening test. Microscopic examination of ACF is very time consuming, because there are so many of them. Furthermore, it is difficult to obtain a proper orientation of the crypts in the histological slides, which is necessary in order to make a correct diagnosis.

Perhaps even more important is the problem that carcinogenesis is a dynamic process, whereas observations can only be made at fixed time points. This raises the question at what time point following carcinogen exposure the ACF should be examined. ACF seem to be studied at arbitrary time points, somewhere between the moment of first appearance of ACF and the moment colorectal tumours appear. We have never seen reports where the time point of examination was based on a rationale, most probably because a valid rationale simply does not exist. Moreover, it is feasible that it is impossible to determine the time point at which ACF have 'the best predictive value', because it may depend on the compound tested. Different compounds probably have a variety of mechanisms determining i) the moment ACF first appear, ii) the moment they start to regress or become dysplastic and iii) their growth rate. Consequently, if these mechanisms are unknown, the best time point for the ACF assay cannot be established.

In a series of experiments described in Chapters 3–6, we investigated whether the number or size (determined at arbitrary time points!) of chemically induced ACF correlated with the ultimate yield of colorectal tumours. In these experiments the animals were fed diets with a variety of compounds with possible modifying effects on colorectal carcinogenesis. We found positive correlations, negative correlations as well as absence of a correlation between ACF and colorectal tumours.

In *conclusion*, our results clearly demonstrate that effects of dietary factors on the number and size of ACF have no predictive value for the effects of these factors on the development of colorectal cancer.

Conclusions

The results of the studies described in this thesis allow the following conclusions:

GOS, RS and WB, which are all fermentable fibres, had protective properties against colorectal carcinogenesis. In contrast, the non-fermentable fibre cellulose generally had no effect or an enhancing effect on carcinogenesis, although the development of adenocarcinomas was suppressed in one study. Regarding the proposed mechanisms of dietary fibre, it was concluded that the protective effects of the fermentable fibres were most probably associated with an increased

production of SCFA in the caecum and a decreased caecal pH. In case of WB, faeces bulking may have added to the protective effect.

Dietary cellulose generally did not confer protection despite marked faeces bulking, which led to the conclusion that faeces bulking as sole factor may fail to protect against the development of colorectal cancer.

The level of dietary GOS during the initiation phase was not relevant for the ultimate tumour yield. Therefore, it was concluded that the protective effect of GOS was exerted during the promotion phase.

Based on the results of two studies it was concluded that high levels of dietary sunflower oil and soya oil had a promoting effect on colorectal carcinogenesis. The promotive effect of the high-sunflower oil diet was possibly related to an increased proliferative activity in colonic crypts. Unexpectedly, the promoting effect of a high-sunflower oil diet could not be reproduced in another study, for unknown reasons. This finding resulted in the inability to draw a conclusion concerning potential effects of dietary fish oil on colorectal carcinogenesis, since the high-sunflower oil group served as positive control. On the other hand, the reduced formation of DNA adducts by dietary fish oil may be an indication of a suppressive effect of fish oil on carcinogenesis at an early stage.

We showed that the combination of fermentable fibre with protecting properties and fat with promoting properties resulted in counteracting effects on colorectal carcinogenesis. However, generalisation of this finding would be an over-simplification of the complex processes taking place during carcinogenesis. Even under well controlled conditions, unknown factors may determine the final effect of the diet on the development of colorectal cancer. Nonetheless, we feel that it is justifiable to state that diets low in fat and high in fermentable fibre most probably have protective properties against colorectal cancer.

In a series of experiments we investigated the predictive value of ACF for colorectal cancer. The results clearly demonstrated that effects of dietary factors on the number and size of ACF had no predictive value for the effects of these factors on the development of colorectal cancer. Therefore, it was concluded that ACF are not suitable as biomarker for colorectal cancer.

Investigation of the influence of various dietary compounds on the expression of a series of genes led to the conclusion that the expression of TIMP-1 in colorectal tissue may be a biomarker for colorectal cancer.

Closing remarks and ideas for future research

It is generally accepted that carcinogenesis is a multihit/multistep process in which a tumour can develop after exposure of cells to an initiator, followed by exposure to a promoter. This results in an accumulation of mutations in specific genes controlling cell division, apoptosis and DNA repair. A logic conclusion would be that a reduced risk of cancer can be accomplished by avoiding exposure to initiators and promoters. Of the avoidable risk factors, tobacco smoking is without doubt the most important one. However, initiating factors are numerous and cannot be avoided in real life, for example exhaust fumes, background atmospheric radiation and food components. Furthermore, spontaneous mutations occur beyond any control and also add to the risk of cancer. This means that life itself is a risk factor, simply because people have to breathe and eat. Individuals with a family history of colorectal cancer will be even at greater risk if they eat a high-risk diet (Little and Faivre, 1999). Thus, complete avoidance of exposure to initiators and promoters is impossible. Nevertheless, if it is possible to avoid a risk factor, there is every reason to do so.

It is clear that no single dietary factor or mechanism can account for all colorectal cancer. Moreover, different factors interact and enhance or counteract each others effects in a complex way irrespective of the mechanisms involved. Results from epidemiological and animal studies and promising results of dietary interventions in high-risk patients show that the development of several cancer types, specifically colorectal cancer, can be influenced by dietary factors. Therefore, dietary intervention strategies may be of great importance for the protection against this disease.

In the past decades the knowledge of mechanisms relating diet and risk of colorectal cancer has increased thanks to continuing investigations and technological innovations. The recent observations that insulin resistance and loss of epithelial barrier function may be determining factors in the risk of colorectal cancer (Bruce *et al.*, 2000) may provide interesting starting-points for new investigations.

A promising novel technology is the development of genomics. Genomics is the technique of investigating the expression of several thousands of genes gathered on a micro-array at the same time. The aim is to find combinations of gene expression

characteristic for certain conditions, such as alteration in tissues following exposure to a toxic or carcinogenic substance. Analyses of the tissue of interest may be confounded if samples are 'contaminated' by the presence of adjacent tissues. But at present, it is possible to isolate specific parts of a microscopic slide for analyses, using a new technique: laser capture microdissection. An example is given in Fig.7.1: using a laser beam, a selection of crypts is cut out from a cryosection of a rat colon and catapulted into a vial for further analyses. Single cells and even organelles from a single cell can be isolated.

For the application of these new techniques investigators are faced with a series of problems. To name a few: what is the optimal way of sampling tissues with minimal loss of RNA? How can minimal amounts of RNA be amplified to yield enough material for analyses? How should multiple changes in the expression of several thousands of genes be interpreted? What about statistics?

Without doubt, these problems will be solved in the near future and the new technologies will provide investigators with exciting and highly promising possibilities for cancer research.

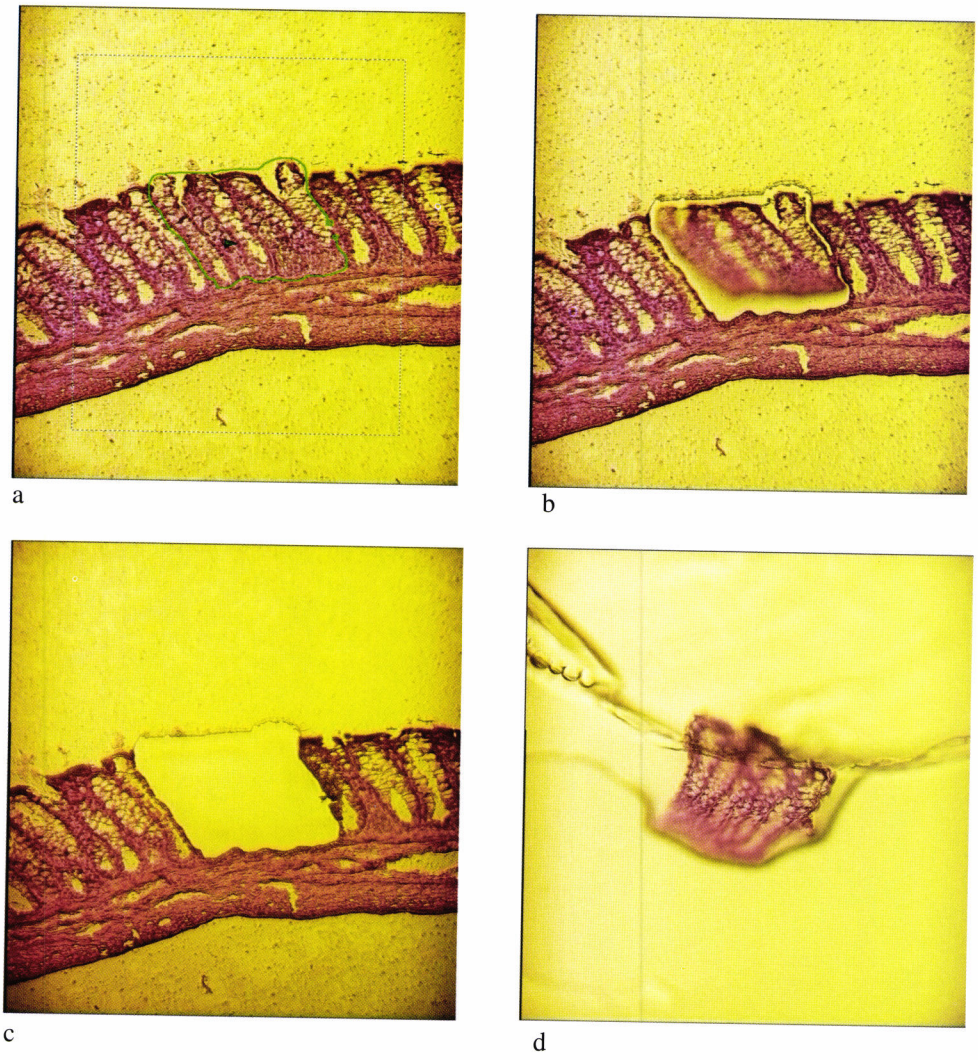

Figure 7.1
Selected crypts in a cryosection of a rat colon outlined with the computer cursor (a). The laser beam follows the outline, cutting the selection from the slide (b). The crypts are catapulted from the slide by a laser blast (c) into a vial (d).

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Summary

Dietary fibre and fat have been implicated as important factors in the etiology of colorectal cancer. In this thesis we investigated the effects of differrent types of fibre and fat on chemically induced colorectal tumours and their precursor lesions in rats.

In the General introduction **(Chapter 1)** the importance of colorectal cancer in humans and the factors which are considered to play a role in the etiology of this disease are presented. The anatomy and function of the colon and rectum are briefly described, as are the events taking place during the development from healthy tissue towards a malignant tumour. Considerable attention is paid to the morphology of ACF and the fact that ACF have been and still are subject of many investigations concerning the questions whether ACF are precancerous lesions, what role they have in the carcinogenic process and whether they are relevant biomarkers with predictive value for the development of colorectal cancer. The animal model used is described. Different types of fibre and fat are described and their possible effects on colorectal carcinogenesis, including the proposed mechanisms of these effects, are discussed. Finally, the objectives of the research described in this thesis are explained and the main questions addressed are presented.

These questions are as follows:

- Does the effect of dietary fibre depend on the type of fibre?
- Does the effect of dietary fat depend on the type of fat?
- Does the combination of fibre and fat influence the effect of the separate factors?
- Do the effects occur during the initiation or the promotion phase?
- Is the occurrence of ACF positively correlated with the occurrence of colorectal tumours, or in other words, are ACF reliable biomarkers for colorectal cancer?
- What are the mechanisms underlying the effects of fibre and fat?

The following experiments were conducted to answer the above questions:

Experiment 1

DMH-treated Wistar rats were fed diets with low or high levels of either *cellulose* (a non-fermentable fibre source), or *GOS* (a fermentable fibre source), combined with different levels of *sunflower oil*, for 9 months **(Chapter 2)**.

Generally, the tumour incidence increased with increasing fat content in the diet. Despite marked faeces bulking, dietary cellulose either had no effect or an enhancing effect on the formation of colorectal tumours in general, although the development of carcinomas was decreased. GOS appeared to be protective against the development of colorectal tumours, as was demonstrated by an inhibitory effect on tumour incidence, multiplicity and size, regardless of the fat content of the diet. The high-fat diets caused an increased BrdU labelling index in colon crypts. In animals fed high-GOS diets, the caecal content was significantly increased in weight and significantly decreased in pH.

It was concluded that tumorigenesis was enhanced by an increased fat content of the diet, and that the diets containing fermentable GOS conferred a greater protection against colorectal cancer than did the diets containing non-fermentable cellulose.

Experiment 2

In AOM-treated F344 rats, the effects of diets low or high in *GOS* on the development of ACF and colorectal tumours were studied **(Chapter 3)**.

The study was designed such that distinction could be made between possible effects being exerted during the initiation or the promotion phase. ACF were scored in subgroups of animals, 7 and 13 weeks after the start. Ten months after the start of the study the remaining animals were killed for scoring colorectal tumours. The aberrant crypt multiplicity, scored after 13 weeks, was predictive for the tumour outcome at the end of the study. However, all other ACF data had no predictive value. The colorectal tumour incidence in rats fed a high-GOS diet was significantly lower than in rats fed a low-GOS diet.

It was concluded that a high-GOS diet had a protective effect against the development of colorectal tumours in rats and that this protective effect was exerted during the promotion phase rather than during the initiation phase of carcinogenesis.

Additionally, in this chapter the results of SCFA measurements were presented. The analyses were conducted in the stored caecal contents sampled from animals of the experiment described in Chapter 2. The concentration of caecal SCFA (produced by bacterial fermentation of fermentable fibre) was significantly higher in high-GOS animals than in low-GOS animals, which was most probably the explanation for the inhibitory effect of GOS on colorectal tumour formation.

Experiment 3

In this study we investigated the effects of diets with low or high levels of either *cellulose* (a non-fermentable fibre source) or *RS* (a fermentable fibre source), combined with different levels of *soya oil*, on the development of ACF and colorectal tumours in AOM-treated F344 rats **(Chapter 4)**.

ACF were scored at 11 weeks. Ten months after the start of the study colorectal tumours were scored and caecal weight and pH, caecal SCFA and faecal bile acids were measured. Animals fed high-cellulose and high-fat diets developed more and larger ACF than animals fed low-cellulose and low-fat diets. RS had no effect on the development of ACF. In general, the development of colorectal tumours was enhanced in rats of the high-cellulose groups, but was inhibited in high-RS-fed animals. A high-fat diet resulted in an increased incidence of colorectal tumours, but had no effect on tumour multiplicity. Caecal weight and the concentration of SCFA were increased in animals fed the high-cellulose diets as well as in those fed the high-RS diets. The caecal pH was decreased in high-RS-fed animals and in low-fat/high-cellulose-fed animals.

It was concluded that colorectal carcinogenesis was enhanced by dietary cellulose and fat, and inhibited by dietary RS. The ACF score was predictive for the tumour outcome in the cellulose groups but not in the RS groups. Therefore, it was concluded that ACF are not suitable as biomarker for colorectal cancer.

Experiment 4

AOM-treated F344 rats were fed diets with low or high levels of *sunflower oil* or with a high level of *fish oil* **(Chapter 5)**.

The fish oil diet was fed only during the initiation phase. During post-initiation these rats were fed the high-fat diet (HFO/HF group). One day after the last AOM treatment DNA adducts were measured in liver and colon. ACF were counted in the colon 8 weeks after the diet switch. Colorectal tumours were scored 9 months

after the study start. The fish oil-fed animals demonstrated less DNA adduct formation in the liver and enhanced development of ACF in comparison with the sunflower oil group. There was no correlation between development of ACF and the occurrence of colorectal tumours. There were no statistically significant differences between the various groups with respect to incidence, multiplicity or size of colorectal tumours.

The lack of an enhanced development of colorectal tumours in the high-fat group, as compared with the low-fat group, was an unexpected result. Since the high-fat group was included to serve as a positive control group, it was considered unjustifiable to draw conclusions with respect to a potential effect on colorectal carcinogenesis of dietary fish oil fed during the initiation phase.

Experiment 5

The effects of diets with *WB, CUR, RUT* or *BIT* on the development of ACF and colorectal tumours were studied in AOM-treated F344 rats. ACF were counted after 7, 15 and 26 weeks. Tumours were scored after 26 weeks and 8 months. In addition the expression was studied of a selection of genes thought to be involved in colorectal carcinogenesis **(Chapter 6)**.

We found that the WB and CUR diets inhibited the development of colorectal tumours. In contrast, the RUT and BIT diets enhanced colorectal carcinogenesis. In addition, the various compounds caused different effects on the development of ACF. In most cases the number or size of ACF were not predictive for the ultimate tumour yield. The expression of some tumour-related genes was significantly different in tumours from the control group as compared to tumours from the treated groups.

It was concluded that WB and CUR, as opposed to RUT and BIT, protected against colorectal cancer. ACF are unsuitable as biomarker for colorectal cancer. The expression of TIMP-1 in colorectal tissue may be a valid biomarker for colorectal cancer.

In **Chapter 7**, the results of all experiments are summarised and discussed and the general conclusions are presented.

The main conclusions are:

- Fermentable fibres protected against colorectal cancer. The protective effects were most probably associated with an increased production of SCFA in the caecum and a decreased caecal pH.
- The non-fermentable fibre cellulose generally did not confer protection despite marked faeces bulking, which lead to the conclusion that faeces bulking as sole factor may fail to protect against colorectal cancer.
- Diets high in sunflower oil or soya oil showed a promoting effect on colorectal cancer; however, the effect of sunflower oil did not occur in another study.
- ACF are unsuitable as biomarker for colorectal cancer.
- It seems justified to state that diets low in fat and high in fermentable fibre most probably protect against colorectal cancer. However, unknown factors may determine the ultimate effect of the diet on colorectal cancer.

Finally, a case of a rare spontaneous rectum carcinoma in a Wistar rat is described in an **Appendix**.

Samenvatting

Inleiding

Dit proefschrift gaat over de invloed van vezel en vet in de voeding op het ontstaan van kanker in het colon en rectum (onderdelen van de dikke darm). In deze samenvatting wordt de in de Nederlandse volksmond gebruikelijke term 'colonkanker' gebruikt.

Voeding en colonkanker

Colonkanker is in de westerse wereld na long-, borst- en prostaatkanker de meest voorkomende soort kanker. In het algemeen wordt aangenomen dat ongeveer eenderde van alle vormen van kanker wordt veroorzaakt door roken, eenderde door de voeding en eenderde door overige oorzaken, bijvoorbeeld erfelijke factoren. Al decennia geleden is vastgesteld dat colonkanker in de westerse wereld veel vaker voorkomt dan in Azië en Afrika. Dit wordt toegeschreven aan verschillen in voedingsgewoonten. In de westerse wereld bevat de voeding gemiddeld weinig vezel en veel vet; in Azië en Afrika bevat de voeding veel vezel en weinig vet. Het gegeven dat de verhouding vezel/vet van invloed is op het risico van colonkanker moet genuanceerd worden. Er bestaan namelijk vele soorten vezel en vet. Sommige vezels blijken een beschermend effect te hebben, andere bieden nauwelijks of geen bescherming. Hetzelfde geldt voor vet. Van een aantal typen vet is bekend dat ze een promoverend effect op kanker hebben. Dat wil zeggen: vet is zelf niet kankerverwekkend, maar bij een vetrijke voeding is er een vergrote kans dat geïnitieerde cellen (pre-kankercellen) gestimuleerd worden om uit te groeien tot een tumor. Dit geldt in het algemeen voor dierlijke vetten, behalve voor visolie dat beschermend blijkt te werken tegen diverse vormen van kanker. Plantaardige vetten kunnen eveneens verschillende effecten hebben op de ontwikkeling van tumoren.

Zeer illustratief voor de invloed van voeding op kanker zijn migratiestudies: zo werd vastgesteld dat de incidentie van colonkanker, die bij Japanners laag is, significant toenam bij de Japanse populatie die was geëmigreerd naar de Verenigde Staten. Dit was terug te voeren op het feit dat deze mensen geleidelijk de westerse eetgewoonten hadden overgenomen.

Hoe kan de relatie tussen voeding en colonkanker onderzocht worden?

Er is veel onderzoek gedaan naar de invloed van voeding op (colon)kanker bij de mens door epidemiologen. Epidemiologisch onderzoek kost veel tijd en geld. Sommige epidemiologische studies strekken zich uit over 10 jaar of langer. De onderzochte groep is doorgaans zeer omvangrijk en heterogeen. Met het laatste wordt bedoeld dat er vele verschillen zijn tussen mensen die invloed kunnen hebben op de resultaten van het onderzoek, bijvoorbeeld verschillen ten aanzien van leeftijd, geslacht, arbeidsomstandigheden, leefomgeving, wel of niet roken, enz. Bij de verwerking van de resultaten kan voor al die factoren weer gecorrigeerd worden. Dit maakt dit type onderzoek ingewikkeld, maar een groot voordeel is dat de mens zelf onderzocht wordt.

Een andere manier van onderzoek is het werken met diermodellen. De meest gebruikte diersoort is de rat. Binnen betrekkelijk korte tijd (minder dan 1 jaar) kan onder goed gecontroleerde condities veel informatie verkregen worden. Proefdieren zijn homogener, correctiefactoren spelen geen rol en het is mogelijk om rechtstreeks het effect op het ontstaan van kanker te bestuderen door de mogelijkheid kanker te induceren met chemische carcinogenen (kankerverwekkende stoffen). Een tekortkoming blijft natuurlijk dat het dieren betreft waardoor de geldigheid van resultaten voor de mens een punt van discussie is.

Colon en kanker

Hieronder volgt een korte beschrijving van de anatomie en functie van het spijsverteringsstelsel, en van het proces dat tot kanker leidt.

Het voedsel komt via de slokdarm in de maag terecht. Daar vindt voorvertering plaats door het maagsap. Vanaf de maag wordt de voedselbrok verder getransporteerd en passeert daarbij achtereenvolgens de dunne darm en de dikke darm. De dunne darm bestaat uit het duodenum (twaalfvingerige darm), het jejunum en het ileum. De dikke darm bestaat uit het caecum (blinde darm), het colon en het rectum.

In de dunne darm worden koolhydraten, eiwitten en vetten door enzymen afkomstig van de alvleesklier en van cellen die de dunne darm bekleden verteerd, d.w.z. gesplitst in moleculen die klein genoeg zijn om door de darmwand te kunnen worden opgenomen. Voor de vetvertering zijn behalve enzymen ook galzouten nodig die geproduceerd worden door de lever en via de galgang in het duodenum

worden uitgescheiden. De dunne darm is de plaats waar het overgrote deel van de voedingscomponenten wordt opgenomen door het lichaam.

De dikke darm verzorgt het verdere transport van de inhoud. Dit deel van de darm bevat geen verteringsenzymen maar is in tegenstelling tot de dunne darm rijk aan bacteriën. Deze zijn in staat om bepaalde bestanddelen van de darminhoud te fermenteren (om te zetten). Ook vanuit de dikke darm worden stoffen opgenomen in het lichaam, bijvoorbeeld sommige vitaminen, bacteriële fermentatieproducten en water. Tenslotte verlaten de restanten het lichaam via de anus.

De opbouw van de wand van de dikke darm wordt in Hoofdstuk 1 met enkele foto's geïllustreerd (Fig.1.1). De darmwand bestaat uit diverse lagen bindweefsel en spierweefsel. De binnenkant is bekleed met slijmvlies waarvan de buitenste laag bestaat uit een enkele laag epitheelcellen, die in feite de enige barrière met de buitenwereld vormen. Het slijmvlies heeft zgn. crypten; dit zijn doodlopende buisvormige voortzettingen van het slijmvlies die loodrecht de darmwand ingaan en met epitheelcellen bekleed zijn. In de onderste helft van de crypten vindt voortdurend celdeling plaats. De nieuw gevormde cellen schuiven langzaam op naar boven, sterven daar op zeker moment af en worden dan in de darmholte afgestoten. Deze cyclus duurt enkele dagen en zorgt ervoor dat de bekleding van de darm continu vernieuwd wordt.

De dikke darm behoort tot de organen waarvan de cellen een relatief hoge delingssnelheid hebben. Daarbij staan de bekledende cellen in nauw contact met de buitenwereld, in dit geval een massa voorbijschuivende darminhoud, waarin potentieel schadelijke stoffen kunnen zitten. Dit kunnen stoffen zijn die rechtstreeks met de voeding zijn opgenomen, stoffen die door de lever uit het bloed zijn gefilterd en via de gal in de darm zijn uitgescheiden, of stoffen die in de darm ontstaan uit andere componenten, bijvoorbeeld door bacteriële omzetting. De combinatie van hoge delingssnelheid en contact met schadelijke stoffen maakt het colon vrij kwetsbaar voor het ontstaan van kanker. Immers, de eerste fase van kanker (de initiatiefase) is schade aan het erfelijke materiaal van een cel, het DNA. Bij frequente deling is de kans op schade groter. Wanneer de schade niet wordt hersteld door het lichaam kan een dergelijke cel ontsporen en ongebreidelde groei gaan vertonen. De vroegst zichtbare veranderingen worden *aberrant crypt foci* (ACF) genoemd (zie Fig.1.2a,b,c in Hoofdstuk 1). ACF zijn aangetoond bij zowel mensen als ratten en worden algemeen beschouwd als voorstadia van tumoren. Herstel is nog steeds mogelijk, maar enkele ACF kunnen verder uitgroeien en echte

tumoren vormen. Zolang die lokaal blijven zijn ze goedaardig: adenomen. Deze kunnen echter ontaarden in kwaadaardige tumoren: carcinomen, gekenmerkt door de eigenschap dat ze het omgevende weefsel infiltreren of doordat kankercellen via het bloed uitzaaien (metastaseren) naar andere organen (zie Fig. 1.2e,f in Hoofdstuk 1). Voordat het zover komt is het noodzakelijk dat de beschadigde cel na de initiatie aan een reeks schadelijke prikkels wordt blootgesteld die er voor zorgt dat uiteindelijk kanker ontstaat. Deze fase van de carcinogenese (het hele proces dat leidt tot kanker) wordt de promotiefase genoemd.

Het proefschrift

In dit proefschrift zijn de resultaten van onderzoeken beschreven die gericht waren op de invloed van vezel en vet in de voeding op het ontstaan van colonkanker bij ratten. Er is gebruik gemaakt van diermodellen, waarbij bij ratten colontumoren werden geïnduceerd door ze een aantal keren middels een onderhuidse injectie een carcinogeen toe te dienen. De dieren werden in groepen ingedeeld die verschillend voer kregen. Na 8-10 maanden werd nagegaan in hoeverre de verschillende diëten van invloed waren geweest op het ontstaan van colontumoren. Hierbij is geprobeerd om de diëten zoveel mogelijk alleen van elkaar te laten verschillen ten aanzien van de onderzochte voedingscomponent.

Aan het eind van elke studie werden de dieren gedood. Hun colon werd verwijderd, in de lengterichting opengeknipt en onderzocht op de aanwezigheid van tumoren (zie Fig.1.2d in Hoofdstuk 1). De tumoren werden microscopisch beoordeeld om vast te stellen of het adenomen of carcinomen betrof. De tumorscore werd weergegeven als *incidentie*: het percentage dieren met (een) tumor(en), en als *multipliciteit*: het aantal tumoren per tumordragend dier. Daarnaast werd de doorsnede van de tumoren gemeten. De resultaten werden steeds statistisch geanalyseerd om vast te stellen of waargenomen verschillen significant waren.

In enkele studies werd ook onderzoek verricht naar de invloed van de verschillende diëten op het ontstaan van DNA adducten (veranderingen van het erfelijk materiaal), op de expressie van een aantal genen (het functionele product van het erfelijk materiaal), of op het ontstaan van ACF.

Gebruikte vezels en vetten

Voedingsvezel is van oudsher gedefinieerd als de bestanddelen van plantencellen die niet verteerbaar zijn in het darmstelsel van de mens. Het wordt ook wel aangeduid als ballast. In de loop der tijd is deze definitie aan verandering onderhevig geweest, vooral door verbeterde analysetechnieken en groeiende kennis over het functioneren van het darmstelsel. De huidige definitie van voedingsvezel luidt: bestanddelen van plantaardige oorsprong die niet gevoelig zijn voor enzymatische vertering in de dunne darm, maar eventueel wel gefermenteerd kunnen worden door bacteriën in de dikke darm.

De gebruikte vezels waren: cellulose, galacto-oligosacchariden, onverteerbaar zetmeel en tarwezemelen. Cellulose is afkomstig van plantaardige celwanden en is noch verteerbaar, noch fermenteerbaar. Galacto-oligosacchariden, onverteerbaar zetmeel en tarwezemelen zijn onverteerbaar in de dunne darm, maar wel fermenteerbaar in de dikke darm. Galacto-oligosacchariden bestaan uit een mengsel van koolhydraten. Interessant is dat ze zijn afgeleid van wei, een restproduct van de kaasbereiding, dus van dierlijke herkomst. Echter, gezien de eigenschappen van dit product past het in de definitie van voedingsvezel. Zetmeel is een koolhydraat dat normaal gesproken goed verteerbaar is in de dunne darm. Onder bepaalde omstandigheden verdwijnt deze eigenschap, bijvoorbeeld wanneer men gekookte aardappelen laat afkoelen. Hierdoor verandert de vorm waarin het zetmeel aanwezig is waardoor de verteringsenzymen er geen vat meer op hebben. Dit wordt onverteerbaar zetmeel (Engels: *resistant starch*) genoemd. Tarwezemelen zijn in feite datgene wat overblijft van tarwekorrels nadat het zetmeel eruit gehaald is (Engels: *wheat bran*).

De volgende typen vet zijn gebruikt: zonnebloemolie, sojaolie en visolie.

Kernvragen in het proefschrift zijn:

- Wat is het effect van de gebruikte vezels en vetten op het ontstaan van colontumoren?
- Welk mechanisme ligt aan dat effect ten grondslag?
- Hebben ACF een voorspellende waarde voor het ontstaan van colontumoren?

Het laatste behoeft een toelichting. Ondanks het feit dat de diermodellen hun waarde hebben bewezen zijn onderzoekers al geruime tijd op zoek naar mogelijkheden om met minder dieren in kortere tijd de invloed van

voedingsfactoren op het ontstaan van colonkanker te kunnen bestuderen. De in 1987 voor het eerst door Bird beschreven ACF leken veelbelovend. Zij kunnen eenvoudig en binnen enkele weken worden geïnduceerd door de dieren met een carcinogene stof te behandelen. ACF zijn voorstadia van tumoren, hoewel slechts enkele daadwerkelijk uitgroeien tot een tumor. Uit onderzoek is gebleken dat het ontstaan en verder ontwikkelen van ACF in veel gevallen beïnvloedbaar is door de voeding. Hiermee leek een snelle screeningmethode beschikbaar te zijn gekomen. Immers, als een stof de ontwikkeling van voorstadia van tumoren remt of stimuleert dan zullen er waarschijnlijk ook respectievelijk minder of meer tumoren ontstaan. ACF zouden in dat geval beschouwd kunnen worden als een zgn. biomarker. De literatuur vermeldt echter tegenstrijdige resultaten, waardoor de meningen hierover zijn verdeeld.

De onderzoeken

Invloed van cellulose op het ontstaan van colontumoren

Uit de resultaten van de onderzoeken beschreven in de **Hoofdstukken 2** en **4** blijkt dat cellulose in het algemeen een promoverend effect had op de ontwikkeling van colontumoren, hoewel de ontwikkeling van carcinomen in het eerste onderzoek was geremd. Dat kwam mogelijk door een verminderde ontaarding van adenomen in carcinomen.

Cellulose wordt niet verteerd, noch gefermenteerd. Dit resulteert in een sterke toename van de faecesproductie en een snellere passage door de darm. Aan dit verschijnsel wordt in het algemeen een beschermende werking toegekend. Het idee is dat schadelijke stoffen worden verdund en door de snelle passage minder kans hebben om schade aan te richten. Uit de onderzoeken blijkt dat dit effect van cellulose niet tot bescherming heeft geleid. Misschien komt dat wel omdat ook stoffen met een gunstig effect worden verdund en sneller worden uitgescheiden.

Invloed van galacto-oligosacchariden, onverteerbaar zetmeel en tarwezemelen op het ontstaan van colontumoren

Deze vezels zijn alle niet verteerbaar in de dunne darm maar wel fermenteerbaar in de dikke darm. Uit de resultaten van de onderzoeken beschreven in de

Hoofdstukken 2, 3, 4 en **6** blijkt dat alle drie vezeltypen een remmend effect hadden op de ontwikkeling van colontumoren.

Bij de fermentatie worden kortketen vetzuren gevormd en de zuurgraad van de darminhoud daalt. De kortketen vetzuren, in het bijzonder boterzuur, hebben een remmend effect op de carcinogenese. Primaire galzouten, die in geringe hoeveelheid vanuit de dunne darm in de dikke darm terechtkomen kunnen door bacteriën worden omgezet in secundaire galzouten, waarvan is aangetoond dat ze schadelijk zijn voor de darmcellen. Door de lagere zuurgraad wordt dit proces geremd.

Invloed van zonnebloemolie, sojaolie en visolie op het ontstaan van colontumoren

Uit de resultaten van de onderzoeken beschreven in **Hoofdstukken 2** en **4** blijkt dat zonnebloemolie en sojaolie een promoverend effect hadden op de ontwikkeling van colontumoren.

Het onderzoek beschreven in Hoofdstuk 5 leidde tot een onverwacht probleem. Het doel van dat onderzoek was om te bestuderen wat het effect van visolie in de voeding op de carcinogenese zou hebben wanneer het alleen tijdens de initiatiefase zou worden gegeven. Als vergelijking waren twee extra groepen meegenomen: de ene groep kreeg voer met een laag, de andere voer met een hoog percentage zonnebloemolie, waarvan eerder was gezien (**Hoofdstuk 2**) dat het een promoverend effect had. Totaal onverwacht bleek het hoog-vet voer in dit experiment geen promoverend effect te hebben. Er bleek helemaal geen verschil te zijn tussen de 3 groepen ten aanzien van de incidentie, multipliciteit of grootte van colontumoren. Het gevolg hiervan was dat het onmogelijk was het effect van het visolie-dieet te interpreteren. Toch is dit experiment niet geheel mislukt. Het benadrukt dat carcinogenese een ingewikkeld proces is, dat op vele manieren kan worden beïnvloed. Er kunnen altijd onbekende factoren een rol spelen, die het uiteindelijke resultaat bepalen. Daarom is het zo belangrijk te zoeken naar de werkingsmechanismen van beïnvloedende factoren en is het nodig onderzoeken te herhalen om resultaten te verifiëren.

Overigens werden bij de dieren die visolie hadden gekregen minder DNA adducten gevormd dan bij de andere dieren. Dit werd in een vroeg stadium van de studie gemeten en zou kunnen wijzen op een beschermend effect van visolie.

Combinaties van vezel en vet

Zoals hierboven is aangegeven kunnen vezel en vet tegengestelde effecten hebben op de carcinogenese. Bij combinatie zou je bijvoorbeeld kunnen verwachten dat het beschermende effect van vezel beter is in combinatie met weinig vet, terwijl de bescherming minder is of misschien wel teniet wordt gedaan in combinatie met veel vet. Helaas is het niet zo eenvoudig dat dit altijd opgaat. Ook hier geldt dat vele (onbekende) factoren het uiteindelijke resultaat mede bepalen.

Aberrant crypt foci

In 4 studies, beschreven in de **Hoofdstukken 3 t/m 6**, werd de voorspellende waarde van ACF voor het uiteindelijke ontstaan van tumoren bestudeerd. Uit de resultaten bleek dat er geen sprake was van een consistente correlatie tussen de ontwikkeling van ACF en het voorkomen van colontumoren. De conclusie is eenduidig: ACF zijn niet bruikbaar als biomarker voor colonkanker. Deze uitspraak geldt in elk geval voor zover het gaat om het aantal en de omvang van de ACF. Het is niet uitgesloten dat een selecte groep ACF wel voorspellende eigenschappen heeft, maar vooralsnog is deze nog niet gekarakteriseerd.

Conclusies

De belangrijkste conclusies van dit proefschrift zijn:

- De fermenteerbare voedingsvezels (galacto-oligosacchariden, onverteerbaar zetmeel en tarwezemelen) lieten een beschermend effect op de ontwikkeling van colontumoren zien. Dit hing waarschijnlijk samen met een verhoogde productie van kortketen vetzuren en een lagere zuurgraad in de dikke darm.
- Cellulose had in het algemeen geen beschermend effect ondanks overduidelijke toename van faecesproductie. Het gunstige effect van een grote faecesproductie is dus discutabel.
- Zonnebloemolie en sojaolie hadden een promoverend effect op colonkanker, hoewel het effect van zonnebloemolie in een tweede studie niet werd bevestigd.
- ACF zijn niet bruikbaar als biomarker voor colonkanker.

- Het lijkt gerechtvaardigd te stellen dat een vetarm dieet, rijk aan fermenteerbare vezels, een beschermend effect heeft op het ontstaan van colonkanker. Echter, onbekende factoren kunnen voor verrassingen zorgen.

Tenslotte

Appendix

Aan het einde van het proefschrift is een appendix opgenomen waarin een geval is beschreven van een spontaan rectumcarcinoom bij een rat, een zeer zeldzame bevinding.

Vanwege de zeldzaamheid van tumoren in de dikke darm bij de rat bestaat er geen gevaar dat onderzoeksresultaten worden verstoord door oncontroleerbare 'spontane gevallen'. Dat betekent dan ook dat het niet nodig is om bij de diermodellen die gebruikt worden in het onderzoek naar colonkanker extra controlegroepen te laten meelopen, die niet met carcinogeen behandeld zijn.

Nawoord

De groeiende kennis op het gebied van de relatie tussen voeding en kanker kan de mens helpen keuzes te maken t.a.v. zijn voedingspatroon om zo weinig mogelijk risico te lopen op ernstige voedingsgerelateerde ziekten. Hierbij kan men soms op het verkeerde been worden gezet; de euforie van de ontdekking dat het nuttigen van meervoudig onverzadigde vetzuren de kans op hart- en vaatziekten zou kunnen verkleinen werd enigszins getemperd door de minstens zo belangrijke ontdekking dat dit type vetzuren het ontstaan van tumoren zou kunnen bevorderen. Je kunt er voor kiezen om wel of niet te roken, maar eten moeten we hoe dan ook. Het veiligst lijkt het om in elk geval bekend risicovol voedsel te vermijden en zo gevarieerd mogelijk te eten. Tenslotte neme men een klassieke uitdrukking ter harte die de oude Griekse Lyrici al hebben bedacht: *μηδέν άγαν* (spreek uit als *midén ágan*), hetgeen zoveel betekent als 'in niets te veel', ofwel 'alles met mate'.

Curriculum vitae

De auteur werd geboren op 5 november 1957 te Heerlen. In 1975 behaalde hij aan het Grotius College te Heerlen het gymnasiumdiploma. In 1977 begon hij met de studie Diergeneeskunde aan de Faculteit Diergeneeskunde van de Rijksuniversiteit Utrecht. In 1986 werd het diploma Dierenarts, differentiatie Gezelschapsdieren behaald. Na enige tijd in de veterinaire praktijk werkzaam te zijn geweest was hij van 1988 tot 1989 wetenschappelijk medewerker bij de Afdeling Bijzondere Dieren van de Vakgroep Pathologie van de Utrechtse Faculteit Diergeneeskunde. In 1990 trad hij als patholoog in opleiding in dienst bij de Sectie Pathologie van de Afdeling Biologische Toxicologie van het Centraal Instituut voor Voedingsonderzoek te Zeist, onderdeel van de Nederlandse Organisatie voor Toegepast Natuurwetenschappelijk Onderzoek (TNO). In 1994 werd hij door de Nederlandse Vereniging voor Pathologie erkend als toxicologisch patholoog. In 1997 werd hij door de Nederlandse Vereniging voor Toxicologie erkend als toxicoloog. De werkgever is nog steeds dezelfde, maar inmiddels omgedoopt tot TNO Voeding, Afdeling Algemene Toxicologie. Hier is de auteur werkzaam als toxicologisch patholoog en projectleider van studies in het kader van protocoltoxicologie en veiligheidsfarmacologie. Daarnaast voert hij nog enkele veterinaire taken uit. Bij TNO Voeding werd ook het onderzoek, beschreven in deze dissertatie, uitgevoerd.

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Bedankt!

Zo, het ei is gelegd. Dit is eindelijk de laatste pagina die ik voor het proefschrift schrijf. Ik wil in dit stukje de vele mensen bedanken die er aan hebben bijgedragen.
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Tenslotte mijn lieve Mariëtte, bedankt voor je steun bij de laatste loodjes.

Marcel

Appendix

Spontaneous adenocarcinoma of the rectum in a male Wistar rat

M.V.W. Wijnands and R.A. Woutersen

TNO Nutrition and Food Research
Department of General Toxicology

Submitted for publication.

Abstract

A male Wistar rat, about 5 months of age, exhibited a tumour of the rectum. Upon microscopical examination the tumour was diagnosed a moderately differentiated tubular adenocarcinoma showing infiltrative growth. This is the first report of a spontaneous rectum carcinoma in a Wistar rat.

Introduction

The animal was one of 96 male Wistar:WU, outbred rats of the F0-generation from an oral standard two-generation reproduction toxicity study. The rat was a high-dose animal, and was 5 months old when killed according to schedule. At necropsy, the rectum showed a local thickening at the serosal side, just cranially of the anus. After cutting the rectum, the thickening was seen to protrude slightly into the lumen of the rectum. The diameter was about 0.5 cm. Despite the size and location of the lesion, no defecation problems or other clinical signs had been noticed.

In order to determine whether or not this highly unusual observation was a fortuitous finding not related to treatment, the rectum of all other 23 top-dose males were examined macroscopically and microscopically. Samples were preserved in a neutral, aqueous, phosphate-buffered, 4% solution of formaldehyde. The tissues were embedded in paraffin wax, sectioned at 5 µm and stained with haematoxylin and eosin for examination by light microscopy.

Results

Upon microscopy, the lesion turned out to be a tumour with a cauliflower-like growth. It did not have a pedicle, but rested on a wide base and slightly protruded into the lumen of the rectum (Fig. A1). The tumour had a glandiform structure with tubular cavities of varying, often irregular shape, lined with cylindrical, cuboidal, or flattened epithelium arranged in one or several layers. Cell nuclei were large, polymorphic and hyperchromatic. Mitotic figures were frequent. The tumour infiltrated locally, as seen from growth through the muscularis mucosae into the

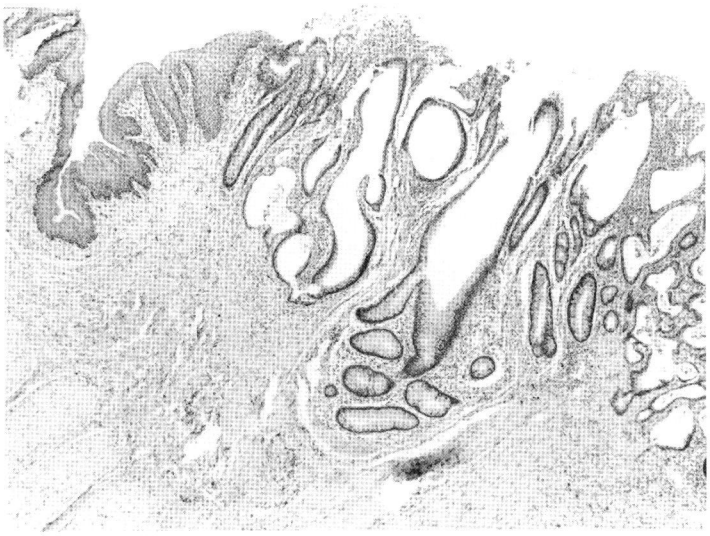

Figure A1. Carcinoma of the rectum, just cranially of the stratified squamous epithelium of the anal canal. H&E, 50x.

submucosa. In the stroma, fibroblasts and inflammatory cells were observed. The lumen of the atypical glandular structures contained amorphous material and some inflammatory cells.

Histopathological examination of the rectum of the other top-dose males was essentially negative. Therefore, it was concluded that the tumour observed was a spontaneous lesion, not related to treatment.

Discussion

Tumours of the colon and rectum frequently occur in man. Furthermore, (colo)rectal cancer is a notorious complication in patients with a prior history of radiation therapy for neoplastic diseases in the pelvic region, such as tumours of the female genital tract (Giacchero *et al.*, 1986; Sekine *et al.*, 1986).

Spontaneous tumours in the gastrointestinal tract of rats are extremely rare. From the beginning of the previous century hundreds of thousands of both wild and

laboratory rats have been examined for the occurrence of intestinal tumours, but only very few have been found and reported (Pozharisski, 1990). However, it cannot be excluded that intestinal tumours have been missed, because routine examination of the entire intestinal tract is not a common procedure. Spontaneous tumours of the intestinal tract in rats of various strains have been found in the caecum, colon, and less frequently in the small intestines (Crain, 1958; Gilbert and Gillman, 1958; Thompson *et al.*, 1961; Burn *et al.*, 1966; Schardein *et al.*, 1968; Maekawa and Odashima, 1975; Sass *et al.*, 1975; Miwa *et al.*, 1976; Goodman *et al.*, 1979; Pozharisski, 1990; Chandra and Frith, 1994). The majority of spontaneous tumours of the rat intestines are of epithelial origin. Most colon tumours are found in the proximal part (Miwa *et al.*, 1976). Colorectal tumours appear not to be particularly associated with old age. Burn *et al.* (1966) described colon tumours in two female Wistar Albino rats of about six months of age. Willis (1935) found colon carcinomas in two nine months old rats. The rat in the present case study was about five months old. Goodman *et al.* (1979) reported two spontaneous rectum neoplasms in F344 rats: an adenocarcinoma in a female, and a fibrosarcoma in a male. To our knowledge, up to now no spontaneous adenocarcinoma in the rectum of a Wistar rat has been reported.

Tumours of the colon and rectum in the rat can easily be induced by a variety of chemical carcinogens, such as 1,2-dimethylhydrazine and its metabolites azoxymethane or methylazoxymethanol acetate, methylnitrosourea, and methylnitronitrosoguanidine (Enker and Jacobitz, 1976; Rogers and Nauss, 1985). Histologic features of carcinogen-induced intestinal adenocarcinomas in rats show great similarity to those of spontaneous adenocarcinomas in rats, dogs, cats and man (Lingeman and Garner, 1972; Pozharisski, 1990).

Induced intestinal tumours in the rat can metastasize through invasion of lymphatic vessels. Metastases appear firstly in the regional lymph nodes (Pozharisski, 1990). Unfortunately, the regional lymph nodes of the present rat were not available for microscopical examination. Therefore, it was not possible to determine whether the tumour had metastasized. However, since there was clear infiltrative growth and great morphological similarity with experimentally induced colorectal tumours, this tumour at least had the potency to metastasize.

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Stellingen

1. De fermenteerbare voedingsvezels galacto-oligosacchariden, onverteerbaar zetmeel en tarwezemelen hebben, in tegenstelling tot het niet-fermenteerbare cellulose, een beschermend effect op de ontwikkeling van colontumoren bij ratten (dit proefschrift).

2. Een hoog sojaoliegehalte in de voeding heeft bij ratten een promoverend effect op colonkanker (dit proefschrift).

3. Het gunstige effect van versnelde darmpassage wordt waarschijnlijk overschat (dit proefschrift).

4. ACF zijn niet bruikbaar als biomarker voor colonkanker (dit proefschrift).

5. Onderzoeksresultaten kunnen op oncontroleerbare wijze beïnvloed worden door onbekende factoren (dit proefschrift).

6. Een negatief resultaat is even belangrijk als een positief resultaat.

7. Diet is a chronic source of both frustration and excitement to epidemiologists (Peto and Doll, 1981).

8. Gezien het feit dat het telefoonboek van pathologen doorgaans onder hun microscoop ligt heeft de introductie van e-mail het contact van pathologen met de buitenwereld bevorderd.

9. Iemand die als argument tegen een stelling de uitspraak 'dat kan ik mij niet voorstellen' bezigt, geeft slechts blijk van een gebrek aan voorstellingsvermogen.

10. Het is dat we niet zonder kunnen, maar slapen is absolute tijdverspilling.

11. Als het proces van volwassen worden inhoudt dat het vermogen om te spelen verloren gaat, dan hoopt de auteur dat hij nooit volwassen wordt.

Stellingen behorend bij het proefschrift
Effects of dietary fibre and fat on colorectal carcinogenesis in rats
Marcel V.W. Wijnands