INTERACTIVE EFFECTS BETWEEN DIETARY FAT AND A VEGETABLES-FRUIT MIXTURE ON COLORECTAL CARCINOGENESIS

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Jolanda M. Rijnkels

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van de Landbouwuniversiteit Wageningen,
Dr. C. M. Karssen,
in het openbaar te verdedigen
op dinsdag 10 maart 1998
des namiddags te vier uur in de Aula.

Cover : *Freeze-dried vegetables and fruit, photograph by Jolanda Rijnkels*
Printing : *Ponsen & Looijen bv, Wageningen*
ISBN : *90-5485-817-6*

These studies were funded by the Dutch Cancer Society (Konining Wilhelmina Fonds; KWF93-485). Financial support for the publication of this thesis by the Wageningen Agricultural University and the Dutch Cancer Society are gratefully acknowledged.

Stellingen

1. Een groenten-fruit mengsel, qua samenstelling en hoeveelheid overeenkomend met de gemiddelde samenstelling- en consumptiepatronen in Nederland en aanwezig in een hoog vetrantsoen, kan een darmtumor promoverende werking hebben, afhankelijk van het al dan niet gemuteerd zijn van het *Apc* gen.
   Dit proefschrift.

2. Bij onderzoek naar de invloed tussen voedingsfactoren op het ontstaan van dikke darmkanker dient rekening te worden gehouden met het feit dat de aard en de mate van het interactieve effect sterk kan worden beïnvloed door chemische carcinogenen en genetische manipulaties.
   Dit proefschrift.

3. Er zijn onvoldoende aanwijzingen dat voedingsvet een belangrijke bijdrage levert aan het ontstaan van darmkanker bij de mens.
   Howe, GR et al. (1997) Cancer Causes and Control 8, 215-228.

4. De theorie, gepostuleerd door Polly Matzinger, inhoudende dat het immuunsysteem reageert op het onderscheid gevaarlijk versus ongevaarlijk, in plaats van lichaamseigen versus niet lichaamseigen, verdient nadere onderbouwing aangezien het een impuls kan geven in de ontwikkeling van nieuwe therapieën ter bestrijding van kanker.
   Matzinger, P (1994) Annu Rev Immunol 12, 991-1045.
   Fuchs, Ej & Matzinger, P (1996) Semin-Immunol 8, 271-280.

5. De factor licht krijgt onvoldoende aandacht bij de beoordeling van toxische stoffen.

6. Wetende dat effecten van bepaalde voedingscomponenten afhankelijk zijn van de fase van het kankerproces, voedingssamenstelling, omgevings- en andere factoren zouden voedingsadviezen, zoals " eet volop groente en fruit" en "eet ruimschoots brood en aardappelen", opgesteld door het Voorlichtingsbureau voor de Voeding, genuanceerd moeten worden.

7. De invoering van beurspromovendi bij enkele Nederlandse universiteiten is niet alleen een gebrek aan waardering voor de promovendus, máár ook een gebrek aan zelfwaardering van zowel de universiteiten áls de promovendus.

8. Gelijke behandeling en verdraagzaamheid worden niet bereikt door naar gelijkheid te streven, maar door te accepteren dat alle mensen ongelijk zijn.
   N.a.v. het boek " Alle mensen zijn ongelijk", Prof. Dr. Hans Galjaard, 1994.

9. We beginnen met verwarring en eindigen met verwarring op hoger niveau.
   Oud gezegde.

10. Een half pak lucht verkopen als luchtig toetje riekt naar munt slaan uit niets.

Stellingen behorende bij het proefschrift "Interactieve effects between dietary fat and a vegetables-fruit mixture on colorectal carcinogenesis" door Jolanda Rijnkels, 10 maart 1998.

Voorwoord

Het proefschrift wordt gezien als bewijs voor het zelfstandig kunnen beoefenen van de wetenschap en vormt de kroon op de onderzoeksperiode als promovendus. Daarbij geeft het proefschrift een wetenschappelijk inhoudelijk overzicht van deze promotieperiode. Maar deze periode is voor mij ook op andere vlakken zeer waardevol geweest, zoals de praktische, organisatorische en onderwijservaringen die ik heb opgedaan.

De studies beschreven in dit proefschrift zouden verder niet mogelijk geweest zijn zonder de inzet, steun en commentaren van vele anderen, waaraan ik veel dank verschuldigd ben en er een aantal in het bijzonder wil noemen. Mijn directe begeleiders, Gerrit Alink, Ruud Woutersen en Jan Koeman, die veel tijd besteed hebben in mijn wetenschappelijke vorming. Bert Weijers, Jo Haas, Hans van den Berg en Victor Hollanders die mij enorm praktisch hebben ondersteund bij de uitvoering van de dierproeven. Johan Zwiesereijn en Peter Cenijn die mij bijzonder hebben geholpen bij het verwerken van duizenden kilo's groenten en fruit. Mijn kamergenoten en direct betrokkenen, Bert Haenen, Arnold Goeptar en Eric Vis, die nogal eens mijn geklaag, als het naar mijn idee niet meezat, 'ongewild' moesten aanhoren. Mijn vriend Henk Jan, die zeker in de laatste fase van het schrijven van dit proefschrift mijn 'aanwezigheid' heeft moeten ontberen. En dan natuurlijk al die anderen die ervoor gezorgd hebben dat ik met plezier mijn promotieonderzoek heb verricht; alle mede-toxers, CKP-ers, studenten, en WAIOO-ers.

Contents

General introduction

Scope of the present thesis

The hypothesis that dietary habits may cause colorectal cancer has been proposed 30 years ago and is still a matter of research. At present, many epidemiological and animal studies have indicated that a range of normal occurring dietary components may influence the risk of developing colorectal cancer (Burnstein, 1993; Cohen, 1987; Doll *et al.*, 1981; Kritchevsky, 1994; Potter, 1996; Steinmetz *et al.*, 1991a; Weisburger, 1991; Wynder *et al.*, 1967; Ziegler *et al.*, 1987; Zimbalist *et al.*, 1995). Prominent among these dietary components are dietary fat (high intake) and vegetables and fruit (low intake). However, not all these studies showed such a correlation and a number of animal experiments revealed also different outcomes (Howe *et al.*, 1997; Kinlen, 1986; Kolonel *et al.*, 1986; Prentice *et al.*, 1990; Trock *et al.*, 1990; Van den Brandt, 1997). This may be partly ascribed to differences in the balance of dietary components (Miller *et al.*, 1994; Reddy, 1995). This is demonstrated, for instance, by Alink *et al.* (1993). They examined specific dietary factors in a long-term animal study, in which diets were used resembling human Western-style diets, including high amounts of fat, raw or fried meat, and a vegetables-fruit mixture. The study showed that a vegetables-fruit mixture protected against colorectal tumor development when added to low-fat animal diets. A vegetables-fruit mixture added to a high-fat human diet, including fried meat, however, enhanced tumor development, whereas fried meat itself did not modify tumor development. These observations led to the hypothesis that the amount of fat may counteract the preventive properties of the vegetables-fruit mixture. This hypothesis formed the starting-point of the studies presented in this thesis, which are performed in order to elucidate possible interactive effects between a vegetables-fruit mixture and dietary fat on colorectal carcinogenesis. The next paragraphs emphasis aspects which are relevant within the context of the studies presented in this thesis, in order to understand the scope of the present thesis.

Dietary fat

A positive association of dietary fat with colorectal cancer was first suggested by Wynder *et al.* in 1967. One of the earliest and still most prominent hypothesis on the relationship between colorectal cancer risk and dietary fat intake is the bile acid hypothesis, formulated in the late seventies by among others Hill *et al.* (1971) and Reddy (1995). According to this hypothesis fat acts as a promoter, through increasing bile acid production. Ultimately, the colonic epithelium is exposed to increased secondary bile acids, which act as a promoter by inducing cell proliferation. Certain food compounds, such as fiber and calcium, may bind bile acids and even fatty acids, and thereby may interfere with the tumor promotion effects of dietary fat (Newmark *et al.*, 1984; Potter, 1996; Van der Meer *et al.*, 1984; Weisburger, 1991). At present the bile acid hypothesis is not the only proposed mechanism by which fat may promote carcinogenesis. Other proposed mechanisms are a) altering membrane phospholipid turnover and prostaglandin synthesis, b) altering the composition of gut flora, c) suppression of immune function, and d) altering initiation events (Potter, 1996; Pariza, 1988; Weisburger, 1991; Yang *et al.*, 1992).

Furthermore, during the last two decades it became evident that not only the amount of fat, but also the type of fat, differing in fatty acid composition, determines colorectal cancer risk (Reddy, 1995). Fats rich in ω-6 polyunsaturated fatty acids, such as sunflower oil, or in saturated fatty acids, such as lard, have stronger tumor promotion potential than fats rich in ω-3 polyunsaturated fatty acids, such as fish oil.

Vegetables and fruit

A variety of vegetables and fruit is suggested to possess anticarcinogenic potential. Among the most discussed vegetables are raw vegetables, green vegetables, cruciferous vegetables, tomatoes and carrots, because of their strong inverse relation with colorectal cancer (Steinmetz *et al.*, 1991a, 1996). The role of fruit is not very well understood, but attention is given to for instance citrus fruits. Vegetables and fruit form a valuable source of many potentially anticarcinogenic constituents, such as fiber, vitamins, minerals and a variety of so-called non-nutrients. Therefore, the mechanism by which vegetables and fruit may prevent carcinogenesis is not uniform, because the preventive mechanisms of each nutrient or non-nutrient may differ and their mechanisms may overlap or counteract.

Of special interest in the prevention of colorectal cancer are non-nutrients, because of their possible use as chemopreventives and as supplements in 'healthy' food products. Many non-nutrients have been considered as anticarcinogenic. For recent reviews see Hartman *et al.* (1990), Morse *et al.* (1993), Steinmetz *et al.* (1991b), and Wattenberg (1992).

Up to now, mechanisms of action of anticarcinogenic non-nutrients are not fully understood and several different mechanisms may be involved. A few examples will demonstrate the state of the art. Flavonoids (e.g. quercetin, rutin, kaempferol), which are widespread found in vegetables and fruit, but also in tea and wine, may act as inhibitors of carcinogenesis by antioxidant activity, modulation of (de)toxification routes, maintenance of DNA repair, inhibition of arachidonic acid metabolism and modulation of immune system (Hartman *et al.*, 1990; Morse *et al.*, 1993; Steinmetz *et al.*, 1991b). Breakdown products of glucobrassicin (isothiocyanates, indole-3-carbinol), which are specifically found in cruciferous vegetables (e.g. broccoli, cauliflower, Brussels sprouts and cabbage), may block carcinogenesis by induction of enzyme activities involved in detoxification of both natural occurring and chemical carcinogens (Hartman *et al.*, 1990; Verhoeven *et al.*, 1997). Further, worth mentioning is that vegetables, fruit, but also legumes are a major source of fiber. In the seventies, Burkitt *et al.* (1974) noted that fiber-rich diets protected against colorectal cancer. Plausible mechanisms by which fiber may protect against colorectal carcinogenesis are binding to bile acids and carcinogens, increasing stool bulk, and production of short chain fatty acids, such as butyrate and proprionate which inhibit cell proliferation (Kim *et al.*, 1996; Scheppach *et al.*, 1995; Steinmetz *et al.*, 1996).

Inconsistency in the results of epidemiological and animal studies

Whether colorectal cancer risk can be modified by a diet low in fat and rich in vegetables and fruit is a matter of debate. Although the positive association between fat and colorectal cancer and the negative association between vegetables or fruit and colorectal cancer have been observed, the findings have not been consistent among epidemiological studies. This inconsistency may have arisen, at least in part, from methodological limitations. Especially case-control studies are susceptible to several confounders, such as recall and selection bias (Miller *et al.* 1994). Furthermore, not always adjustments for age, sex, or smoking habits were made. Even when these limitations were taken into account, human evidence for fat as a cause of colorectal

cancer is weak (Howe *et al.*, 1997; Kinlen, 1986; Kolonel *et al.*, 1986; Prentice *et al.*, 1990). On the contrary, evidence for vegetables and fruit as protective against colorectal cancer is much stronger (Kim *et al.*, 1996; Miller *et al.*, 1994; Steinmetz *et al.*, 1991a; Trock *et al.*, 1990; Van den Brandt, 1997).

Laboratory animal studies were carried out to further establish the causal relationships between dietary factors and colorectal cancer. Only a few animal studies investigated food items or human diets as a whole. Rozen *et al.* (1996) compared human diets, of which the dietary composition was based on diets of patients having colorectal adenomas, with standard rat chow to study the feasibility of this complete human diet in rodents, and observed enhanced colonic epithelial proliferation in rats maintained on human diets. These human 'adenoma' diets included meat, eggs, milk products, and vegetables. Studies from a number of laboratories evaluated specific amounts and types of fat or fiber as supplements to standard rodent diets. These studies revealed inconsistent results concerning the influence of these dietary components (Newberne *et al.*, 1986; Potter *et al.*, 1986; Reddy, 1995; Rogers *et al.*, 1993). The inconsistencies may be partly ascribed to the dietary compositions used, which differed markedly from human diets in their source, preparation, and content. Animal studies investigating specified amounts of vegetables or fruit in relation to colorectal carcinogenesis are almost absent. A few animal studies have demonstrated inhibition of mammary carcinogenesis by cruciferous vegetables, such as cabbage, broccoli and Brussels sprouts (Stoewsand *et al.*, 1988; Wattenberg *et al.*, 1989). Several studies have shown anticarcinogenic properties of vegetables and fruit, especially those of cruciferous origin, or isolated plant constituents on the mechanisms at which these constituent may prevent carcinogenesis, which are reviewed by Rogers *et al.* (1993), Steinmetz *et al.* (1991b, 1996), and Wattenberg (1992). In these studies however, rather unrealistic high amounts of dietary constituents have been used, which may have limited the predictive value concerning colorectal cancer risk in humans. The predictive value may also be limited by the fact that the mechanisms of the anticarcinogenic effects of these dietary constituents have been studied in rather short-term animal experiments only, whereas cancer development is a long-term event by which the body has most probably adapted to changes caused by altered dietary composition.

Overall, animal studies show that inconsistent findings may be partly ascribed to the dietary composition of the experimental diets used. Because diet is complex and dietary factors probably interact, it is likely that the balance between dietary components, rather than individual components, is an important risk factor in developing colorectal cancer.

Interactive effects among dietary factors on colorectal cancer

Interaction between dietary factors has also been studied. In a case-control study, the highest risk of developing colorectal cancer was associated with a diet high in fat and low in fiber, whereas a reduction in risk was observed with a diet low in fat and high in fiber (Potter *et al.*, 1986). Comparable interactive effects between dietary fat and fiber were observed in chemically-induced colorectal carcinogenesis models in animals maintained on standard rodent diets (Kroes *et al.*, 1986; Nigro *et al.*, 1979; Sinkeldam *et al.*, 1990). Alabaster *et al.* (1995) combined beta-carotene with high-fat/low-fiber and high-fat/high-fiber diets in an animal study. Beta-carotene is present in orange vegetables and fruits (e.g. carrots, mango) and is considered to be inversely associated with colorectal cancer. It appeared that beta-carotene and fiber, individually as well as combined, protected against colorectal cancer in animals fed high-fat diets. Alink *et al.* (1993) examined specific dietary factors in an animal study, in which diets were used resembling human Western-style diets, as was mentioned earlier in the introduction. In the study of Alink *et al.*, a vegetables-fruit mixture protected against colorectal tumor development in low-fat animal diets, but when present in high-fat human diets including fried meat, enhanced tumor development was observed.

In summary, the results of previously mentioned studies demonstrate that dietary factors may interact with each other, indicating that the balance between dietary components, rather than individual components, may be an important risk factor in developing colorectal cancer.

Objectives and approach of the present thesis

The study of Alink *et al.* (1993) led to the hypothesis that the preventive potency of a vegetables-fruit mixture may be counteracted by the amount of fat in the diet. To substantiate the hypothesis that interactive effects among dietary constituents play an important role in their effects on colorectal carcinogenesis, we have performed seven *in vivo* and *in vitro* studies using dietary fat and a vegetables-fruit mixture in diets resembling the mean composition and consumption values in The Netherlands.

In the first part of the thesis the results have been summarized of studies with four different long-term animal models for colorectal cancer. We have chosen for this approach, since we were interested in the effects of the vegetables-fruit mixture on colorectal carcinogenesis induced by a direct (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine,

MNNG) and an indirect (1,2-dimethylhydrazine, DMH) acting carcinogen as well as on the initiation and promotion phase of the carcinogenesis process (azoxymethane, AOM). DMH and AOM are used, because these compounds induce colorectal cancer that closely imitates the type and location of colorectal cancer in man. Finally, we were highly interested in the effects of the dietary factors in animals genetic susceptible for developing intestinal cancer (Apc^{Min} mice).

The second part of the thesis was aimed to elucidate the mechanisms by which the interactive effects of dietary fat and vegetables and fruit influenced the development of colorectal cancer. Emphasis was given to xenobiotic enzyme activities and immune function (long-term DMH-study), xenobiotic enzyme activities (short-term DMH-study) and *in vitro* studies. In these *in vitro* studies, interactive effects between stearic acid, indole-3-carbinol, and crude extracts of the vegetables-fruit mixture were studied on cytotoxicity, gap junctional intercellular communication and cytochrome P450-1A activities.

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Part I

Interactive effects of dietary factors on colorectal tumorigenesis

1

Interaction of dietary fat with a vegetables-fruit mixture on 1,2-dimethylhydrazine-induced colorectal cancer in rats

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Nutrition and Cancer Int J 27, 261-266, 1997

Abstract

The aim of the present investigation was to study the interaction of dietary fat in combination with a vegetables-fruit mixture on 1,2-dimethylhydrazine-induced colorectal carcinogenesis in rats. For this purpose, 120 weanling male Wistar rats received a semisynthetic diet without (groups A and C) or with a vegetables-fruit mixture (groups B and D; vegetables and fruit content 19.5% wt/wt), for 35 weeks. Diets of groups A and B contained 20 energy% (e%) fat, whereas diets of groups C and D contained 40e% fat. The vegetables and fruit used, the amount of fat and its fatty acid composition were chosen according to the mean consumption figures of The Netherlands. After the animals were maintained for four weeks on the respective diets, they were given ten weekly s.c. injections of 1,2-dimethylhydrazine at a dose rate of 50 mg/kg body weight. After sacrifice, their colons were removed and examined macroscopically and microscopically for the presence of tumors. Rats fed high-fat diets developed significantly more tumors than rats fed low-fat diets. Furthermore, although not statistically significant, a lower number of colorectal tumors was observed in rats fed either a low- or a high-fat diet containing the vegetables-fruit mixture compared to rats given diets without the vegetables-fruit mixture. No differences were observed in intestinal tumor incidences among all groups. The results suggest that a vegetables-fruit mixture used in this experiment, present in an amount comparable with the mean consumption in The Netherlands, has no significant inhibitory effect on the development of colorectal tumors induced by 1,2-dimethylhydrazine in rats maintained on diets either low or high in fat.

Introduction

Colon cancer is one of the leading cancers in the Western world (Doll *et al.*, 1981). Although the intestinal cancer risk depends on various etiological factors, such as inflammatory bowel diseases, genetic constitution and exposure to environmental agents, especially the Western diet seems to be the predominant cause of colorectal cancer (Doll *et al.*, 1981; Weisburger, 1991; Wynder *et al.*, 1967).

Based on epidemiological data, the Western diet is characterized by a high degree of fat/meat and low amounts of vegetables/fruit (Potter, 1995, 1996). However, epidemiological studies do not find consistent positive or negative associations between fat or vegetables and fruit for colorectal cancer (Potter, 1995; Schatzkin *et al.*, 1995; Smith *et al.*, 1985). This seems mainly due to the limitations of the study design, such as recall bias, uncertainties about the quality of the food consumed and (unknown) confounding factors (Tau, 1995). Animal studies performed under controlled conditions do not have such limitations. However, although animal data generally support the epidemiologic observation that Western diet contributes to a high risk for developing colorectal cancer, the results of

the animal studies are not unanimous (Newberne *et al.*, 1986; Willett, 1995). This may be ascribed, at least partly, to the study design. In most studies only one isolated dietary component is examined, while in real life human diet contains hundreds of different components. It is well known from experimental studies, that the effects of mixtures of compounds interact, resulting in a final effect, which can be completely different from the effects of the individual components (Newberne *et al.*, 1986; EPA, 1986). Consequently, extrapolation of the results, obtained with one dietary component in animal models to the actual human colon cancer risk is rather difficult and is considered to be questionable. Furthermore, in several animal studies doses of dietary micronutrients or non-nutrients are used far exceeding daily human intake, making extrapolation to the effects in real life rather doubtful.

The above mentioned shortcomings of studying the effects of isolated dietary components support the need to perform colorectal carcinogenesis experiments, using diets, which are comparable with regular human diets. Using such an experimental design, interactive effects among dietary constituents can be expected. Furthermore, in these diets dietary components are present in realistic amounts (Newberne *et al.*, 1986; Rozen *et al.*, 1996).

Previously, we have studied colorectal tumor modulation in rats using heat processed human diets, high in fat (40 energy% (e%)) and including a vegetables-fruit mixture (Alink *et al.*, 1993). In that study, it was observed that the vegetables-fruit mixture significantly increased the number of adenocarcinomas compared to human diets without a vegetables-fruit mixture. However, animal diets low in fat (20e%) including a vegetables-fruit mixture had an inhibitory effect on colorectal cancer development compared to animal diets without a vegetables-fruit mixture. As the fat content was the most important difference between the human and animal diets used in that study, it was suggested that fat interacted with the inhibitory potency of the vegetables-fruit mixture. Therefore, the present study was carried out in order to further investigate the interactive effects of dietary fat with a vegetables-fruit mixture.

The possible interaction of fat with a vegetables-fruit mixture was studied on DMH-induced colon cancer in Wistar rats using 'human-based' animal diets. In these 'human-based' animal diets, the choice of vegetables and fruit, the amount of fat and its fatty acid composition were based on the mean consumption figures in The Netherlands.

Materials and methods

Animals

Five-week-old male Wistar rats (Crl:(WI)WU BR; Charles River, Sulzfeld, Germany) were randomly divided in four groups of thirty animals each - A, B, C and D - corresponding with the diets (see below).

The animals were housed in plastic cages (three per cage) with wire tops and sawdust bedding. The cages were randomly placed in an animal isolator (Isotec; Harlan, Zeist, The Netherlands) under negative pressure, in a temperature- (± 22°C) and humidity- (50-60%) controlled room, with artificial lighting (12 h light/12 h dark). The animals had free access to diet and tap water.

Diets

The following four diets were used: a diet low in fat (20e%, Diet A), a diet low in fat (20e%) to which a vegetables-fruit mixture was added (19.5% wt/wt, Diet B), a diet high in fat (40e%, Diet C) and a diet high in fat (40e%) to which the same vegetables-fruit mixture was added (19.5% wt/wt, Diet D) as in diet B.

The Muracon-SSP/tox animal diet (Hope Farms, Woerden, The Netherlands) served as the basal diet for the four different diets. The fat in the diets was a mixture of lard and sunflower oil, resulting in a fatty acid composition similar to an average human diet in the Netherlands, with 34.3e% saturated fatty acids, 42.1e% mono-unsaturated fatty acids and 17.2e% poly-unsaturated fatty acids. In diets B and D, a vegetables-fruit mixture was supplied to the Muracon-SSP/tox.

Table 1. Vegetables and fruit composition.

Component	Level (% wet wt)	Component	Level (% wet wt)
potatoes	35.10	spinach	2.50
bananas	3.00	leek	2.50
oranges	9.00	red cabbage	2.50
apples	19.10	white cabbage	2.50
lettuce	3.75	sauerkraut	2.50
green pepper	1.25	carrots	1.25
tomatoes	3.75	Brussels sprouts	1.25
cucumber	3.75	beetroot	2.50
cauliflower	3.75		

The choice of the vegetables and fruit used (Table 1) approached the mean vegetables and fruit consumption in The Netherlands (Van den Berg, 1981). Vegetables and fruit were prepared under household conditions. The following products were cooked; potatoes, cauliflower, spinach, leek, red and white cabbage, sauerkraut, carrots, brussels sprouts and beet. After freezing (-40°C) the mixture was freeze-dried, ground and homogenized. Before the mixture was added to the basal diet it was analyzed for nutrient, vitamin and mineral content (data not shown). Furthermore, total glucosinolate content (Department of Food Science, Agricultural University, Wageningen, The Netherlands) and flavonoid contents (Institute of Food Chemistry and Nutrition, Søborg, Denmark) were measured in the vegetables-fruit mixture (Table 2). The vegetables-fruit mixture was tested on possible pathogenic contamination (BioConsult B.V., Mijdrecht, The Netherlands) and appeared to be negative.

Table 2. Composition of the four experimental diets.

In % (wt/wt)	Diet A	Diet B	Diet C	Diet D
protein[a]	23.19	23.19	26.50	26.50
carbohydrate[b]	48.43	30.39	30.78	10.14
fat[c]	8.50	8.50	20.00	20.00
fiber[d]	10.67	9.80	12.20	11.20
vegetables-fruit mixture[e]	0.00	19.50	0.00	22.30
premix SSP/tox[f]	1.02	1.02	1.17	1.17
rest of SSP/tox[g]	8.28	7.55	9.47	8.65

a: Acid casein (ac) and soy protein (sp); A/B: 18.55 gram ac and 4.64 gram sp; diet C/D: 21.20 gram ac and 5.30 gram sp. Per 100 gram of diet.

b: Cerelose (ce) and corn starch (cs); A: 23.33 g ce and 25.10 g cs, B: 24.79 g ce and 5.60 g cs, C: 2.08 g ce and 28.70 g cs, D: 3.74 g ce and 6.40 g cs. Per 100 gram of diet.

c: 84 gram lard and 16 gram sunflower oilper 100 gram fat.

d: Cellulose.

e: Total glucosinolate content near detection level (i.e. glucobrassicin), flavonoid content 640 mg/100 g (i.e. quercetin, catechin, naringenin, hesperetin).

f: Composed of vitamins E, A, D_3, K, B_1, B_2, B_6, B_{12}, C, niacin/nicotine acid, Ca-pantothenate, folic acid, PABA, inositol, MgO, $FeSO_4.H_2O$, $ZnSO_4.5H_2O$, $CoSO_4$, ammonium heptamolybdate, NaF, Ca-jodate, $AlK(SO_4)_2.12H_2O$, $CrCl_3.6H_2O$, Siliciumoxid. This composition was adjusted for the presence of the vegetables-fruit mixture (see materials and methods).

g: Composed of $CaHPO_4.2H_2O$, KCl, NaCl, Na_2CO_3, methionine synthetase, choline Cl. This composition was adjusted for the presence of the vegetables-fruit mixture (Materials & Methods).

The composition of the diets is shown in Table 2. The extra 20e% fat added to diets C and D was at the expense of carbohydrates, whereas proteins and micronutrients in diets A and B were adjusted to diets C and D, as the caloric composition of the four diets differed. Consequently the diets only differed in the fat/carbohydrate content and in the presence of specific constituents (type of fiber, non-nutrients) in the vegetables-fruit mixture.

Diets were stored at -40°C in air closed plastic bags during the experiment. No antioxidants or other preservatives were added.

Experimental design

All animals were fed one of the four experimental diets during the whole experiment, which lasted up to 35 weeks. Four weeks after the start of the experiment all rats were subcutaneously injected with 1,2-dimethylhydrazine (DMH: Aldrich Chemical Co.Ltd.; Gillingham, Dorset, UK) at a dose of 50 mg/kg body weight, once a week for ten weeks. DMH was dissolved in 1.5% solution of EDTA in 0.9% NaCl, that was adjusted to pH 6.5 (final solution of 1 ml/kg body weight).

Growth rate and food intake per cage were monitored weekly. The animals were checked daily for their health. Those who were in poor health or moribund condition were killed and autopsied. At the end of the experiment all animals were killed by exsanguination via the abdominal aorta under ether anaesthesia and subjected to detailed gross examination.

Histopathology

The colon and rectum were opened longitudinally and number, size, gross appearance and location of tumors were registered. Each tumor was collected and individually processed. The rest of the colon and rectum was prepared as Swiss rolls. For light microscopy, all tumorous lesions and Swiss rolls were fixed in 4% phosphate buffered formalin, embedded in paraffin, cut in 5 mm thin sections and stained with haematoxylin and eosin. Tumors were classified as sessile adenomas, polypoid adenomas, adenocarcinomas, mucus-producing adenocarcinomas or signet-ring cell carcinomas, according to the criteria previously described (Alink *et al*, 1993). In assessing the colorectal tumor incidence, number of tumors and multiplicity, a distinction was made between adenomas (sessile and polypoid adenomas) and (adeno)carcinomas (adenocarcinomas, mucus-producing adenocarcinomas and signet-ring cell carcinomas). The multiplicity is a relative measure of the tumor rate, which is defined as the number of tumors divided by the number of tumor bearing animals.

Statistics

Differences in weight gain were compared by analysis of variance (ANOVA) and

Differences in weight gain were compared by analysis of variance (ANOVA) and differences in tumor incidences by logistic regression with binomial distribution and logit link. The number of tumors were compared by generalized linear regression with Poisson distribution and log link. For all tests a confidence interval of 95% ($p<0.05$) was chosen.

Results

General observations

No significant differences in body weights were observed between the groups (Figure 1). Although the caloric composition of the four groups differed, the animals adjusted their daily food intake accordingly (Figure 2); rats maintained on a high-fat diet (Groups C and D) showed a lower food intake than rats maintained on a diet low in fat (Groups A and B). The slight increase in food intake and in body weight, seen in week 15 in all four groups, is most probably related to finishing the DMH treatment.

At the end of the study mortality amounted to 24.1% and was almost similar in the four groups, except group A, which demonstrated a slightly higher mortality than the other groups (A: 34.4%, B: 20.1%, C: 25.4% and D: 20.1%). The cause of dead of three rats could not be established. Hence, the total number of effective animals was 117.

Of the colorectal tumors 78.3% were located in the distal part of the colon.

Table 3. Tumor incidence and multiplicity in colon and rectum in rats fed the experimental diets.

Diet[a]	No. of	Animals with tumors			No. tumors/tumor bearing rat		
	animals	Total	Adenoma	Carcinoma	Total	Adenoma	Carcinoma
A	30	23 (79)[b]	19 (63)	16 (70)	3.30	2.30	1.00
B	29	25 (86)	18 (62)	18 (62)	2.52	1.20	1.32
C	28	24 (86)	22 (79)	20 (71)	5.13[c*]	3.50[c**]	1.63
D	30	29 (97)	20 (67)	25 (83)	3.79[d*]	2.28[d**]	1.52

a: Group A: 20 e% fat, Group B: 20 e% fat + veg/fruit, Group C: 40 e% fat, Group D: 40 e% fat + veg/fruit

b: Numbers in parentheses, percentage of tumors per dietary group

c: Group A versus C: * $0.01>p>0.005$, ** $0.05>p>0.02$

d: Group B versus D: * $0.01>p>0.005$, ** $0.005>p>0.002$

Figure 1. Mean body weights of DMH treated rats fed one of the four different diets during the experiment. (O) Diet A: 20e% fat, (●) diet B: 20e% fat including a vegetables-fruit mixture, (■) diet C: 40e% fat, (□) diet D: 40e% fat including a vegetables-fruit mixture.

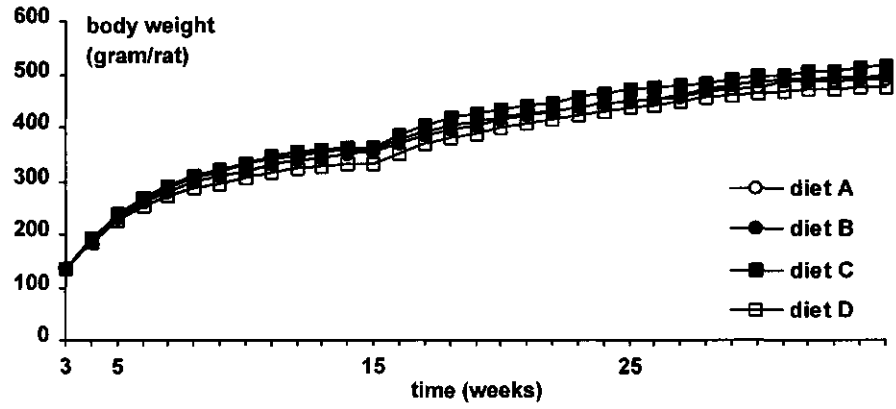
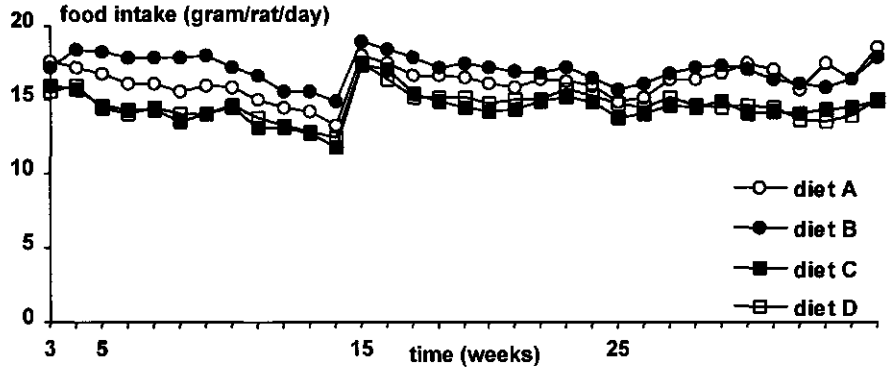


Figure 2. Mean daily food intake per rat fed one of the four different diets during the experiment. (O) Diet A: 20e% fat, (●) diet B: 20e% fat including a vegetables-fruit mixture, (■) diet C: 40e% fat, (□) diet D: 40e% fat including a vegetables-fruit mixture.



Colorectal tumor incidence

The majority (87%) of the animals developed one or more colorectal tumors. Group A demonstrated the lowest number of tumor-bearing animals (79%), whereas group D showed the highest number of tumor-bearing animals (97%) (Table 3). However, no significant differences in colorectal tumor incidences were found among the four groups.

Number of tumors and multiplicity

Type and number of large bowel tumors observed are shown in Table 4. Of the tumors observed 62.6% were adenomas and 37.4% (adeno)carcinomas. Of the adenomas 64.8% were classified as polypoid adenomas. The number of polypoid adenomas was slightly higher in animals fed the vegetables-fruit mixture (Group B: 73.3%, Group D: 72.7%) than in animals maintained on a diet without a vegetables-fruit mixture (Group A: 62.3%, Group C: 57.1%). Of the (adeno)carcinomas 86.3% were classified as adenocarcinomas, 8.6% as mucus-producing adenocarcinomas and 5.1% as signet-ring cell carcinomas.

Animals on a high-fat diet (Groups C and D) exhibited a significant increase in number of adenomas ($p<0.005$) and adenocarcinomas (Group A versus C: $p<0.05$). The number of tumors was, although not statistically significant, lower in the groups maintained on diets with a vegetables-fruit mixture compared to the groups maintained on diets without this mixture but with the same amount of fat (Table 4). This effect was due to a lower number of (total) adenomas in these groups.

The multiplicity (Table 3) showed the same trends as observed for the number of tumors; diets high in fat clearly enhanced the multiplicity, while the presence of a vegetables-fruit mixture in the diets only resulted in a small inhibition. That the multiplicity showed the same trends was because no differences in tumor incidences were found between the four groups.

Other Tumors

Although DMH is known as a specific colon cancer inducer, tumors at other sites than colon and rectum were observed, as shown in Table 5. Most of these tumors were located in duodenum (55.3%), jejunum (11.1%) and Zymbal glands (9.1%). Statistical analysis revealed no differences among the different groups. Also no trends were observed. Malignancies observed in liver and abdomen were all diagnosed to be secondary lesions originating from primary intestinal tumors.

Table 4. Number and types of DMH-induced tumors in colon and rectum.

Diet	Total no. of	Adenomas			(Adeno)carcinomas			
group[a]	tumors	Sessile	Polypoid	Total	Adeno	Mucus-prod.	Signet-ring cell	Total
A	**76**	20	33	**53**	19	2	2	**23**
B	**63**	8	22	**30**	28	4	1	**33**
C	**123**[b*]	36[b****]	48[b****]	**84**[b**]	35[b**]	3	1	**39**[b***]
D	**110**[c*]	18	48[c**]	**66**[c*]	38	3	3	**44**

a: Group A: 20 e% fat, Group B: 20 e% fat + veg/fruit, Group C: 40 e% fat, Group D: 40 e% fat + veg/fruit

b: Group A versus C: *$p<0.001$, ** $0.005>p>0.002$, *** $0.02>p>0.01$, **** $0.05>p>0.02$

c: Group B versus D: *$p<0.001$, ** $0.005>p>0.002$

Table 5. Number of tumors, other than of colon and rectum, found in DMH-treated rats.

Group[a]	Duodenum	Jejunum	Caecum	Liver	Pancreas	Stomach	Prostate	Abdomen	Zymbal gland
A	17	5	1	3	3	-	1	3	5
B	18	5	-	1	-	-	-	2	7
C	24	2	-	1	3	1	-	-	7
D	25	5	1	2	-	-	-	-	10

a: Group A: 20 e% fat, Group B: 20 e% fat + veg/fruit, Group C: 40 e% fat, Group D: 40 e% fat + veg/fruit

Discussion

A highly relevant observation in the present study was that rats given a vegetables-fruit mixture in combination with a low or a high-fat diet developed less, although statistically not significant, colorectal adenomas then those on a diet without a vegetables-fruit mixture. Although the low-fat diets had the same fat percentage as the animal diets in our previous study (Alink *et al.*, 1993), the significant protective effect of the vegetables-fruit mixture on colorectal adenoma development was not found in the present study. The reason for the discrepancy between the results of those studies can not easily been given since the experimental conditions were almost similar in both studies, but might be ascribed to the slight differences in the fatty acid composition. Moreover, in contrast with our previous study with processed human diets, the present vegetables-fruit mixture in combination with the high-fat diet did not exhibit an enhancing effect on colorectal carcinogenesis (Alink *et al.*, 1993). Although no definite conclusions can be drawn on the interactive effects of fat and of the vegetables-fruit mixture, because no significant inhibitory effects with the vegetables-fruit mixture were obtained, the results suggest that fat does not interact with the inhibitory capacity of the vegetables-fruit mixture. Some literature exists on dietary components present in vegetables and fruit, showing protection against the development of colon tumors even in combination with high-fat diets (Alabaster *et al.*, 1995; Hardman *et al.*, 1995; Sinkeldam *et al.*, 1990). Deschner *et al.* (1993) observed a lower incidence of focal areas of dysplasia in colonic epithelia in mice maintained on high-fat diets in combination with the flavonoid, quercetin or rutin. Other investigators, however, have demonstrated that a high-fat diet can completely abolish the inhibitory effect of dietary factors (Nigro, 1981).

It is not illogical that the present results with the vegetables-fruit mixture are less pronounced than those obtained with studies where the modulating effects of only one dietary constituent, present in a high concentration, is investigated. In the present study, realistic "human-based" animal diets were used, which contained both promoting components apart from inhibitory constituents, in realistic quantities. Moreover, the vegetables and fruit have been processed under household conditions (e.g. cut in pieces and cooked), which may result in an overall lowering of the anticarcinogenic properties by physical and enzymatic depletion of several non-nutrients (Fenwick *et al.*, 1983). We measured flavonoids in reasonable amounts in our diets to which a vegetables-fruit mixture was added, but the glucosinolate content was very low. Finally, the DMH treatment induced a rather high number of tumors per animal, indicating that the carcinogen treatment might have been too effective to find a protective effect of the vegetables-fruit mixture used in the present study.

The most meaningful observation of the present study with DMH-treated rats is that diets high in fat (40e%), either with or without a vegetables-fruit mixture, almost resulted in a doubling ($p<0.05$) in number of colorectal tumors, compared to low (20e%) fat diets. Since the energy intake among all animals was comparable, this enhancing effect is most probably due to the fat content of the diets. This is in agreement with the results of several other studies focussing on fat as a risk factor in colorectal cancer (Reddy *et al.*, 1977; Weisburger, 1986). Also the type of fat used (mainly lard) could have positively influenced the number of tumors, since it has been found that lard, rich in saturated fatty acids, possess tumor promoting capacity (Ma *et al.*, 1996; Reddy *et al.*, 1986).

It may be concluded that, the vegetables-fruit mixture, consumed in an amount representative for the mean consumption in The Netherlands, accounted for only a slight but non-significant inhibition of the development of DMH-induced colorectal tumors in rats, whereas fat consumed in an amount and with a fatty acid composition representative to the mean consumption and composition figures in The Netherlands, significantly enhanced colorectal carcinogenesis.

Acknowledgements

We thank J. Haas and B. Weijers of the Centre for Small Laboratory Animals of the Agricultural University for expert technical assistance. We also acknowledge E. Schoenen of the TNO Toxicology and Nutrition Institute for statistical advice. This study was supported by Grant LUW93-485 of the Dutch Cancer Society. The Central Horticultural Auctions in The Netherlands is gratefully acknowledged for providing most vegetables and fruit.

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2

Modulation of dietary fat-enhanced colorectal carcinogenesis in *N*-methyl-*N*'-nitro-*N*-nitroso-guanidine-treated rats by a vegetables-fruit mixture

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Nutrition and Cancer Int J 29, 90-95, 1997

Abstract

In the present investigation the modulation of a vegetables-fruit mixture on *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced colorectal carcinogenesis was studied in rats maintained on a low- or high-fat diet. For this purpose, 120 rats received a semi-synthetic diet without (Groups A and C) or with a vegetables-fruit mixture (19.5% wt/wt; Groups B and D), for 35 weeks. Diets of Groups A and B contained 20 (low) energy percent (20e%) fat, whereas diets of Groups C and D contained 40e% (high) fat. Between week 4 and 9 the animals were given weekly intrarectal instillations of 6 mg MNNG/kg body wt. The colorectal adenocarcinoma incidences showed a significant decrease in animals fed high-fat diets with a vegetables-fruit mixture compared to animals fed a high-fat diet alone. Furthermore, without a vegetables-fruit mixture diets high in fat caused a significant increase in adenocarcinoma incidence compared to animals fed diets low in fat. Although not significant, the adenoma incidences in animals fed a vegetables-fruit mixture tended to be lower than in animals maintained on a diet without this mixture. The results demonstrate that a vegetables-fruit mixture has a significant inhibitory potency on the development of colorectal tumors induced by MNNG in rats fed diets high in fat.

Introduction

The high incidence of colorectal cancer in the Western world is mainly ascribed to dietary habits (Doll *et al.*, 1981; Wynder *et al.*, 1967; Zimbalist *et al.*, 1995). Diets high in fat/meat and low in vegetables/fruit have been found to be associated with a high risk of colorectal cancer (Potter, 1995, 1996; Sian, 1987). Several epidemiologic studies, however, did not confirm the association (Bingham *et al.*, 1979; Schatzkin *et al.*, 1995; Trock *et al.*, 1990). Although epidemiological studies investigate dietary habits as a whole, these different results are partly due to confounders, such as recall-bias and uncertainties about the quality of the food consumed (Burnstein, 1993; Tau, 1995). Animal studies performed under controlled conditions do not have such limitations. The results of animal studies generally support the above mentioned association, but the results of these studies are not unanimous (Nauss *et al.*, 1984; Newberne *et al.*, 1986). This may be ascribed, at least partly, to differences in study design (Nauss *et al.*, 1987). In most studies, the effect of only one isolated dietary component or constituent on colon carcinogenesis is examined, whereas in real life the human diet contains hundreds of different components, which can interact with each other resulting in a final effect that can be completely different from that expected from the effects of the isolated components (Ma *et al.*, 1996; Marian, 1996; EPA, 1986). Furthermore, in several animal studies dietary micronutrients or non-nutrients are used in doses that far exceed human intake making extrapolation to the effects in humans under realistic circumstances rather doubtful. These shortcomings in animal studies warrant

the need to perform colorectal carcinogenesis experiments using diets as much as possible imitating realistic regular human diets. Using such study design, interactive effects among dietary constituents can be expected.

Previously, we have studied the modulation of 1,2-dimethylhydrazine (DMH)-induced colorectal carcinogenesis in rats fed heat-processed human diets, high in fat (40 energy percent; 40e%) and including a vegetables-fruit mixture, or fed animal diets, low in fat (20e%) and including a vegetables-fruit mixture (Alink *et al.*, 1993). In that study, the vegetables-fruit mixture added to the processed human diets increased the development of colorectal cancer, while animal diets containing the vegetables-fruit mixture clearly inhibited colorectal carcinogenesis. As the fat content was a main difference between the diets, it was suggested that fat counteracted with the inhibitory potency of the vegetables-fruit mixture. Recently, we studied this hypothesis, using a DMH-induced colorectal carcinogenesis animal model (Rijnkels *et al.*, 1997). In that study a non-significant inhibitory effect of the vegetables-fruit mixture was found on colorectal cancer development, either in rats fed low- (20e%) or high- (40e%) fat animal diets. Fat, however, clearly enhanced the development of colorectal tumors. From those results, there were no indications of the existence of any interaction between fat and a vegetables-fruit mixture, with regard to their effect on colorectal carcinogenesis.

The present study was designed to investigate whether the effect of a vegetables-fruit mixture on fat-enhanced colorectal carcinogenesis is influenced by the way colorectal tumors are induced in rats. In the present study, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is used to induce colorectal tumors in rats. In contrast to DMH, MNNG is a direct acting carcinogen, which is administered intrarectally in stead of subcutaneously. Apart from the carcinogen used, the experimental design of the present study was similar to the previous study with DMH (Rijnkels *et al.*, 1997).

Materials and methods

Animals

Five-week-old male Wistar rats [Crl:(WI)WU BR; Charles River, Sulzfeld, Germany] were randomly divided into four groups of 30 animals each (A, B, C, and D) corresponding with the diets (see below).

The animals were housed in plastic cages (3/cage) with wire tops and sawdust bedding. The cages were randomly placed in an animal isolator (Isotec, Harlan, Zeist, The Netherlands) under negative pressure in a temperature- (± 22°C)- and humidity- (50-60%)

controlled room, with artificial lighting (12:12-hour light-dark cycle). The animals had free access to diet and tap water. The diets were refreshed weekly.

Diets

The following four diets were used: a diet low in fat (20e%; Diet A), a diet low in fat (20e%) to which a vegetables-fruit mixture was added (19.5% wt/wt; Diet B), a diet high in fat (40e%; Diet C) and a diet high in fat (40e%) to which the same vegetables-fruit mixture was added (19.5% wt/wt; Diet D) as in Diet B. The semi-synthetic Muracon-SSP/tox animal diet (Hope Farms, Woerden, The Netherlands) served as the basal diet for the four different diets.

The fat in the diets was a mixture of lard and sunflower oil, resulting in a fatty acid composition similar to an average human diet in The Netherlands, with 34.3e% saturated fatty acids, 42.1e% monounsaturated fatty acids and 17.2e% polyunsaturated fatty acids. The extra 20e% fat added to Diets C and D was at the expense of carbohydrates. Through this expension, the caloric density of the high-fat diets was higher than of the low-fat diets. To allow for decreased food consumption in animals consuming diets of higher caloric density, the protein, mineral and vitamin contents of the basal Muracon-SSP/tox in the high-fat diets were adjusted to the low-fat diets, in such a way that all animals in experiment had approximately the same intake of these dietary constituents.

In Diets B and D, a vegetables-fruit mixture was supplied to the Muracon-SSP/tox diet. The choice of the vegetables and fruit used (Table 1) approached the mean vegetables and fruit consumption in The Netherlands (Van den Berg, 1981). Vegetables and fruit were prepared under household conditions. The following products were cooked; potatoes, cauliflower, spinach, leek, red and white cabbage, sauerkraut, carrots, Brussels sprouts, and beet. After freezing (-40°C) the mixture was freeze-dried, ground, and homogenised.

Table 1. Vegetables and fruit composition.

Component	Level (% wet wt)	Component	Level (% wet wt)
potatoes	35.10	spinach	2.50
bananas	3.00	leek	2.50
oranges	9.00	red cabbage	2.50
apples	19.10	white cabbage	2.50
lettuce	3.75	sauerkraut	2.50
green pepper	1.25	carrots	1.25
tomatoes	3.75	brussels sprouts	1.25
cucumber	3.75	beetroot	2.50
cauliflower	3.75		

Before the vegetables-fruit mixture was mixed with the basal Muracon-SSP/tox and pelleted, it was analysed for nutrient, vitamin and mineral content (data not shown). These analyses were done to adjust the amounts of these dietary constituents in the Muracon-SSP/tox, used for the vegetables-fruit containing diets, in that these diets did have approximately the same final nutrient, mineral and vitamin content as did the diets not containing a vegetables-fruit mixture.

Table 2. Final composition of the four experimental diets[a]

Ingredients	**Diet A**[b] *low fat*	**Diet B** *low fat + VF*[i]	**Diet C** *high fat*	**Diet D** *high fat + VF*[i]	**VF-*mixture***[h]
fat[c]	8.50	8.50	20.00	20.00	0.40
carbohydrate[d]	48.34	30.39	30.78	10.14	63.00
protein[e]	23.19	23.19	26.50	26.50	8.80
fiber (cellulose)	10.67	9.80	12.20	11.20	14.20
vitamins[f]	0.35	0.31	0.40	0.35	0.80
minerals[g]	8.95	8.31	10.12	9.51	1.30
VF-mixture[i]	-	19.50	-	22.30	
water					11.50
kcal/kg	**3603**	**3608**	**4126**	**4132**	**3548**

a: Values (% wt/wt) are calculated from analyses and represent approximate composition.

b: Diet A is the basal Muracon-SSP/tox diet. In Diets C and D the carbohydrate, protein, fiber, vitamin and mineral contents of the Muracon-SSP/tox were adjusted to allow for decreased food consumption in animals consuming diets of high caloric density. In Diets B and D the amounts of carbohydrate, fiber, vitamins and minerals of the Muracon-SSP/tox were adjusted for the presence of these constituents in the vegetables-fruit mixture (VF-mixture: see **Materials and Methods**)

c: 84 % lard and 16 % sunflower oil.

d: Composition Diet A: 23.33 g/100 g cerelose and 25.10 g/100g corn starch.

e: Composition Diet A: 18.55 gram/100 g acid casein and 4.64 gram/100 g soy protein.

f: Composition of Muracon-SSP/tox (Diet A): cholin Cl (50%) 0.303 g/100g, vitamin A 0.34 mg/kg, vitamin A/D_3 1.50 mg/kg, vitamin E (50%) 6.54 mg/kg, vitamin K_3 0.360 mg/kg, thiamin 0.650 mg/kg, riboflavin 0.920 mg/kg, pyridoxine HCl 0.270 mg/kg, niacin/nicotin 4.60 mg/kg, calcium pantothenate 2.86 mg/kg, vitamin B_{12} 2.02 mg/kg, folic acid 0.300 mg/kg, biotin 2% 1.375 mg/kg, inositol 13.79 mg/kg, β-carotene 10% 2.86 mg/kg, vitamin C 12.39 mg/kg.

g: Composition of Muracon-SSP/tox (Diet A): $CaHPO_4.2H_2O$ 3.34 g/100g, KCl 0.93 g/100g, NaCl 0.37 g/100g, Na_2CO_3 0.46g/100g, methionine synthetase 0.09 g/100g, PABA 18.18 mg/kg, MgO 90.93 mg/kg, $FeSO_4.H_2O$ 13.24 mg/kg, $ZnSO_4.5H_2O$ 7.00 mg/kg, $CoSO_4$ 0.225 mg/kg, ammonium heptamolybdate 0.01 mg/kg, NaF 0.01 mg/kg, Ca-iodate 0.05 mg/kg, $AlK(SO_4)_2.12H_2O$ 6.59 mg/kg, $CrCl_3.6H_2O$ 0.02 mg/kg, Siliciumoxid 2.79 g/100g.

h: Total glucosinolate content near detection level (i.e. glucobrassicin), flavonoid content 640 mg/100 g quercetin, catechin, naringenin, hesperetin).

i: VF = vegetables-fruit mixture.

Furthermore, total glucosinolate content (Dept. of Food Science, Agricultural University, Wageningen, The Netherlands) and flavonoid contents (Institute of Food Chemistry and Nutrition, Søborg, Denmark) were measured in the vegetables-fruit mixture (Table 2). The vegetables-fruit mixture was tested for possible pathogenic contamination (BioConsult, Mijdrecht, The Netherlands) and appeared to be negative.

The final composition of the diets is shown in Table 2. As a consequence of adjusting the diets for protein, mineral and vitamin contents, the diets differed only in the fat/carbohydrate content and in the presence of specific constituents (type of fiber, non-nutrients) in the vegetables-fruit mixture.

Finally, the pelleted diets were stored at -40°C in air-closed plastic bags during the experiment. No antioxidants or other preservatives were added.

Experimental Design

All animals were fed one of the four experimental diets during the whole experiment, which lasted up to 35 weeks. Four weeks after the start of the experiment, all rats received intrarectal instillations of MNNG (Aldrich Chemical; Gillingham, Dorset, UK) at 6 mg/kg body wt, once a week for 5 weeks. MNNG was dissolved in a 1% solution of carboxymethylcellulose in 0.9% NaCl (pH 6.5). The MNNG solution was instilled intrarectally in an amount of 1 ml/kg body wt, at a distance of 6 cm from the anal orifice. Individual growth rate was monitored weekly. Also, average food consumption for each cage was measured weekly. The animals were checked daily for their health. Those who were in poor health or moribund condition were killed and autopsied. At the end of the experiment, all animals were killed by exsanguination via the abdominal aorta under ether anesthesia and subjected to detailed gross examination.

Histopathology

The colon and rectum were opened longitudinally and number, size, gross appearance, and location of tumors were registered. Each tumor was collected and individually processed. The rest of the colon and rectum was prepared as Swiss rolls. For light microscopy all tumorous lesions and Swiss rolls were fixed in 4% phosphate-buffered formalin, embedded in paraffin, cut in 5 μm thin sections, and stained with hematoxylin and eosin. Tumors were classified as sessile adenomas, polypoid adenomas, adenocarcinomas, mucus-producing adenocarcinomas, or signet-ring cell carcinomas, according to the criteria previously described (Rijnkels *et al.*, 1993). In assessing the colorectal tumor incidence, and number of tumors, a distinction was made between adenomas (sessile and polypoid adenomas) and (adeno)carcinomas (adenocarcinomas, mucus-producing adenocarcinomas, and signet-ring cell carcinomas).

Statistics

Differences in weight gain were compared by analysis of variance and differences in tumor incidences by chi-square analysis-of-contingency. The number of tumors was compared by generalised linear regression with Poisson distribution and log link. For all tests a confidence interval of 90% was chosen.

Results

General Observations

The mean body weights of the group fed a high-fat diet without a vegetables-fruit mixture were significantly higher, compared to the other three groups (Table 3). Between the other three groups no significant differences in body weights were observed. Although the caloric composition of the four groups differed, the animals adjusted their daily food intake accordingly (Table 3); rats maintained on a high-fat diet (Groups C and D) showed a lower food intake than rats maintained on a diet low in fat (Groups A and B).

At the end of the study mortality amounted to 2.5%. Ninety-five percent of the colorectal tumors were located less than 5 cm from the rectal orifice.

Table 3. Mean body weight and average food consumption in rats fed the experimental diets.

Diet[a]	No.	Body Weight (g/animal)			Food Consumption (g/day/animal)		
	Rats	Week 1[c]	Week15	Week30	Week 1	Week 15	Week 30
A	30	134±7	432±35	541±39	18.35±1.53	17.80±3.37	16.65±3.13
					(66.1±5.5)[d]	*(64.1±12.1)*	*(60.0±11.2)*
B	30	134±8	432±28	523±41	17.65±0.01	17.70±1.93	17.45±1.48
					(63.7±0.0)	*(63.8±6.9)*	*(62.9±5.3)*
C	30	133±8	480±30[b]	584±47[b]	16.75±0.33	16.65±0.02	16.20±3.30
					(69.1±1.3)	*(68.7±0.1)*	*(66.8±13.6)*
D	30	133±8	440±23	541±31	16.05±0.24	16.40±0.90	16.50±0.78
					(66.3±1.0)	*(67.7±3.7)*	*(68.2±3.2)*

a: Group A: 20e% fat; Group B: 20e% fat + vegetable-fruit mixture; Group C: 40e% fat; Group D: 40e% fat + vegetable-fruit mixture.

b: Statistical significant from Groups A, B and D ($p<0.05$; ANOVA)

c: ± standard deviation.

d: Data in parentheses represent the mean caloric intake (kcal) ± standard deviation per animal per day.

Colorectal Tumor Incidence

Overall, 65% of the animals developed colorectal tumors (Table 4). At a confidence interval of 93%, a vegetables-fruit mixture added to a high-fat diet significantly ($p<0.01$) lowered the number of animals having adenocarcinomas in comparison with animals maintained on a high-fat diet alone. Furthermore, the adenoma incidences tended to be lower for groups fed a vegetables-fruit mixture compared to groups fed no vegetables-fruit mixture.

High-fat diets significantly increased the adenocarcinoma incidence compared to diets low in fat ($p<0.05$). No such enhancement was observed for adenoma incidences.

Number of Colorectal Tumors

Type and number of large bowel tumors are shown in Table 4. Of the animals who developed tumors, 79% did develop no more than one or two tumors. Of all the types of tumors observed 68% were classified as adenomas, of which 75% were of the polypoid type. The remainder type of tumors were adenocarcinomas; no mucus-producing adenocarcinomas or signet-ring cell carcinomas were observed.

Although animals fed a vegetables-fruit mixture containing diet high in fat developed slightly fewer colorectal tumors compared to the other animal groups and animals fed low-fat diets with a vegetables-fruit mixture developed slightly more adenocarcinomas than animals fed the low-fat diet alone, no statistically differences were found among these groups.

Other Tumors

Tumors or tumor like lesions at other locations than colon and rectum were not observed.

Table 4. Colorectal tumor incidence, number and types of MNNG-induced colorectal tumors[a].

Diet	Incidence (%)			Number and Type of tumors			
	Total	Adeno-mas	Adeno-carcino-mas	sessile adenomas	polypoid adenomas	Total No. adenomas	Adeno-carcino-mas
A	**77**	67	23	9	18	**27**	8
B	**60**	50	27	6	19	**25**	15
C	**73**	60	43[b]	4	21	**25**	16
D	**50**	40	13[c]	4	11	**15**	5

a: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; see Table 3 footnote for composition of diets.

b: Statistical significant from Group A ($p<0.05$; Chi-square analysis-of-contingency)

c: Statistical significant from Group C ($p<0.01$; Chi-square analysis-of-contingency)

Discussion

The most relevant observation of the present study with MNNG-treated rats is that adding a vegetables-fruit mixture to a high-fat diet resulted in a significantly lower colorectal adenocarcinoma incidence in comparison to groups maintained on a high-fat diet without a vegetables-fruit mixture. This result demonstrates that the vegetables-fruit mixture interacts with the effect of fat on colorectal carcinogenesis. Furthermore, feeding a vegetables-fruit mixture to the animals tended to lower the adenoma incidences in a non-significant way. Regarding the slight differences in number of colorectal tumors among the groups, especially in number of adenocarcinomas, interpretation of these differences are difficult, not only because no significancies were found, but also because relative few number of tumors did develop.

This anticarcinogenic potency of the vegetables-fruit mixture was even present after the vegetables and fruit used had been processed (e.g. cut in pieces and cooked) and added to the diets in amounts, representative for the mean consumption values for The Netherlands. It is not illogical to assume that the effect observed is attributed to the non-nutrient fraction of the vegetables-fruit containing diets, since nutrients in the diets were adjusted to each other experimental diet. Which non-nutrients in the vegetables-fruit mixture caused this protection cannot be determined from the results of the study, because a whole vegetables-fruit mixture was used and not specific non-nutrients of it. The inhibitory potency of vegetables and fruit or their components on the development of cancer has been described in several studies (Sinkeldam *et al.*, 1990; Srisangnam *et al.*, 1980), including studies in which the animals were maintained on high-fat diets (Alabaster *et al.*, 1995; Deschner *et al.*, 1993). However, it is also been observed that fat counteracts the anticarcinogenic effects of dietary components, such as fiber and cabbage (Bresnick *et al.*, 1990; Nigro, 1981).

The results of the present MNNG-study point more clearly to an inhibiting effect of vegetables and fruit on colon carcinogenesis than the results of our previous similarly designed experiment (Rijnkels *et al.*, 1997), in which colorectal tumors were induced by the indirect acting carcinogen DMH. In the DMH-study, the vegetables-fruit mixture caused a non-significant lower number of colorectal adenomas. There may be two explanations for the difference between the MNNG- and DMH-study. Firstly, MNNG and DMH differ in the way they act as a carcinogen, in that MNNG is a direct acting carcinogen, whereas DMH needs to be metabolized to become carcinogenic. The vegetables-fruit mixture may influence metabolism of DMH. Secondly, although the design of both experiments were almost similar, a new bulk of vegetables and fruit were processed for the MNNG-study. It is known, for example, that the amount of certain

compounds present in vegetables and fruit depends on the harvesting time and the character of the soil (Fenwick *et al.*, 1983; Hocman, 1989). Therefore, the composition of the vegetables-fruit mixture might have been slightly different, hence influencing the results.

The present results were also not in accordance with the results of another previous study, performed in our laboratory (Alink *et al.*, 1993), in which a significant protective effect of a same vegetables-fruit mixture was observed on adenoma incidence only in combination with low-fat diets, while in heat processed human diets high in fat a vegetables-fruit mixture increased rather than decreased the colorectal adenocarcinoma incidence. In that study, the combination of heat processing and the presence of a vegetables-fruit mixture could have enhanced the colorectal tumor development. This may implicate that more dietary factors than fat alone could have interacted with the inhibitory potency of the vegetables-fruit mixture.

In the present study, fat showed a significant enhancing effect on the adenocarcinoma incidence and to a slight non-significant degree on the number of colorectal adenocarcinomas in animals fed diets without a vegetables-fruit mixture. This observation is in accordance with those of Sinkeldam and coworkers (Sinkeldam *et al.*, 1990), who studied combined effects between dietary fat and fiber. In MNNG-treated animals, they observed a distinct increase in colorectal tumor incidence with an increasing level of fat, which was only found when the fiber content of the diet was low. Many animal studies performed by other investigators, focussing exclusively on fat, show that the amount and type of fat is a risk factor for colorectal cancer (Reddy *et al.*, 1987, 1988). Evaluation of their results indicate that fat rich in saturated fatty acids, such as lard, which is the main type of fat in the present study, has tumor promoting capacity. This was also seen in our previous study with DMH-treated rats (Rijnkels *et al.*, 1993). In that study, however, fat also promoted colorectal cancer in the presence of a vegetables-fruit mixture, which was not seen in the present study. The difference between the results of both studies may be explained by the types of carcinogens used to induce colorectal tumors, in that MNNG acts as a direct and DMH as a indirect acting carcinogen.

It is concluded that a vegetables-fruit mixture, added to a high-fat diet in an amount representative for the mean consumption values in The Netherlands, has a significant inhibitory effect on the development of colorectal adenocarcinomas in MNNG-treated rats. Remarkably, this effect was less pronounced when the vegetables-fruit mixture was added to a low-fat diet, pointing to an interaction between vegetables and fruit and the enhancing effect of fat on the development of colorectal cancer.

Acknowledgements

The authors thank J. Haas and B. Weijers (Centre for Small Laboratory Animals, Agricultural University) for technical assistance, and Central Horticultural Auctions (The Netherlands) for providing vegetables and fruit. This study was supported by Dutch Cancer Society Grant LUW93-485.

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Effects of dietary fat and a vegetables-fruit mixture on the development of intestinal neoplasia in the *Apc*^{Min} mouse

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Submitted to Carcinogenesis

Abstract

We have investigated possible interactive effects between dietary fat and a vegetables-fruit mixture in a mouse model for multiple intestinal neoplasia, the Apc^{Min} mouse. For this study, four different diets (A-D) were compared, which either were low in fat, 20 energy percentage (e%; diets A/B) or high in fat, 40e% (diets C/D). 19.5% (wt/wt) of diets B and D were replaced by a freeze-dried vegetables-fruit mixture. The diets were balanced as such that they only differed among each other in fat/carbohydrate content and the presence of specific plant constituents. Because the initiation of intestinal tumors in Apc^{Min} mice occurs relatively early in life, exposure to the diets was already started *in utero*. Mice maintained on a high-fat diet did not develop statistically significant higher numbers of small or large intestinal tumors than mice maintained on a low-fat diet, after three months. A vegetables-fruit mixture added to a low-fat diet significantly lowered multiplicity of small intestinal polyps, but not of colon tumors in male Apc^{Min} mice. Strikingly, addition of a vegetables-fruit mixture to female mice maintained on a low-fat diet and to both sexes maintained on a high-fat diet significantly enhanced intestinal polyp multiplicity. In conclusion, our results indicate that neither a lower fat intake nor consumption of a vegetables-fruit mixture included in a high-fat diet decreases the development of polyps in mice genetically predisposed to intestinal tumor development.

Introduction

Colorectal cancer is one of the leading cancers in the Western world, with significant differences in incidence worldwide (Doll *et al.*, 1981; Steinmetz *et al.*, 1996). Several epidemiological studies have associated these differences mainly to lifestyle, in particular dietary habits (Steinmetz *et al.*, 1996). In general, increased risk is associated with high dietary intake of fat, meat and alcohol, whereas a decreased risk is associated with a high intake of vegetables and to a lesser extent fruits (Bueno-de Mesquita*et al.*, 1997; Reddy, 1995). However, whether diets low in fat and rich in vegetables and fruit may reduce the risk for developing colorectal cancer is still a matter of debate, because both epidemiological and animal studies revealed inconsistent results (Giovannucci *et al.*, 1994; Wynder *et al.*, 1992). Concerning the epidemiological studies, these conflicting results are partly due to confounders, such as recall-bias, uncertainties about the quality of the food consumed and to the genetic heterogeneity within the populations studied (Taubes, 1995). Animal studies performed under controlled conditions do not suffer from these limitations. However, in most of these animal studies dietary compositions are used, which differ markedly from human diets in their source, preparation and content. Furthermore, isolated dietary components are many times used in animal models in amounts far

exceeding daily human intake. Such unrealistic high amounts may limit the predictive value concerning colorectal cancer risk in humans. Further, because diet is complex and dietary factors probably interact, it is likely that these interactions among the different dietary components, rather than individual components, are important risk factors in developing colorectal cancer (Alabaster *et al.*, 1995; Sinkeldam *et al.*, 1990). Apart from the differences in dietary composition, animal studies vary in the chemical carcinogens used for inducing cancer and vary among the species used, which may be different in genetic susceptibility for intestinal carcinogenesis (Pories *et al.*, 1993). These dietary and methodological shortcomings in animal studies warrant the need to perform colorectal carcinogenesis experiments using diets as much as possible imitating realistic regular human diets, taken interactive effects into account. Such an attempt was reported previously (Rijnkels *et al.*, 1997a, 1997b). Recently, in these studies interactive effects between dietary fat and a vegetables-fruit mixture (VFM) were studied on 1,2-dimethylhydrazine (DMH)- and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-induced colorectal carcinogenesis in rats. For these studies, amounts of fat (40 energy percentage, e%) and a freeze-dried vegetables-fruit mixture (19.5% wt/wt) were used resembling the mean human consumption and composition values in The Netherlands. A vegetables-fruit mixture, however, only decreased colorectal carcinogenesis in the MNNG model, whereas dietary fat alone enhanced colorectal tumor development in both the DMH and the MNNG animal models. Therefore, it is suggested that the preventive properties of the vegetables-fruit mixture depended mainly on the chemical carcinogen used to induce colorectal cancer, which ascribes the importance to take methodological limitations into account (Pories *et al.*, 1993; Reddy, 1995).

The results of the DMH- and MNNG-study focussed our interest to study possible interactive effects between dietary fat and a vegetables-fruit mixture in an animal model using mice with a genetically altered gene, which is known to be rate-limiting in the initiation of both inherited and somatic colorectal cancer. The *adenomatous polyposis coli* (*Apc*) gene on chromosome 5q is considered to be a gatekeeper gene for the intestine, contributing to the formation of a benign adenoma (polyp), one of the first stages of colorectal cancer (Kinzler *et al.*, 1997; Powell *et al.*, 1992). In the majority of colorectal neoplasia, the *Apc* tumor suppressor gene is either mutational inactivated by the introduction of premature stop codons or deleted by loss of heterozygosity or both. In recent years introducing specific germ line mutations in the genes known to be involved in colorectal cancer has generated several inbred mouse models. Among these intestinal mouse models, mice with defects in the *Apc* gene are at present among the most promising because of their genotype-phenotype correlation (Dove *et al.*, 1995; Fodde *et al.*, 1995). To date, several mouse lineage's heterozygous for specific mutations at the endogenous *Apc* gene have been

developed and characterized with respect to their intestinal tumor multiplicity (Bilger*et al.*, 1996; Shoemaker *et al.*, 1997). Two of these strains of mutant *Apc* mice, Apc^{Min} (multiple intestinal neoplasia) and $Apc^{\Delta 716}$ are characterized by a relatively high tumor multiplicity in an identical inbred C57BL6 genetic background (approx. 20-90 and 200-500 small intestinal tumors per mice in Apc^{Min} and $Apc^{\Delta 716}$ respectively; Moser *et al.*, 1989; Oshima *et al.*, 1995).

The present study was performed to study the interactive effects between dietary fat (20- and 40e%) and a vegetable-fruit mixture (19.5% wt/wt), with exactly the same dietary composition as the DMH- and the MNNG-study (Rijnkels*et al.*, 1997a, 1997b) on intestinal neoplasia in Apc^{Min} mice. Because the onset of intestinal carcinogenesis in Min mice is assumed to occur relatively early in life (Laird *et al.*, 1995; Shoemaker *et al.*, 1995) exposure to the four experimental diets was already started during mating, continued through weanling and lasted till they were three months old.

Materials and methods

Animals, housing and clinical observations

Min (C57BL/6J-$Apc^{Min/+Apc}$) heterozygote mice were obtained from a colony at Leiden University (R. Fodde, MGC-Dept. Human Genetics), established with *Min* mice obtained from the original colony at the McArdle Laboratory (W.F. Dove, McArdle labs, University of Wisconsin, Madison). They were bred with C57BL/6JIco (+/+) females purchased from Broekman Institute B.V. in the Netherlands. Using DNA isolated from mouse tails, progeny were genotyped before weaning by an allele-specific PCR for the nonsense mutation at codon 850 (Su *et al.*, 1992) as described by Jacoby *et al.* (1996). The total number of 120 *Min* mice (60 males and 60 females) for this experiment were obtained from 56 breeding couples, each containing one male C57BL/6J-$Apc^{Min/+Apc}$ mouse and two female C57BL/6J-$^{+Apc/+Apc}$ mice. Already from mating these breeding couples were randomly divided in the 4 dietary groups to which the different diets (A, B, C, D) were allocated *ad libitum*. After weaning, mice were housed in groups of 2-6 animals of the same sex per cage under controlled environmental conditions (temperature 21 ± 1°C, relative humidity 53% ± 2%, 12/12 light-dark cycle, air changed 8 times/h) and had free access to food and tap water. Body weight and food intake was recorded weekly. All mice were observed at least once a day for any abnormalities in clinical appearance until sacrifice.

Diets

A detailed description of the diets tested is described elsewhere (Rijnkels *et al.*, 1997a). In brief, the following four diets were used: a diet low in fat (20e%; Diet A), a diet low in fat (20e%) to which a vegetables-fruit mixture was added (19.5% wt/wt; Diet B), a diet high in fat (40e%; Diet C) and a diet high in fat (40e%) to which the same vegetables-fruit mixture was added (19.5% wt/wt; Diet D) as in Diet B. The semi-synthetic Muracon-SSP/tox animal diet (Hope Farms, Woerden, The Netherlands) served as the basal diet for the four different diets.

The fat in the diets was a mixture of lard and sunflower oil, resulting in a fatty acid composition similar to an average human diet in The Netherlands. The extra 20e% fat added to Diets C and D was at the expense of carbohydrates. In Diets B and D, a vegetables-fruit mixture was supplied to the Muracon-SSP/tox diet. The choice of the vegetables and fruit used approached the mean vegetables and fruit consumption in The Netherlands. The following products were separately cooked; potatoes, cauliflower, spinach, leek, red and white cabbage, sauerkraut, carrots, Brussels sprouts, and beet. After freezing (-40°C) the mixture was freeze-dried, ground, and homogenised. Before the vegetables-fruit mixture was mixed with the basal Muracon-SSP/tox and pelleted, it was analysed for nutrient, vitamin and mineral content (data not shown). The final composition of the diets is shown in Table 1. As a consequence of adjusting the diets for protein, mineral and vitamin contents, the diets differed only in the fat/carbohydrate content and in the presence of specific constituents (type of fiber, non-nutrients) in the vegetables-fruit mixture. Finally, the pelleted diets were stored at -40°C in air-closed plastic bags before use in the experiment. No antioxidants or other preservatives were added.

Tissue Sampling and Scoring Tumors

Mice were sacrificed by euthanasation with KRA around day 90 (range 85-93 days) after birth. This point in time was chosen to minimize the risk of intercurrent mortality due to severe progressive anemia, rectal prolaps or intestinal obstruction typically for Apc^{Min} mice around day 120 (Moser *et al.*, 1989). At post mortems, the intestines (except the rectum) were isolated, spread onto filter paper, dissected longitudinally with fine scissors and mucus and faeces were removed. The small intestine was separated from the colon and divided in three approximately equal sections (length ± 11 cm). These sections were macroscopically examined by a biotechnician unaware of the animal's diet, to record polyp number, size and location. After this macroscopic examination, Swiss rolls were made of the small intestines and colon, fixed overnight at 4°C in 4% cold neutral buffered formaldehyde, washed twice with 70% ethanol, and embedded in paraffin.

Table 1. Final composition of the four experimental diets[a]

Ingredients	Diet A[b] *low fat*	Diet B *low fat + VF*	Diet C *high fat*	Diet D *high fat + VF*	VF-mixture[h]
fat[c]	8.50	8.50	20.00	20.00	0.40
carbohydrate[d]	48.34	30.39	30.78	10.14	63.00
protein[e]	23.19	23.19	26.50	26.50	8.80
fiber (cellulose)	10.67	9.80	12.20	11.20	14.20
vitamins[f]	0.35	0.31	0.40	0.35	0.80
minerals[g]	8.95	8.31	10.12	9.51	1.30
VF-mixture	-	19.50	-	22.30	
water					11.50
kcal/kg	**3603**	**3608**	**4126**	**4132**	**3548**

a: Values (% wt/wt) are calculated from analyses and represent approximate composition.

b: Diet A is the basal Muracon-SSP/tox diet. In Diets C and D the carbohydrate, protein, fiber, vitamin and mineral contents of the Muracon-SSP/tox were adjusted to allow for decreased food consumption in animals consuming diets of high caloric density. In Diets B and D the amounts of carbohydrate, fiber, vitamins and minerals of the Muracon-SSP/tox were adjusted for the presence of these constituents in the vegetables-fruit mixture (VF-mixture).

c: 84 % lard and 16 % sunflower oil.

d: Diet A: 23.33 g/100 g cerelose and 25.10 g/100g corn starch.

e: Diet A: 18.55 gram/100 g acid casein and 4.64 gram/100 g soy protein

f: Diet A: cholin Cl (50%) 0.303 g/100g, vitamin A 0.34 mg/kg, vitamin A/D$_3$ 1.50 mg/kg, vitamin E (50%) 6.54 mg/kg, vitamin K$_3$ 0.360 mg/kg, thiamin 0.650 mg/kg, riboflavin 0.920 mg/kg, pyridoxine HCl 0.270 mg/kg, niacin/nicotin 4.60 mg/kg, calcium pantothenate 2.86 mg/kg, vitamin B$_{12}$ 2.02 mg/kg, folic acid 0.300 mg/kg, biotin 2% 1.375 mg/kg, inositol 13.79 mg/kg,β-carotene 10% 2.86 mg/kg, vitamin C 12.39 mg/kg.

g: Diet A: $CaHPO_4.2H_2O$ 3.34 g/100g, KCl 0.93 g/100g, NaCl 0.37 g/100g, Na_2CO_3 0.46g/100g, methionine synthetase 0.09 g/100g, PABA 18.18 mg/kg, MgO 90.93 mg/kg, $FeSO_4H_2O$ 13.24 mg/kg, $ZnSO_4.5H_2O$ 7.00 mg/kg, $CoSO_4$ 0.225 mg/kg, ammonium heptamolybdate 0.01 mg/kg, NaF 0.01 mg/kg, Ca-iodate 0.05 mg/kg, $AlK(SO_4)_2.12H_2O$ 6.59 mg/kg, $CrCl_3.6H_2O$ 0.02 mg/kg, SiO_2 2.79 g/100g.

h: Composition (% wet wt): potatoes (35.1), bananas (3.0), oranges (9.0), apples (19.10), lettuce (3.75), green pepper (1.25), tomatoes (3.75), cucumbers (3.75), cauliflower (3.75), spinach (2.50), leek (2.50), red (2.5), and white cabbage (2.5), sauerkraut (2.5), carrots (1.25), Brussels sprouts (1.25), beet (2.50). Total glucosinolate content near detection level (i.e. glucobrassicin), flavonoid content 640 mg/100 g (i.e. quercetin, catechin, naringenin, hesperetin).

Hematoxylin/eosin-stained 5 μm sections were histopathologically examined (degree of dysplasia was scored as slight, moderate or severe). A selection of colon tumors were collected separately, snap frozen in liquid nitrogen and stored for further analysis.

Statistical analysis

Differences in body weights were compared by analysis of variance and differences in tumor multiplicity by linear regression with log-link and Poisson distribution. For all tests a confidence interval of 95% was chosen ($p \leq 0.05$).

Results

General observations

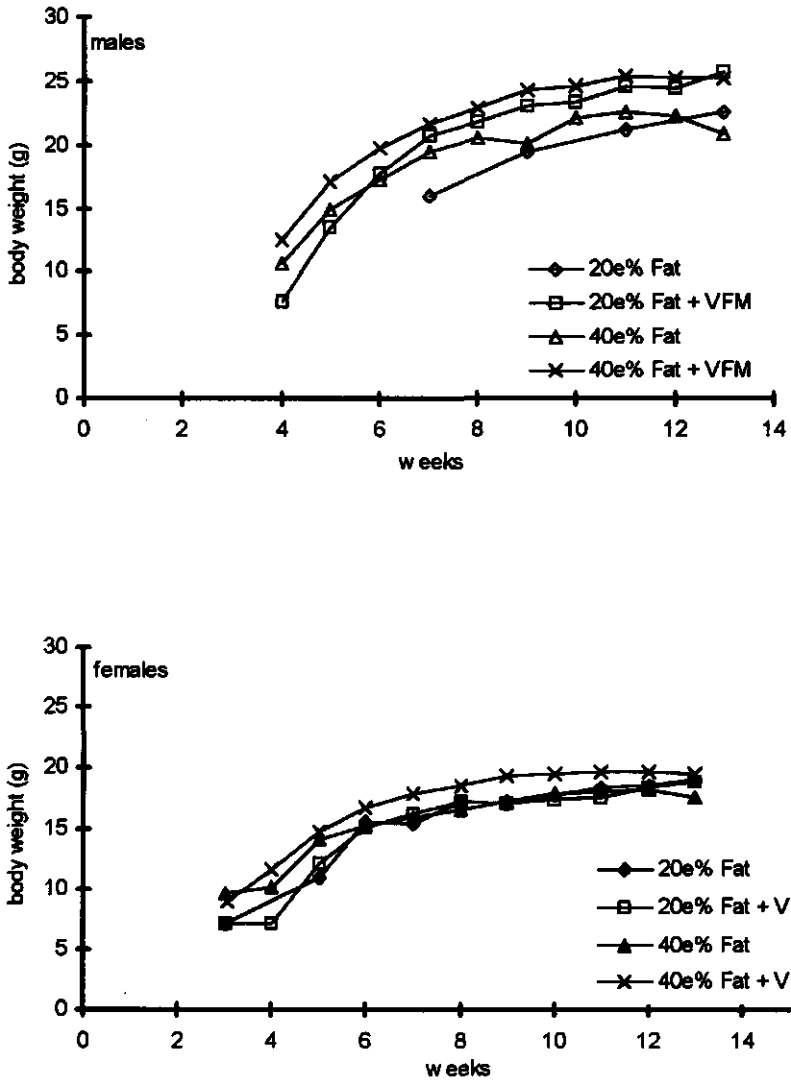
Although the body weights of mice consuming a vegetables-fruit mixture in their diets (B, D) were in general somewhat higher (most pronounced for female mice), no statistically significant differences in body weights were observed between the four diet groups (Figure 1). This is in line with the data on daily food-intake (not shown), which indicate that in particular the high-fat diet, to which a vegetables-fruit mixture was added, was consumed on average 15% (wt/wt) more than the other diets. Although the diets A and B are isocaloric, and this also holds for diets C and D, the differences in food-intake of the diets containing a vegetables-fruit mixture could have resulted in a slightly increased caloric intake for mice on respectively diets B and D compared to diets A and C.

Intestinal tumor incidence and multiplicity (Table 2)

All tumors in the small intestine as well as in the colon were classified as benign adenomas (polyps) with no local invasion of the lamina propria. On average, mice developed between 10 and 30 polyps in the small intestine, while in colon it ranged between 0 and 2 tumors per mice.

As expected, almost all mice (71-100%) developed multiple small intestinal tumors. Therefore, no significant effects of fat or a vegetables-fruit mixture on tumor incidence values for the small intestine were observed. For the colon, the tumor incidences were generally lower (40-80%). The colon tumor incidence was decreased, although not statistically significant, for male mice maintained on a low-fat diet including a vegetables-fruit mixture, whereas it was increased, again not statistically significant, in mice maintained on a high-fat diet including a vegetables-fruit mixture.

Figure 1. Mean body weights of male and female *Apc*^{Min} mice fed diets low in fat (Diet A), high in fat (Diet C) or fed diets including a vegetables-fruit mixture (VFM) in low (Diet B)- or high (Diet D)-fat diets.



Mice consuming a high-fat diet, without further additions, did not develop different numbers of small or large intestinal tumors than mice consuming a low-fat diet. A vegetables-fruit mixture added to a low-fat diet caused a significant lower multiplicity of small intestinal polyps, but not of colon tumors, in male Apc^{Min} mice. In contrast, in female mice maintained on a low-fat diet and in both sexes maintained on a high-fat diet, addition of a vegetables-fruit mixture significantly enhanced the small intestinal polyp multiplicity. In the colon, this observation was also significant in mice fed a vegetables-fruit mixture added to high-fat diets. Furthermore, in females fed a low-fat diet, a vegetables-fruit mixture tended to enhance colon tumor development.

Intestinal tumor size, localization and the degree of dysplasia (Table 2)

The mean size of the polyps in the small intestine averaged 2 mm in both males and females, while in the colon it averaged 2.6±0.4 (SD) mm in males and 3.2±0.9 mm in females. The mean size of small and large intestinal polyps differed not significantly among the dietary groups.

Although not significant, small intestinal polyps tended to be more localized in the duodenum in the females of Groups B, C, and D and in males of Group B, whereas in the males of Group A polyps tended to be more localized in the ileum. In the colon, more than 80% of the tumors observed in females of Groups B, C, and D and in males of Group C were found proximal, between 4 and 7 cm from caecum.

Although the tumor localization and size were not influenced by the diets applied, the degree of dysplasia of the polyps was not the same for all four diets (data not shown). In the small intestine, more polyps with slight dysplasia developed in animals maintained on a high-fat diet compared with the groups maintained on low-fat diets. In the colon, a small (low-fat) to substantial (high-fat) increase in the number of moderate dysplastic polyps was observed upon addition of a vegetables-fruit mixture.

Discussion

In the present study, dietary fat, consumed in an amount and with a fatty acid composition representative to the mean consumption and composition values in The Netherlands, showed no effect on the development of intestinal neoplasia in *Apc* deficient mice. Furthermore, the study of Kranen *et al.* revealed no effect of the same high-fat diets (40e%) on intestinal carcinogenesis in *Apc1638N* mice (Submitted). However, the present result was not similar with findings of Hioki *et al.* (1997) and Lipkin *et al.* (1996), who

Table 2. *Intestinal polyp incidences, multiplicity and localization of intestinal polyps in Apc^{Min} mice maintained on the experimental diets.*

Diet[a]	Sex	No. of Animals	Incidence, %		Multiplicity [b,d]		Localization polyps, %[b,c,d]		
			Small Intestines	Colon	Small Intestines	Colon	Section 1	Section 2	Section 3
A	Male	15	100	80	16.4±0.3	1.2±0.01	30.9±13.7	23.6±35.9	45.5±32.0
B		15	100	53	9.5±0.2**	1.0±0.02	50.0±27.7	18.2±35.3	32.8±51.6
C		16	88	63	14.9±0.2	1.1±0.02	31.4±19.1	31.0±21.7	37.6±21.4
D		17	100	76	27.7±0.3*	2.1±0.03*	34.4±14.7	30.0±22.9	35.6±24.2
A	Female	14	71	71	11.9±0.3	0.9±0.01	34.2±26.8	34.1±48.8	31.7±60.5
B		16	94	63	15.8±0.2**	1.4±0.03	48.6±13.0	20.2±18.8	31.2±28.6
C		15	80	40	12.8±0.2	0.5±0.01	42.7±20.0	31.3±27.5	26.0±36.4
D		16	100	75	25.8±0.2*	1.3±0.02***	42.4± 9.2	29.1±18.7	28.5±25.7

a: Diet A, 20e% fat; Diet B, 20e% fat + VFM; Diet c, 40e% fat; Diet D, 40e% fat + VFM.

b: Mean values ± standard error of the mean.

c: Section 1: duodenum+first part jejunum (length 11 cm); section 2: jejunum (length 11 cm); section 3: last part jejunum+ileum (length 11 cm).

d: Statistical significance is as follows: * $p<0.01$ between Group B/C and D; ** $p<0.01$ between Group A and B; *** $p<0.01$ between Group C and D.

provided Western-style diets to $Apc^{\Delta 716}$ and *Apc1638N* mice respectively. They reported increased intestinal polyp numbers and colonic polypoid hyperplasia respectively. Overall, the amount and composition (saturated and omega-6 polyunsaturated fatty acids) of fat in Western-style diets, such as consumed in The Netherlands, are associated with higher risk for developing colorectal cancer, whereas diets containing mainly fats composed of omega-3 fatty acids (fish oil) are associated with a lower risk (Reddy, 1995). In addition, it is reported that Apc^{Min} and $Apc^{\Delta 716}$ mice, maintained on diets enriched with omega-3 fatty acids, were protected against development of intestinal neoplasia (Paulsen*et al.*, 1997). The presently observed absence of effect by dietary fat on intestinal tumor development may be partly ascribed to differences in dietary composition of the commercial rodent diets, apart from the dietary fat composition, in comparison with diets used by other investigators. Because interactive effects between dietary components are proposed, effects of fat on colorectal cancer may be differentially modulated depending on the type of rodent diets used. Furthermore, the present study differed with most other studies in that exposure of the diets started *in utero*, in order to include possible effects of fat as early as possible during the life span of the Apc^{Min} mice. However, in most other studies, diets were distributed to *Apc* deficient mice and other rodent species after weaning.

Remarkably, the present study shows that a reasonable amount of specific plant constituents present in the vegetables-fruit mixture hardly protected against intestinal tumor development in Apc^{Min} mice. To the contrary, the mixture enhanced rather then inhibited tumor development in mice maintained on high-fat diets. This observation is not in accordance with some epidemiological and other animal experiments, in which an inverse association is suggested between vegetables and fruit and intestinal cancer risk. Furthermore, protective effects on intestinal neoplasia in *Apc* deficient mice have been reported for specific plant constituents, such as for the soybean-derived Bowman-Birk inhibitor and less pronounced for high fiber administered as short-chain fructo-oligosaccharides (Kennedy *et al.*, 1996; Pierre *et al.*, 1997). In the present study no clear protection was observed by the vegetables-fruit mixture against intestinal carcinogenesis. This may be ascribed to the use of whole vegetables and fruit, whereas in the aforementioned studies isolated constituents were used. Moreover, vegetables and fruit were used in regular amounts. Furthermore, it cannot be excluded that a vegetables-fruit mixture enhanced intestinal carcinogenesis in mice due to an increased caloric intake in comparison with mice fed no vegetables-fruit mixture. However, this explanation is less likely, because it appears not valid for the low-fat diet results. Therefore, it is suggested that vegetables and fruit may have stimulatory activities in high-fat diets, whereas it may not stimulate or even prevent effects of low amounts of fat on intestinal carcinogenesis in *Apc* deficient male mice, pointing to an interaction between dietary fat and a vegetables-fruit mixture.

The presently used experimental diets revealed different outcomes when provided to rats in which colorectal cancer was induced by 1,2-dimethylhydrazine or MNNG, as reported previously (Rijnkels *et al.*, 1997a, 1997b). In both animal models, fat significantly enhanced colorectal carcinogenesis, whereas a vegetables-fruit mixture showed only a pronounced protection against colorectal carcinogenesis in the MNNG-study. Apart from the use of chemical carcinogens, differences in species and the duration of the experiments, the different outcomes between these two studies and the presently used *Apc* deficient mice may be ascribed to differences in genetic susceptibility. In the human situation, deficiencies at the *Apc* gene are found in patients having familial adenomatous polyposis and in many patients having somatic colorectal cancer. *Apc* deficient mice are a valuable model for these human diseases. Therefore, the present results indicate that consumption of reasonable amounts of vegetables and fruit in diets high in fat may enhance tumor progression in persons who are genetically predisposed. To further elucidate the relationship between genetic defects on the *Apc* gene and dietary habits additional research is needed.

In conclusion, the results of the present study showed absence of an effect by dietary fat in mice having deficiencies on the *Apc* gene. Notably, a vegetables-fruit mixture added to low-fat diets did not consistently protect against intestinal carcinogenesis, whereas the same mixture added to high-fat diets may have enhanced rather than inhibited small and large intestinal carcinogenesis.

Acknowledgements

We thank C. Moolenbeek, J. Assink, and J. Wijnands for skillful technical assistance, Central Horticultural Auctions (The Netherlands) for providing most vegetables and fruit, and the Dutch Cancer Society for partly financial supporting this study (Grant LUW93-485).

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4

Absence of an inhibitory effect of a vegetables-fruit mixture on the initiation and promotion phase of azoxymethane-induced colorectal carcinogenesis in rats fed low-or high-fat diets

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Nutrition and Cancer Int J. 30, issue 2, 1998.

Abstract

The potential inhibitory effects of a vegetables-fruit mixture on the initiation and promotion phase of azoxymethane (AOM)-induced colorectal carcinogenesis were examined in rats fed either low- or high-fat diets. Therefore, rats, randomly divided in eight groups, were fed diets low in fat (20 energy percentage (e%); Diets A/B) or high in fat (40e%; Diets C/D), either supplemented with a vegetables-fruit mixture (19.5% wt/wt; Diets B/D) or not (Diet A/C) for 36 weeks. The animals were maintained on the respective diets for four weeks, thereafter, they were given three weekly subcutaneous injections of AOM at 15 mg/kg body wt. Eight weeks after the start of the study, diets of animals maintained on Diet A were replaced by Diet B (Group A→B), Diet C (Group A→C) or were maintained on the same diet (Group A→A), as indicated by the different characters, which stand for the type of diets given. Diets of animals maintained on Diet B or Diet D, were replaced by Diet A or Diet C respectively (Groups B→A, D→C). Furthermore, diets of animals maintained on Diet C were replaced by Diet A (Group C→A), Diet D (Group C→D), or were maintained on the same diet (Group C→C). Multiplicity of colorectal tumors did not differ between groups fed a vegetables-fruit mixture given either during the initiation phase or either during the promotion phase (B→A vs. A→B; D→C vs. C→D). However, multiplicity was significantly lower in animals fed low-fat diets in comparison with animals fed high-fat diets, in combination with a vegetables-fruit mixture (A→B/B→A vs. C→D/D→C). Furthermore, multiplicity was significantly increased in groups fed a high-fat diet during the promotion phase only, in comparison with animals fed a low-fat diet during the whole experiment (A→C vs. A→A). No other differences in multiplicity, or tumor incidences were observed among the eight experimental groups.

Introduction

It is assumed that consumption of high amounts of fat or meat and low amounts of vegetables and fruit are associated with the development of colorectal cancer in men. However, whether these dietary factors are indeed causative related with colorectal cancer is a matter of debate, since epidemiological and animal studies do not consistently report such a relationship (Doll *et al.*, 1981; Potter, 1995, 1996; Schatzkin *et al.*, 1995; Smith *et al.*, 1985; Weisburger, 1991). The discrepancies between the results of the various animal studies performed may be ascribed, at least partly, by the fact that meals are composed of various food components, whereas many investigators used standard chow supplemented with an isolated component of the food. A mixture of food components can either positively or negatively interact with each other, resulting in either and enhancing or inhibitory effect on carcinogenesis (Newberne *et al.*, 1986). For instance, it has been reported that the inhibitory effect of fiber on colorectal carcinogenesis was completely abolished when present in high-fat diets (Sinkeldam *et al.*, 1990), and the inhibitory effect of a vegetables-

fruit mixture on 1,2-dimethylhydrazine (DMH)-induced colorectal cancer completely disappeared when present in heat-processed human diets high in fat (Alink*et al.*, 1993). Up to now, only a few studies have been published about combination effects of dietary components on colorectal carcinogenesis.

In assessing and understanding interactive effects between food components, we have conducted a series of studies, in which the possible interactive effects of low- and high-fat diets combined with a vegetables-fruit mixture on colorectal carcinogenesis were examined in more detail. We have found previously that diets containing a vegetables-fruit mixture did not have a pronounced inhibitory effect on DMH-induced colorectal tumor development, irrespective of the fat content of the diets (Rijnkels *et al.*, 1997a). However, a similar vegetables-fruit mixture added to a high-fat diet caused a significant decrease in adenocarcinoma incidence in *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)-treated animals (Rijnkels *et al.*, 1997b). Based on these results we concluded that the vegetables-fruit mixture used in these experiments has an inhibitory effect on colorectal carcinogenesis, depending on the carcinogen used to induce colorectal cancer. The most marked difference between DMH and MNNG is that DMH, an indirect-acting carcinogen, needs to be metabolized to become an ultimate carcinogen, whereas MNNG reacts directly with DNA (Druckrey *et al.*, 1972; IARC Monographs, 1987). Vegetables and fruit modulate xenobiotic metabolism and as such interfere with carcinogenesis. But also other possible inhibitory mechanisms of vegetables, fruit or plant constituents are reported, such as radical scavenging, stimulation of DNA-repair, antiproliferative and -progressive activities (Hartman *et al.*, 1980; Morse *et al.*, 1993; Wattenberg, 1992; Webster *et al.*, 1996). These other potential inhibitory mechanisms of the vegetables-fruit mixture may have resulted in decreased colorectal tumor incidence in animals of the MNNG-study. Concerning the DMH-study, DMH metabolism was not influenced by the vegetables-fruit mixture only, but DMH metabolism may have furthermore restricted other inhibitory effects of this vegetables-fruit mixture.

To examine the effect of the vegetables-fruit mixture used in the DMH- and MNNG-study on the carcinogenesis process in more detail, an experiment was designed in which the inhibitory potency of a vegetables-fruit mixture was investigated on the initiation and the promotion phase, using both low- and high-fat diets. The same diets were used as in the DMH and MNNG studies (Rijnkels *et al.*, 1997a, 1997b). Azoxymethane (AOM), a DMH metabolite, was used to induce colorectal cancer, since this carcinogen needs only two or three injections to induce colorectal cancer, which leads to a better differentiation between the initiation and the promotion phase.

Materials and Methods

Animals

Five-week-old male Fischer-344 rats [CDF/CrlBR, Charles River GmbH, Sulzfeld, Germany] were randomly divided into eight groups of thirty animals each. Each group was given one of the four experimental diets (see below). The animals were housed in plastic cages (3/cage) with wire tops and sawdust bedding. The cages were randomly placed in an animal isolator (Isotec; Harlan, Zeist, The Netherlands) under negative pressure. One week after the AOM injection, the animals were placed in a normal animal room. Furthermore, during the experiment they were kept in a temperature (±22°C)- and humidity (50-60%)-controlled room with artificial lighting (12:12-hour light-dark cycle). They had free access to diet and tap water. Diets were refreshed weekly.

Table 1. Final composition of the four experimental diets[a]

Ingredients	Diet A[b] low fat	Diet B low fat+VF	Diet C high fat	Diet D high fat+VF	VF-mixture[g]
Fat[c]	8.50	8.50	20.00	20.00	0.40
Carbohydrate[d]	48.34	30.39	30.78	10.14	63.00
Protein[e]	23.19	23.19	26.50	26.50	8.80
Fiber (cellulose)	10.67	9.80	12.20	11.20	14.20
Vitamins[f]	0.35	0.31	0.40	0.35	0.80
Minerals[f]	8.95	8.31	10.12	9.51	1.30
VF-mixture	-	19.50	-	22.30	
kcal/kg	3603	3608	4126	4132	3548

a: Values are expressed as % (wt/wt), and are calculated from analyses, and represent approximate composition.

b: Diet A is the basal Muracon-SSP/tox diet.

c: 84 % lard and 16 % sunflower oil.

d: Diet A: 23.33 g/100 g cerelose and 25.10 g/100g corn starch.

e: Diet A: 18.55 gram/100 g acid casein and 4.64 gram/100 g soy protein.

f: See Rijnkels *et al.* (1997b) for exact vitamin and mineral contents.

g: Composition (% wet wt): potatoes (35.1), bananas (3.0), oranges (9.0), apples (19.10), lettuce (3.75), green pepper (1.25), tomatoes (3.75), cucumbers (3.75), cauliflowers (3.75), spinach (2.50), leek (2.50), red cabbage (2.5), white cabbage (2.5), sauerkraut (2.5), carrots (1.25), Brussels sprouts (1.25), beetroot (2.50). Total glucosinolate content near detection level (i.e. glucobrassicin), flavonoid content 640 mg/100 g (i.e. quercetin, catechin, naringenin, hesperetin).

Diets

The following four diets were used: a diet low in fat (20e%, Diet A); a diet low in fat (20 energy% (e%)) to which a vegetables-fruit mixture was added (19.5% wt/wt, Diet B); a diet high in fat (40e%, Diet C); a diet high in fat (40e%) to which the same vegetables-fruit mixture used in Diet B was added (19.5% wt/wt, Diet D). The semisynthetic Muracon-SSP/tox animal diet (Hope Farms, Woerden, The Netherlands) served as basal diet for these four different diets.

The composition of fat, the choice of the vegetables and fruit, and the preparation of the vegetables-fruit mixture are described in detail elsewhere (Rijnkels *et al.*, 1997a). In short, the fatty acid composition was similar to an average human diet in The Netherlands. The extra 20e% fat added to Diets C and D was at the expense of carbohydrates. Through this expansion, the caloric density of the high-fat diets was higher than of the low-fat diets. To allow for decreased food consumption, in animals consuming diets of higher caloric density, the protein, mineral and vitamin contents of the basal Muracon-SSP/tox in the high-fat diets were adjusted to the low-fat diets, in such a way that all animals had approximately the same intake of these dietary constituents. The vegetables-fruit mixture was analyzed on nutrient, mineral and vitamin content to adjust these amounts in the Muracon-SSP/tox, used for the vegetables-fruit containing diets. Therefore, diets containing a vegetables-fruit mixture had approximately the same nutrient, mineral and vitamin content as diets without a vegetables-fruit mixture. Consequently, the diets differed only in fat/carbohydrate content and in the presence of specific constituents (type of fiber, non-nutrients) in the vegetables-fruit mixture. Table 1 shows a detailed composition of the diets used.

Experimental Design

All animals were maintained on one of the four experimental diets (Diets A, B, C, or D) from the beginning of the experiment, which lasted up to 36 weeks. Four weeks after the start of the experiment all rats were subcutaneously injected with AOM (cas.no. 25842-45-2; Sigma Chemical, St.Louis, MO, USA) at 15 mg/kg body wt once a week for 3 weeks. AOM was dissolved in 0.9% saline (final solution of 1 ml/kg body wt). Two weeks after the last AOM injection, diets of groups maintained on Diet A were replaced by Diet B (Group A→B) or C (Group A→C), or were maintained on Diet A (Group A→A; Figure 1). Diets of groups maintained on Diet B were replaced by Diet A (Group B→A). Diets of groups maintained on Diet C were replaced by Diet A (Group C→A) or D (Group C→D), or were maintained on Diet C (Group C→C). Diets of groups maintained on Diet D were replaced by Diet C (Group D→C).

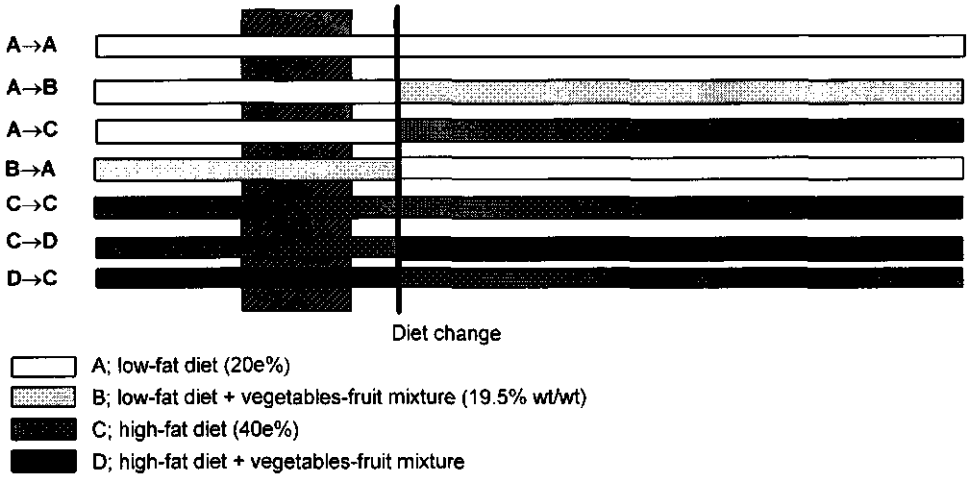
Individual growth rate was monitored weekly. Also, average food consumption for each cage was measured weekly. The animals were checked daily for their health. Those who

were in poor health or in moribund condition were killed and autopsied. At the end of the experiment, all animals were killed by exsanguination via the abdominal aorta under ether anaesthesia and subjected to detailed gross examination.

Histopathology

The colon and rectum were opened longitudinally, and number, size, gross appearance, and location of tumors were registered. Each tumor was collected and individually processed. The rest of the colon and rectum was prepared as Swiss rolls. For light microscopy, all tumorous lesions and Swiss rolls were fixed in 4% phosphate-buffered formalin, embedded in paraffin, cut in 5-mm thin sections, and stained with hematoxylin and eosin. Tumors were classified as sessile adenomas, polypoid adenomas, adenocarcinomas, mucus-producing adenocarcinomas, or signet-ring cell carcinomas, according to the criteria previously described (Alink *et al*, 1993). In assessing the colorectal tumor incidence, number of tumors and multiplicity, a distinction was made between adenomas (sessile and polypoid adenomas) and (adeno)carcinomas (adenocarcinomas, mucus-producing adenocarcinomas, and signet-ring cell carcinomas).

Figure 1. Experimental design. The horizontal bars represent the time axis of the experiment. Diets were provided from the start of the experiment. Shadings of the horizontal bars represent the type of diets which were given. Eight weeks after the start of the experiment, diets were replaced, as indicated by altered shadings of the bars. The vertical gray bar represents the period animals were treated with AOM. Of each group, the first character stands for type of diet given in the first eight weeks of the experiment, the second character stands for the type of diet given for the rest of time the experiment lasted. See also **Materials and Methods.**



The multiplicity is a relative measure of the tumor rate, which is defined as the number of tumors divided by the number of tumor-bearing animals.

Statistics

Differences in body weight were compared by analysis of variance, and differences in tumor incidences by chi-square analysis-of-contingency-table. The numbers of tumors were compared by Kruskall-Wallis statistic, and multiplicity by linear regression with log-link and Poisson distribution. For all tests a confidence interval of 95% ($p<0.05$) was chosen.

Results

General Observations

No significant differences in body weights were observed among the eight different groups (Table 2). Although the caloric composition of the diets differed, the animals roughly adjusted their daily food intake accordingly (Table 2); animals maintained on a high-fat diet (Diets C/D) showed a slightly lower food intake than rats maintained on a low-fat diet (Diets A/B). At the end of the experiment, mortality amounted to 25%, which was not significantly different among the eight groups. The effective number of animals per group was thirty.

Table 2. Mean body weights and food intake of animals in experiment.

Group[a]	Body Weight, g/animal[b]			Food consumption, g/day/animal[b]		
	Week 1	Week 8	Week 24	Week 1	Week 8	Week 24
A→A	122.5±7.3	265.2±12.8	380.8±19.2	12.7±0.5	13.6±0.5	13.4±0.5
A→B	119.6±5.9	265.0±10.3	369.4±17.6	12.3±0.5	13.5±0.4	13.9±0.3
A→C	121.7±6.9	272.0±12.8	380.8±19.2	12.7±0.5	13.6±0.2	12.9±0.5
B→A	119.7±7.1	265.0± 9.1	366.4±23.7	12.4±0.5	13.7±0.4	13.7±0.3
C→A	122.2±7.4	269.8±11.9	375.3±22.1	12.2±0.5	12.8±0.4	13.5±0.6
C→C	120.6±8.1	268.9±15.0	380.0±26.7	11.8±0.5	12.3±0.6	12.6±0.6
C→D	123.3±6.7	278.5±12.0	391.5±24.7	12.2±0.5	12.9±0.3	13.0±0.3
D→C	122.0±6.4	268.8±14.1	380.9±29.7	11.9±0.3	12.5±0.6	12.5±0.9

a: First character indicates type of diet given in the first eight weeks of the experiment, the second character indicates type of diet given for the rest of time the experiment lasted. A; low-fat diet (20e%), B; low-fat diet + vegetables-fruit mixture (19.5% wt/wt), C; high-fat diet (40e%), D; high-fat diet + vegetables-fruit mixture.

b: Values are means ± standard deviation.

Colorectal Tumor Incidence

No significant differences in colorectal tumor incidence were observed among the eight groups (Table 3). Almost all animals developed tumors (94.6%): 87.9% of all animals developed adenomas, whereas 72.5% developed (adeno)carcinomas.

Type, Number, and Multiplicity of Colorectal Tumors

Adenomas were the most frequently observed (66.4%) type of tumors. The other tumors were (adeno)carcinomas. Of the adenomas 35.5% were characterised as sessile, and 64.5% as polypoid. Most carcinomas were classified as adenocarcinomas (93.2%), 1.2% were mucus-producing adenocarcinomas and 5.6% signet-ring cell carcinomas. The number of colorectal adenomas and adenocarcinomas did not differ among the eight groups (data not shown).

Significant enhanced tumor multiplicity was observed in groups fed high-fat diets in comparison with groups fed low-fat diets, containing a vegetables-fruit mixture during the initiation or promotion phase (Group A→B/B→A versus C→D/D→C; $0.02<p<0.05$; Table 3). This significant difference was mainly due to the lower number of adenomas (Group A→B versus C→D; $0.05<p<0.10$, Group B→A versus D→C; $0.02<p<0.05$).

Table 3. Tumor incidence, and multiplicity in colon and rectum.

Group[a]	Incidence, %[b]			Multiplicity[c]		
	Total	Adenoma	Adeno-carcinoma	Total	Adenoma	Adeno-carcinoma
A→A	93.3 (28)	90.0 (27)	76.7 (23)	3.89±0.35	2.48±0.32	1.83±0.28
A→B	93.3 (28)	90.0 (27)	73.3 (22)	3.61±0.43	2.41±0.31	1.64±0.36
A→C	96.7 (29)	93.3 (28)	76.7 (23)	4.86±0.42[d]	3.46±0.34[d]	1.91±0.37
B→A	96.7 (29)	86.7 (26)	63.3 (19)	3.34±0.37	2.50±0.34	1.68±0.39
C→A	96.7 (29)	86.7 (26)	66.7 (20)	4.13±0.43	3.19±0.41	1.85±0.41
C→C	100.0 (30)	86.7 (26)	80.0 (24)	4.23±0.39	3.23±0.47	1.79±0.31
C→D	93.3 (28)	86.7 (26)	76.7 (23)	4.89±0.59[d]	3.42±0.47	2.09±0.35
D→C	86.7 (26)	83.3 (25)	66.7 (20)	5.04±0.58[d]	3.60±0.53[d]	2.05±0.42

a: See Table 2 footnote for dietary composition of groups.

b: Values are percentages of animals bearing that specific type of tumor. Values in parentheses represent absolute number of animals having tumors.

c: Values are means ± standard error of the mean expressed in number of tumors/tumor bearing animal.

d: Statistical significance is as follows: $0.002<p<0.005$, A→A vs. A→C (total); $0.005<p<0.01$, A→A vs. A→C (adenoma); $0.02<p<0.05$, A→B/B→A vs. C→D/D→C (total).

Furthermore, multiplicity was significantly increased in animals fed low-fat diets during the initiation phase and high-fat diets during the promotion phase in comparison with animals maintained on low-fat diets during the whole experiment (Group A→C versus A→A; $0.002<p<0.005$). This was mainly caused by a significant increase of adenomas (Group A→A versus A→C; $0.005<p<0.010$). No differences in multiplicity were observed among other dietary groups.

Other Observations

The mean size of colorectal adenomas and (adeno)carcinomas did not differ among the dietary groups (adenomas, 2.4±0.3 mm^2; (adeno)carcinomas, 5.2±0.6 mm^2). Similarly, the mean distance of these tumors, measured from distal point of rectum did not differ (adenomas, 5.2±0.6 cm; (adeno)carcinomas, 5.4±0.6 cm).

Intestinal tumors located at sites other than colon and rectum were mainly located in duodenum and jejunum, which were identified as adenomas or adenocarcinomas (Table 4). No significant differences in number of these tumors were observed among the groups. Furthermore, tumors were occasionally observed in caecum.

Discussion

The results of the present study demonstrate that a vegetables-fruit mixture does not have inhibitory effects on AOM-induced colorectal carcinogenesis in rats, either given during the initiation or in the promotion phase, irrespective of the fat contents of the diets.

Table 4. Intestinal tumors other than observed in colon and rectum[a,b].

Group	Duodenum		Jejunum		Caecum
	Adenoma	Adenocarcinoma	Adenoma	Adenocarcinoma	Adenocarcinom
A→A	7	24	-	2	-
A→B	4	21	-	7	2
A→C	6	27	-	8	-
B→A	5	22	1	7	-
C→A	8	21	-	5	1
C→C	6	24	-	6	-
C→D	7	25	-	4	1
D→C	7	18	-	8	-

a: See Table 2 footnote for dietary composition of groups.

b: Values are total numbers of tumors.

This confirms the outcome of the previous study with DMH-treated rats, in which we found that exactly the same mixture did not cause a pronounced inhibitory effect on DMH-induced colorectal tumor development, when present during both initiation and promotion phase, either in combination with low- or with high-fat diets (Rijnkels *et al.*, 1997a). Hence, a vegetables-fruit mixture does not have a strong potency to modulate colorectal carcinogenesis, induced in rats by DMH or its metabolite, AOM. Because the main difference in composition between vegetables-fruit containing diets and diets not containing such a mixture was the presence of non-nutrients, such as flavonoids (see Table 1, subscript g), any effect of the vegetables-fruit mixture in the present study is suggested to be caused by these plant constituents. Apparently, the plant constituents, some of which were measured in reasonable amounts in our vegetables-fruit mixture, were not able to modify AOM-induced colorectal carcinogenesis, although some compounds were suggested to have anticarcinogenic potential (Hartman *et al.*, 1990; Wattenberg, 1992). However, we have found an inhibitory potential of the same mixture on MNNG-induced colorectal carcinogenesis (Rijnkels *et al.*, 1997b). A justifiable explanation may be that the anticarcinogenic potency of the vegetables-fruit mixture in the MNNG-study, is mainly due to anti-initiating properties, rather then to antipromotion properties. As is known from other reports, the inhibitory effects of vegetables, fruit or plant constituents on the initiation phase of the carcinogenesis process may also be ascribed to radical scavenging or maintenance of the DNA-repair mechanisms (Hartman *et al.*, 1980; Morse *et al.*, 1993; Wattenberg, 1992; Webster *et al.*, 1996; Williams *et al.*, 1991).

The different findings between the present study and the MNNG-study may be mainly due to the route of administration, and the type of DNA-damage induced. Concerning the route of administration, AOM was injected systemically, while MNNG was administered locally in the colon. AOM is metabolized both in liver and colon. In liver AOM-metabolites are partly excreted in the gut and transported to the colon. Therefore, colon epithelium is not only exposed systemically to AOM-metabolites, but also locally from the colon lumen, whereas colon epithelium is only locally exposed to MNNG. The vegetables-fruit mixture is consumed orally. Therefore, it can be assumed that the main part of its anti-initiation effect will occur locally in the colon. In that case, animals treated with DMH will only be partly protected against DMH exposure by the vegetables-fruit mixture, whereas animals treated with MNNG will be protected more effectively.

Concerning the type of DNA-damage induced by DMH- or AOM-metabolites and MMNG, an important difference is that DMH- or AOM-metabolites form among others O^6-methylguanine adducts, which have been suggested to be the most important promutagenic lesion in AOM/DMH carcinogenesis, while MNNG hardly induces this type of DNA adduct (Netto *et al.*, 1992; Nivard, 1991, Moloney *et al.*, 1983). Repair of O^6-methylguanine

adducts is, however, almost lacking in colon epithelium. Hence, assuming that the anticarcinogenic capacity of a vegetables-fruit mixture is related to DNA-repair, this stimulation will not influence repair activity of O^6-methylguanine adducts (Wattenberg, 1992; Webster *et al.*, 1996).

In the present study high amounts of fat, representative for the Western diet, stimulated colorectal carcinogenesis. This finding is in accordance with many other reports, which demonstrated that a high-fat diet enhances carcinogenesis (Potter, 1996; Weisburger, 1991). Remarkably in the present study is that stimulation of colorectal carcinogenesis by high-fat diets was only found in those groups fed diets containing a vegetables-fruit mixture (A→B/B→A vs. C→D/D→C). Multiplicity of colorectal tumors of the eight different groups, was increased in the order of Group B→A, having the lowest multiplicity, Group A→B, Group A→A, Group C→C, Group C→D, and Group D→C, having the highest multiplicity. Although, multiplicity between Groups A→B/B→A and A→A, and between Groups C→D/D→C and C→C were non-significant, these data point to an interaction between a vegetables-fruit mixture with dietary fat-promoted carcinogenesis in such a way that vegetables and fruit tended to inhibit carcinogenesis when present in low-fat diets, but tended to enhance carcinogenesis when present in high-fat diets. This observation supports findings of other investigators, demonstrating that the inhibitory effects of vegetables or fruit constituents are limited by the amount of fat (Alink *et al.*, 1993; Sinkeldam *et al.*, 1990).

Finally, the results of the present study demonstrate that the enhancing effect of fat on AOM-induced colorectal carcinogenesis merely occurred during the promotion phase. This conclusion is based on the observation that colorectal tumor multiplicity in the group fed low-fat diets during the whole experiment was significantly lower than in the group fed a high-fat diet during the promotion phase only (A→A vs. A→C), whereas tumor multiplicity in the group fed a high-fat diet during the initiation phase was similar to that in the group fed a low-fat diet during the initiation phase (C→A/C→C vs. A→A/A→C). These findings are in accordance with the conclusion of many other investigators that high-fat diets enhance carcinogenesis mainly during the promotion phase (Ma *et al.*, 1996; Reddy *et al.*, 1977, 1986; Weisburger, 1986).

In conclusion, a vegetables-fruit mixture, consumed in amounts representative for the mean consumption in The Netherlands, did not affect AOM-induced colorectal tumorigenesis either when present during the initiation or the promotion phase. Fat, however, showed an enhancing effect on AOM-induced colorectal carcinogenesis, which was not inhibited by a vegetables-fruit mixture.

Acknowledgements and Notes

The authors thank J. Haas and B. Weijers (Centre for Small Laboratory Animals, Agricultural University Wageningen) for technical assistance, and Central Horticultural Auctions in The Netherlands for providing most vegetables and fruit. This study was supported by Grant LUW93-485 of the Dutch Cancer Society.

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Part II

Interactive effects of dietary factors on (anti)carcinogenic mechanisms *in vivo* and *in vitro*

5

A vegetables-fruit mixture hardly influenced xenobiotic enzyme activities and immune function in rats fed low- or high-fat diets

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Submitted to Food and Chemical Toxicology

Abbreviations: Con A, Concanavalin A; BSA, bovine serum albumin; cpm, counts per minute; DMH, 1,2-dimethylhydrazine; EROD, ethoxyresorufine-*O*-deethylation; GST, cytosolic glutathion-S-transferase; LPS, lipopolysaccharide; LST, lymphocyte stimulation test; MLR, mixed lymphocyte reaction; NDMA-d, *N*-nitrosodimethylamine-demethylase; NK cell activity, natural killer cell activity; PHA, phytohemagglutinin; PROD, pentoxyresorufine-*O*-deethylation; PWM, pokeweed mitogen; sem, standard error of the mean; UDP-GT, UDP-glucuronyltransferase; VF-mixture, vegetables-fruit mixture.

Abstract

The influences of a vegetables-fruit mixture (19.5% wt/wt) added to low- or high-fat diets (20 or 40 energy percent) were investigated on xenobiotic enzyme activities in liver, and immune function in spleen and mesenteric lymph nodes, in rats having 1,2-dimethylhydrazine-induced colorectal cancer. Enzyme activities and immune function were determined after animals were fed the diets for 36 weeks. A vegetables-fruit mixture added to low-fat diets significantly decreased glutathion-S-transferase (GST) activity compared with low-fat diets only. Further, high-fat diets significantly decreased ethoxyresorufine-*O*-deethylation and GST activity compared with low-fat diets. No differences among the dietary groups were measured concerning pentoxyresorufine-*O*-deethylation, NDMA-demethylase and UDP-glucuronyl transferase activities. No effects were found on natural killer cell activities, mitogen stimulated lymphoproliferation and mixed lymphocyte reactions. These data indicate that both a vegetables-fruit mixture and dietary fat have only negligible effects on xenobiotic enzyme activities and no effects on the measured immune parameters in rats with colorectal cancer.

Introduction

Dietary compounds may modulate colorectal carcinogenesis by altering xenobiotic metabolism or immune function. For instance, cruciferous vegetables (e.g. Brussels sprouts, broccoli), are known to enhance glutathion-S-transferase, cytochrome P-450-IA and -IIB enzyme activities (Nijhoff *et al.*, 1993,Vang *et al.*, 1991). And, although fat is merely known as a promoter of tumorigenesis, modulation of xenobiotic metabolism by fat has been described (Chinery *et al.*, 1993; Yoo *et al.*, 1990). In addition, dietary fat and flavonoids (plant constituents present in various vegetables and fruit) have been shown to modulate immune function (Locniskar *et al.*, 1986; Maki *et al.*, 1992; Middleton *et al.*; 1992, Steerenberg *et al.*, 1997).

In assessing the mechanisms by which dietary compounds modulate colorectal carcinogenesis, it has to be realised that diet is complex and dietary factors probably interact. Therefore, investigation of the interactive effects among dietary compounds on colorectal carcinogenesis is of importance. Recently, we performed an animal study in which rats were fed low- or high-fat diets (20 or 40 energy%), whether or not combined with a vegetables-fruit mixture (19.5% wt/wt) for 9 months, to investigate possible interactive effects between these dietary factors on 1,2-dimethylhydrazine (DMH)-induced colorectal carcinogenesis. In this study, fat enhanced DMH-induced colorectal tumorigenesis, whereas protection of a vegetables-fruit mixture was not pronounced, irrespective of the fat content (Rijnkels *et al.*, 1997).

The present paper reports the effects of a vegetables-fruit mixture and dietary fat on xenobiotic enzyme activities in liver, and immune function in spleen and mesenteric lymph nodes, determined in the aforementioned animal study. This study was carried out with the aim to investigate whether there is a relationship between the effects of these dietary factors on liver enzyme activities and immune function, and their modulation of colorectal carcinogenesis.

Materials and methods

Experimental design

The experimental design is described in detail elsewhere (Rijnkels *et al.*, 1997). In short, 120 weanling male Wistar rats received a semisynthetic Muracon-SSP/tox diet without (Groups A and C) or with a vegetables-fruit mixture (Groups B and D; vegetables and fruit content 19.5% wt/wt), for 36 weeks. Diets of Groups A and B contained 20 energy percent (e%) fat, whereas diets of Groups C and D contained 40e% fat. The vegetables and fruit used, the amount of fat and its fatty acid composition were chosen according to the mean consumption values in The Netherlands.

Table 1. Final composition of the four experimental diets, expressed as percentage wet weight.

Ingredients[a]	Diet A[b]	Diet B	Diet C	Diet D	VF-mixture[c]
Fat	8.50	8.50	20.00	20.00	0.40
Carbohydrate	48.34	30.39	30.78	10.14	63.00
Protein	23.19	23.19	26.50	26.50	8.80
Fiber	10.67	9.80	12.20	11.20	14.20
Vitamins	0.35	0.31	0.40	0.35	0.80
Minerals	8.95	8.31	10.12	9.51	1.30
VF-mixture	-	19.50	-	22.30	
kcal/kg	**3603**	**3608**	**4126**	**4132**	**3548**

a: For exact dietary composition see Rijnkels*et al.* (1997).

b: Diet A is the basal Muracon-SSP/tox diet.

c: Composition (% wet wt): potatoes (35.1), bananas (3.0), oranges (9.0), apples (19.10), lettuce (3.75), green pepper (1.25), tomatoes (3.75), cucumbers (3.75), cauliflowers (3.75), spinach (2.50), leek (2.50), red (2.5) and white cabbage (2.5), sauerkraut (2.5), carrots (1.25), Brussels sprouts (1.25), and beet (2.50). Total glucosinolate content near detection level (e.g. glucobrassicin), flavonoid content 640 mg/100 g (e.g. quercetin, catechin, naringenin, hesperetin).

Table 2. Hepatic xenobiotic enzyme activities in rats fed low- or high-fat diets, whether or not combined with a vegetables-fruit mixture.

Group[a]	n	P450content[b]	EROD[c]	PROD[c]	NDMA-d[b]	GST[b]	UDP-GT[b]
Diet A	10	0.45±0.06	47.39±3.59	28.93±1.48	1.67±0.3	253.1±18.8	8.03±1.56
Diet B	10	0.45±0.05	47.90±3.35	25.43±0.85	2.64±0.73	197.9±13.7[d]	5.02±0.91
Diet C	10	0.44±0.03	28.94±3.35[e]	33.54±5.00	2.68±0.73	185.4±17.0[e]	7.06±0.84
Diet D	10	0.48±0.03	44.39±2.42	34.26±2.60	2.74±0.80	209.5±18.5	5.98±0.81

a: Diet A: low fat, Diet B: low fat including a vegetables-fruit mixture, Diet C: high fat, Diet D: high fat including a vegetables-fruit mixture.

b: Values ± standard error of the mean (sem) are expressed as nM/min/mg microsomal protein or nM/min/mg cytosolic protein (GST).

c: Values ± sem are expressed as pM/min/mg microsomal protein.

d: Significantly different from Group A ($p < 0.05$).

e: Significantly different from Groups A, B and D ($p < 0.05$).

Results

DMH metabolizing enzyme activities in liver (Table 2)

A vegetables-fruit mixture added to low-fat diets caused a significantly lower GST activity when compared with the low-fat diets without vegetables and fruit (Diet B versus Diet A; GST, 197.9±13.7 vs. 253.1±18.8 nM/min/mg cytosolic protein). Further, diets high in fat showed significantly lower EROD and GST activities in comparison with low-fat diets (Diet C versus Diet A; EROD, 28.94±3.35 vs. 47.39±3.59 pM/min/mg microsomal protein; GST, 185.4±17.0 vs. 253.1±18.8 nM/min/mg cytosolic protein). No significant differences in PROD, NDMA-d, and UDP-GT activities were measured among the four dietary groups.

Immune response (Table 3)

The number of mesenteric lymph node cells averaged between 41±4 (sem, Diet B) and 55±5 (Diet D) million counts, and the number of spleen lymphocytes averaged between 951±61 (Diet B) and 1088±90 (Diet C) million counts. These cell numbers differed not significantly among the four dietary groups. In addition, NK cell activity (showing only the ratio target:effector cells 100:1), the rate of mitogen stimulation with con A, PHA, PWM and LPS, and the MLR, determined in both spleen and mesenteric lymph nodes differed not significantly among the four dietary groups.

Discussion

Of the five different enzyme activities measured, the only responses of the vegetables-fruit mixture and dietary fat were found on hepatic GST activity and, for dietary fat only, EROD activity. No dietary effects were observed on the immune parameters. As reported elsewhere, the same vegetables-fruit mixture had no pronounced effect on colorectal tumorigenesis in the same animals, whereas fat clearly enhanced colorectal tumorigenesis (Rijnkels *et al.*, 1997).

The absence of an effect on hepatic enzyme activities, except on GST activity, and immune parameters by the vegetables-fruit mixture does not support the suggestion that vegetables and fruit or specific plant constituents may stimulate metabolic activity of certain liver enzymes (e.g. cytochrome P450-IA (EROD), -IIB (PROD), -IIE1 (NDMA-d)), stimulate detoxifying routes (e.g. GST and UDP-GT), and activate immune function (Kubena *et al.*, 1996; Middleton *et al.*, 1992; Nakachi *et al.*, 1992; Nijhoff *et*

Table 3. MLR, NK cell activity, and LST in rats fed low- or high-fat diets, whether or not combined with a vegetables-fruit mixture.

Group[a]	Cell Origin	No. of	NK Cell Activity[c]	LST				MLR[b]
		lymphocytes	100:1[d]	Con A	PHA	PWM	LPS	
Diet A	Mesenteric	46±6	12.2±2.1	45.2± 1.0	9.7± 2.1	5.9±1.3	1.4±0.3	3.7±1.2
Diet B	Lymph	41±4	11.1±2.0	21.3±16.0	4.9± 0.6	3.4±0.7	1.6±0.3	2.1±0.7
Diet C	Nodes	50±6	10.8±2.0	42.4± 6.8	12.7± 2.6	6.3±0.9	1.5±0.1	2.9±0.7
Diet D		55±5	9.1±1.6	34.9± 5.7	8.6± 1.5	5.1±0.7	1.5±0.1	2.1±0.8
Diet A	Spleen	959±92	37.0±4.6	39.8± 7.4	26.7±11.0	11.5±2.1	4.2±1.2	2.7±0.7
Diet B		951±61	31.5±3.4	38.2± 7.7	20.0± 8.4	10.1±2.1	4.0±1.3	2.0±0.5
Diet C		1088±90	34.9±4.0	46.3± 9.1	29.5±12.5	10.9±1.8	3.7±0.9	2.0±0.5
Diet D		1060±83	29.8±3.9	43.6± 9.6	37.4±14.4	12.1±2.8	3.8±1.0	1.6±0.6

a: See Table 2 footnote for composition diets.

b: Values are means ± sem, expressed as stimulation index (thousands cpm).

c: Values ± sem are expressed as % specific release. Calculated as follows: $\frac{\text{sample cpm - spontaneous cpm}}{\text{maximum cpm - spontaneous cpm}}$ x 100%.

d: Effector:target ratio.

al., 1993; Vang *et al.*, 1991; Wortelboer *et al.*, 1992). It should be noticed that in the present study this absence of effect may have partly arisen from the long adaptation period, and that the amounts of the vegetables and fruit used for the mixture, resembled the mean human composition and consumption values in The Netherlands. Most other studies refer to exposure times of maximal four weeks and to 'unrealistic' high amounts of one selected vegetable or specific plant constituent. Therefore, the present study maybe more realistic than the short-term studies.

The present effects of fat on EROD activity and the absence of an effect on immune parameters are supported by Chinery *et al.* (1993) and Lockniskar *et al.* (1986,1987). To what degree inhibition of GST and EROD activities by dietary fat is, however, related to the enhanced colorectal tumor development, as observed in these animals (Rijnkels *et al.*, 1997), is unknown, because the most important tumor promotion mechanism of dietary fat is assumed to be stimulation of bile acid production. Other investigators, however, have found effects of dietary fat on xenobiotic enzyme activities and on immune function (Barone *et al.*, 1989; Dannenberg *et al.*, 1992; Newberne *et al.*, 1981; Wade *et al.*, 1985; Yoo *et al.*, 1990, 1991,1992).

In conclusion, the present data show that both a vegetables-fruit mixture and dietary fat had negligible modulating effects on xenobiotic liver enzyme activities and no influence on immune parameters in rats with colorectal cancer. This may be ascribed to the long adaptation period and the realistic composition of the experimental diets used.

Acknowledgements

B. Verlaan, H. vd Vliet and D.Q. Mol (National Institute of Public Health and the Environment, Bilthoven) are acknowledged for their work on the immunological assays. The authors thank Dr. R.A. Woutersen (Dept. of General Toxicology, TNO Nutrition and Food Research, Zeist) and Prof. Dr. J.H. Koeman (Dept. of Toxicology, Agricultural University Wageningen) for critical reading of the manuscript. This study was supported by Grant LUW93-485 of the Dutch Cancer Society.

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6

Effects of a vegetables-fruit mixture on liver and colonic 1,2-dimethylhydrazine metabolizing enzyme activities in rats fed low- or high-fat diets

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Submitted to Cancer Letters

Abbreviations: DMH, 1,2-dimethylhydrazine; EROD, ethoxyresorufine-*O*-deethylation; GST, cytosolic glutathion-S-transferase; NDMA-d, *N*-nitrosodimethylamine-demethylase; PROD, pentoxyresorufine-*O*-deethylation; sem, standard error of the mean; UDP-GT, UDP-glucuronyl transferase; VF-(mixture), vegetables-fruit (mixture).

Abstract

Effects of a vegetables-fruit mixture (19.5 % wt/wt) were studied on hepatic (h) and colonic (c) 1,2-dimethylhydrazine (DMH)-metabolizing enzyme activities (ethoxy- and pentoxyresorufine-*O*-deethylation, NDMA-demethylase, glutathion-S-transferase, UDP-glucuronyltransferase) in rats fed low- or high-fat diets (20 or 40 energy%). A vegetables-fruit mixture added to the diets resulted in altered enzyme activities in animals either treated or not treated with DMH. Remarkably, the vegetables-fruit mixture given to DMH-treated rats decreased glutathion-S-transferase (h) and increased NDMA-demethylase activities (c), whereas the mixture given to controls increased glutathion-S-transferase (h) and decreased NDMA-demethylase activities (c). The high-fat diets only modulated enzyme activities in animals not treated with DMH.

Introduction

Dietary habits play an important role in the development of colorectal cancer (Peto *et al.*, 1981; Weisburger, 1991). One of the proposed mechanisms of action of dietary compounds, such as vegetables, specific plant constituents and dietary fat, on colorectal carcinogenesis is modulation of enzyme activities involved in activating or deactivating procarcinogens (Guengerich *et al.*, 1995; Vang *et al.*, 1991; Wade, 1986; Wortelboer *et al.*, 1992; Yang *et al.*, 1988, 1992). Whether this will result in decreased or increased risk for developing colorectal cancer will be determined by the dietary composition and the type of natural or chemical procarcinogens present in food.

At our laboratory we are interested in combination effects of food factors, such as vegetables, fruit and dietary fat, on 1,2-dimethylhydrazine (DMH)-induced colorectal tumorigenesis. DMH is a procarcinogen which needs to be metabolized to become an ultimate carcinogen (Druckrey, 1972). Therefore, effects of dietary compounds on DMH metabolizing enzymes are supposed to influence tumor development. The present study was performed to investigate the interactions between a vegetables-fruit mixture (19.5% wt/wt) and different amounts of dietary fat (20 and 40 energy percent) on hepatic and colonic metabolizing enzyme activities in rats, during DMH-treatment.

Materials and methods

Experimental design

Male Wistar rats (N=40, aged 3 months) were randomly assigned to one of the four dietary groups. The composition of the four Muracon-SSP/tox diets has been described in detail elsewhere (Rijnkels *et al.*, 1997). In short, diets of Groups A and B contained 20e% fat, whereas diets of Groups C and D contained 40e% fat (Table 1). Furthermore, in diets of Groups B and D a vegetables-fruit mixture (19.5% wt/wt) was included. The vegetables and fruit used, and the amount and composition of fat were chosen according to the mean consumption values in The Netherlands.

Animals were housed as described elsewhere (Rijnkels *et al.*, 1997), and fed one of the 4 experimental diets during 6 weeks. After 1 week of acclimatization, half of the animals of each group received weekly injections of DMH at 50 mg/kg body wt sc. for four weeks. The remaining half of the animals in each dietary group was given a vehicle (control animals). DMH (Aldrich Co. Ltd.; Gillingham, Dorset, UK) was dissolved in 1.5% solution of EDTA in 0.9% NaCl, that was adjusted to pH 6.5 (final solution of 1 mg/kg body wt). One week after the last DMH-treatment, rats were killed by exsanguination via the abdominal aorta under ether anesthesia.

Table 1. Final composition of the four experimental diets (% wt/wt).

Ingredients	Diet A[a]	Diet B	Diet C	Diet D
Fat[b]	8.50	8.50	20.00	20.00
Carbohydrate[c]	48.34	30.39	30.78	10.14
Protein[c]	23.19	23.19	26.50	26.50
Fiber[c]	10.67	9.80	12.20	11.20
Vitamins/minerals[c]	9.30	8.62	10.52	9.86
VF-mixture[d]	-	19.50	-	22.30

a: Diet A is the Muracon-SSP/tox diet. In Diets C and D the carbohydrate, protein, fiber, vitamin and mineral contents of the Muracon-SSP/tox were adjusted to allow for decreased food consumption in animals consuming diets of high caloric density. In Diets B and D the amounts of carbohydrate, fiber, vitamins and minerals of the Muracon-SSP/tox were adjusted for the presence of these constituents in the vegetables-fruit mixture (VF-mixture).

b: 84 % Lard and 16 % sunflower oil.

c: See Rijnkels *et al.* (1997) for exact composition

d: Composition (% wet weight): potatoes (35.1), bananas (3.0), oranges (9.0), apples (19.10), lettuce (3.75), green pepper (1.25), tomatoes (3.75), cucumbers (3.75), cauliflowers (3.75), spinach (2.50), leek (2.50), red cabbage (2.5), white cabbage (2.5), sauerkraut (2.5), carrots (1.25), Brussels sprouts (1.25), beetroot (2.50). Total glucosinolate content near detection level (e.g. glucobrassicin), flavonoid contact 640 mg.100 g (e.g. quercetin, catechin, naringenin, hesperetin).

The liver was immediately frozen in liquid N_2 and stored at -80°C until further preparation. The colon was longitudinally cut from caecum to rectum and flushed with ice-cold saline solution. Next, colonic mucosal cells were scraped from the tissue with the edge of a sterile glass slide. Cells were frozen in liquid N_2 and stored at -80°C until further preparation for enzyme assays.

Enzyme assays

Liver cytosolic and microsomal fractions were prepared under chilled conditions. Livers were homogenized in ice-cold 50 mM tris-HCl/0.25 M sucrose (pH 7.4) and centrifuged at 12000 *g* for 30 minutes. The supernatant was centrifuged at 105 000 *g* for 75 minutes. The microsomal pellet was suspended in 0.1 M $K_2HPO_4 \cdot 3H_2O$-KH_2PO_4/20% (v/v) glycerol (pH 7.4). Both cytosolic and microsomal fractions were stored at -80°C until further analysis. Per dietary group, colonic mucosal cells of all animals were pooled, then dissolved in ice-cold 50 mM tris-HCl/0.25 M sucrose (pH 7.4) and centrifuged at 105 000 *g* for 75 minutes. Colonic cytosolic and microsomal fractions were prepared as described for the liver.

EROD and PROD activities were fluorimetrically measured according to Burke *et al.* (1985) using a CytoFluor™2350 (Millipore, Isle of Man, British Islands) autoanalyzer. The resorufin formed was measured at 572 nm. *N*-nitrosodimethylamine-demethylase (NDMA-d) activity was assayed according to the method described by Snell *et al.* (1987). N-demethylation of N-nitrosodimethylamine resulted in the formation of formaldehyde, which was colorimetrically detected at 412 nm using the Nash reagent. UDP-glucuronyl transferase (UDP-GT) activity was measured according to the method described by Bock *et al.* (1983). The disappearance of *p*-nitrophenol was colorimetrically determined at 405 nm. Cytosolic glutathion-S-transferase (GST) activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was measured at 25°C (pH 6.5) according to Habig *et al.* (1974). The reaction was monitored spectrophotometrically at 340 nm. Protein concentrations in the cytosolic and microsomal fractions were determined with the Pierce method (Smith, 1985) using BSA as standard. For liver only, microsomes were assayed for contents of cytochrome P450 (Omura *et al.*, 1964).

Statistics

Data were analyzed with one-way analysis of variance and student-t-test. A confidence interval of 95% ($p \leq 0.05$) was chosen. Statistical analyses on colonic samples were limited to those samples in which cytosolic GST activity was measured. For colonic microsomal enzymes, samples were pooled per group, to improve sensitivity of the measurements.

Results

Effects of DMH on hepatic and colonic enzyme activities.

In liver of DMH-treated animals, higher basic enzyme activities were measured than in control animals, concerning EROD (Group B), PROD (Group B), NDMA-d (Groups B and C), UDP-GT (Groups A, B and C), and GST (Groups A and B) (Table 2). In colon, higher basic enzyme activities were measured concerning PROD, NDMA-d, UDP-GT and GST (all groups except Group A) (Table 3).

Effects of a vegetables-fruit mixture and fat on hepatic DMH-metabolizing enzymes (Table 2).

In control animals fed a low-fat diet, a vegetables-fruit mixture caused a significant decrease in NDMA-d and increased GST activities, whereas in DMH-treated animals maintained on a low-fat diet, the vegetables-fruit mixture caused a significant increased PROD and, notably, decreased GST activities. No altered hepatic enzyme activities were measured concerning the presence of a vegetables-fruit mixture in high-fat diets.

High-fat diets in comparison with low-fat diets, only altered hepatic DMH-metabolizing enzyme activities measured in control animals (vehicle treated) and not in DMH-treated animals. In control animals maintained on high-fat diets, significantly increased EROD, PROD and GST activities, and significantly decreased NDMA-d activity were measured.

Effects of a vegetables-fruit mixture and fat on colonic DMH-metabolizing enzymes (Table 3).

Neither the presence of a vegetables-fruit mixture in low-fat diets (Diet B) nor the high-fat diets (Diet C) did result in significantly altered GST activities in DMH-treated and control animals. Concerning colonic microsomal DMH-metabolizing enzyme activities, the most pronounced effects of the vegetables-fruit mixture and dietary fat were found on PROD, NDMA-d, and UDP-GT activities. In DMH-treated animals maintained on a vegetables-fruit mixture, increased NDMA-d and decreased UDP-GT activities were measured, whereas in control animals decreased PROD, UDP-GT, and, notably, decreased NDMA-d activities were measured. Remarkably, a vegetables-fruit mixture given to DMH-treated rats maintained on a high-fat diet decreased NDMA-d activity, whereas such a mixture added to low-fat diets increased NDMA-d activity. Finally, a high-fat diet caused decreased NDMA-d and UDP-GT activities in control animals, whereas in DMH-treated animals only a decreased UDP-GT activity was found.

Table 2. DMH-metabolizing enzyme activities in liver.

Diet group[a]	n	DMH treat.[b]	P450 content[c]	EROD[d]	PROD[d]	NDMA-d[c]	GST[e]	UDP-GT[c]
A	5	-	0.97±0.07	29.93±2.89	14.38±0.60	4.72±0.11	48.59± 8.81	6.86±0.67
A	5	+	0.93±0.16	35.74±5.42	13.95±1.42	4.34±0.37	99.25±19.54[g]	10.11±0.60[g]
B	5	-	0.98±0.10	27.93±4.00	13.20±1.59	3.14±0.53[f]	103.30± 4.15[f]	6.31±0.82
B	5	+	1.38±0.15	47.12±3.34[g]	19.47±1.06[f,g]	5.86±0.68[g]	54.39± 1.95[f, g]	10.36±0.80[g]
C	5	-	1.18±0.13	48.94±5.25[f]	18.65±1.45[f]	2.92±0.44[f]	100.94± 7.93[f]	5.89±0.35
C	5	+	1.36±0.13	43.98±7.74	15.00±1.45	5.05±0.33[g]	79.42± 8.54	10.64±0.63[g]
D	5	-	1.26±0.12	46.66±3.83[f]	20.66±1.84[f]	3.84±0.24[f]	95.94± 9.71[f]	7.22±0.65
D	5	+	1.26±0.12	44.58±4.58	18.26±1.02[f]	3.52±0.60	101.83±10.58	10.01±0.49[g]

a: Diet A: low fat, Diet B: low fat including vegetables-fruit mixture, Diet C: high fat, Diet D: high fat including a vegetables-fruit mixture.

b: Vehicle-treatment (-) or DMH-treatment (+).

c: P450 content, NDMA-d, and UDP-GT activity are expressed as nM/min/mg microsomal protein ± standard error of the mean .

d: EROD and PROD activity expressed as pM/min/mg microsomal protein ± standard error of the mean.

e: GST activity expressed as nM/min/mg cytosolic protein ± standard error of the mean.

f: Significantly different from dietary Group A with respective vehicle (control)- or DMH-treatment ($p<0.05$).

g: Significantly different from respective dietary Group with vehicle-treatment ($p<0.05$).

Table 3. DMH-metabolizing enzyme activities in colon.

Diet group[a]	n	DMH treatment[b]	EROD[c]	PROD[c]	NDMA-d[d]	GST[e]	UDP-GT[d]
A	5	-	1.36	2.85	0.57	3.66±0.42	1.28
A	5	+	1.38	4.04	0.28	4.48±0.33	2.10
B	5	-	1.01	1.50	nd[f]	3.41±0.37	0.71
B	5	+	1.62	5.45	0.52	5.35±0.18[g]	1.60
C	5	-	1.15	2.13	0.10	3.10±0.07	0.86
C	5	+	1.22	5.48	0.22	5.39±0.83[g]	1.27
D	5	-	0.83	1.63	nd	2.93±0.11	1.10
D	5	+	0.90	4.89	0.10	5.59±0.34[g]	1.38

a: Diet A: low fat, Diet B: low fat including vegetables-fruit mixture, Diet C: high fat, Diet D: high fat including a vegetables-fruit mixture.

b: Vehicle-treatment (-) or DMH-treatment (+).

c: EROD and PROD activity expressed as pM/min/mg microsomal protein.

d: NDMA-d, and UDP-GT activity are expressed as nM/min/mg microsomal protein .

e: GST activity expressed as nM/min/mg cytosolic protein ± standard error of the mean.

f: nd = not detected.

g: Significantly different from respective dietary group not treated with DMH ($p<0.05$).

Discussion

Vegetables and fruit may protect against DMH-induced colorectal tumorigenesis by reducing the formation of the ultimate DMH carcinogen (methylazoxymethanol), through inhibition of DMH-metabolizing enzyme activities, such as cytochrome P450-IA (PROD), -IIB (EROD) and -IIE1 (NDMA-d), and through stimulating detoxification via for instance GST. In control animals, this phenomenon was observed for PROD (colon), NDMA-d (liver and colon) and GST (liver) activities. In DMH-treated animals, however, the opposite was observed: PROD (liver) and NDMA-d (colon) activities were increased, whereas GST (liver) activity was decreased. These findings suggest that the effects of a vegetables-fruit mixture on xenobiotic metabolism are strongly influenced by the presence of DMH, indicating that DMH-induced tumorigenesis may be stimulated rather then prevented by a vegetables-fruit mixture. This supports the findings of a previous study, performed in our laboratory, in which a vegetables-fruit mixture added to diets with the same composition as in the present study, showed no pronounced protection against DMH-induced colorectal carcinogenesis in rats (Rijnkels *et al.*, 1997).

Remarkably, the results of the present study showed that the effects of fat on hepatic and colonic enzyme activities were strongly reduced by DMH-treatment. This observation supports the findings of Pence *et al.* (1991), who reported no effects of increasing fat content on hepatic cytochrome P450-IIB (PROD) and -IIE1 (NDMA-d) activities during DMH-treatment, whereas altered cytochrome P450-IIE1 was observed with increased fat content without DMH-treatment. The conclusion may be drawn that fat-enhanced DMH-induced colorectal carcinogenesis is not mediated by biotransformation. However, it may not be excluded that fat influences carcinogenesis by modulation of xenobiotic metabolism on a more indirect way. Fat may interact with effects of other dietary compounds on xenobiotic metabolism, thereby influencing their potential to inhibit or stimulate colorectal carcinogenesis by this mechanism. Interactive effects among dietary compounds may be expected, because diet is complex. Interestingly, in the present study, the effects of a vegetables-fruit mixture on colonic NDMA-d activity were modulated by high amounts of fat.

In conclusion, the results of the present study demonstrate that the effects of a vegetables-fruit mixture and dietary fat on metabolizing enzyme activities were strongly influenced by DMH-treatment and were further influenced by modulatory effects of dietary compounds.

Acknowledgments

The authors thank Jo Haas and Bert Weijers (Center for Small Laboratory Animals, Agricultural University Wageningen) for technical assistance, and Dr. Ruud Woutersen (Dept. of General Toxicology, TNO Nutrition and Food Research, Zeist) and Prof. Dr. Jan Koeman (Dept. of Toxicology, Agricultural University Wageningen) for critical reading this manuscript. This study was financially supported by Grant LUW93-485 of the Dutch Cancer Society.

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Effects of indole-3-carbinol and vegetables-fruit extracts on stearic acid modulated cytotoxicity, intercellular communication and cytochrome P450-IA activity

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Submitted to Environmental Toxicology & Pharmacology

Abbreviations: EROD, 7-ethoxyresorufin-O-demethylase; GJIC, gap junctional intercellular communication; I3C, indole-3-carbinol; LDH, lactate dehydrogenase; TCDD, 2,3,7,8-tetrachloordibenzo-p-dioxine; TPA, 12-O-tetradecanoyl-phorbol-13-acetate.

Abstract

Effects were investigated of indole-3-carbinol (I3C) and extracts of a vegetables-fruit mixture on stearic acid modulated cytotoxicity, gap junctional intercellular communication (GJIC) and cytochrome P450-IA activity (EROD), in V79-wild type, V79-rIA1, and Caco-2 cells. Interestingly, I3C (25 μg/ml) did not prevent stearic acid-induced cytotoxicity (300 μM, V79-wild type; 200 μM, V79-rIA1; 1000 μM Caco-2), whereas it did prevent inhibition of GJIC caused by stearic acid (10 μM). Furthermore, I3C induced EROD activity in the presence of 500 μM stearic acid. Water and hexane extracts of a vegetables-fruit mixture protected against decreased GJIC caused by 10 μM stearic acid and induced EROD activity when co-exposed with 500 μM stearic acid in Caco-2 cells. Further, the order at which I3C, vegetables-fruit extracts and stearic acid were added to the cells and the type of cells used had an influence on the observed combined effects. In conclusion, the present *in vitro* study showed that I3C and vegetables-fruit extracts modulate effects of stearic acid on cytotoxicity, intercellular communication, and cytochrome P450-IA activity.

Introduction

A variety of vegetables and fruit have been suggested to play a role in preventing carcinogenesis (Steinmetz *et al.*, 1991a; 1996). In addition, many vegetable and fruit constituents, such as flavonoids and indole-3-carbinol (I3C), are thought to have chemoprevention properties (Hartman *et al.*, 1990; Morse *et al.*, 1993; Wattenberg, 1992). I3C is formed by hydrolysis of glucobrassicin, and is present in cruciferous vegetables (e.g. cabbages, Brussels sprouts; Steinmetz *et al.*, 1991b; Verhoeven *et al.*, 1997). I3C was shown to inhibit carcinogenesis, but also enhanced tumor formation has been reported (Jongen, 1996). There are several proposed mechanisms of action of I3C on carcinogenesis, such as modulation of xenobiotic metabolism and of tumor promotion (Bailey *et al.*, 1987; Birt *et al.*, 1986; McDanell *et al.*, 1987,1988;).

By studying mechanisms of the effects of isolated plant constituents, such as I3C, one should bear in mind that anticarcinogenic properties of vegetables and fruit are most probably the result of the presence of several types of plant constituents apart from I3C. It is not illogical to assume that these constituents may interact with each other, thereby enhancing or inhibiting one another's anticarcinogenic properties. Therefore, mechanistic studies should be extended using whole vegetables or fruit. Furthermore, anticarcinogenic properties of vegetables and fruit may be limited by the presence of food components with tumor promoting properties. A much-discussed food component, which is associated with increased cancer risk, is dietary fat, especially fat rich in

saturated and omega-6 polyunsaturated fatty acids (Ma *et al.*, 1996; Weisburger, 1986). It is proposed that fat acts mainly as a promoter, but also modulation of cytochrome P450-isoenzymes, is described after changing fatty acid composition in diets (Yang *et al.*, 1992;Yoo *et al.*, 1992). Up to now, only few reports described combined effects of dietary components. At least two studies, one dealing with dietary fiber and one dealing with a vegetables-fruit mixture, reported suppression of the preventive properties of these dietary components on carcinogenesis by dietary fat (Rijnkels *et al.*, 1997; Sinkeldam *et al.*, 1990).

In order to understand interactive effects between food components, it is of paramount importance to elucidate the mechanisms through which interactions may take place in the carcinogenic process. *In vitro* studies are a valuable tool to reach this goal.

The aim of the present *in vitro* study was to assess modulation of I3C and vegetables-fruit extracts on effects caused by stearic acid. I3C and stearic acid, a saturated fatty acid, were chosen as model constituents. The vegetables-fruit extracts were made from a mixture of vegetables and fruit used previously in our colorectal carcinogenesis animal model (Rijnkels *et al.*, 1997). Combined effects were studied on cytotoxicity, gap junctional intercellular communication (GJIC) and cytochrome P450-IA activity (7-ethoxyresorufin-O-demethylase, EROD). GJIC is important in control of cell proliferation and differentiation (Holder *et al.*, 1993) and, therefore, inhibition of GJIC is related to tumor promotion. Cytochrome P450-IA monooxygenase, a phase I enzyme, is involved in activating or deactivating procarcinogens. As a result, modulation of its activity may prevent formation of ultimate carcinogens. Although cytotoxicity is not directly related to the carcinogenesis process, tissues chronically experiencing cytotoxicity are more susceptible to develop tumors.

Materials and Methods

Cells, chemicals, and biochemicals

Human colon carcinoma Caco-2 cell line was a gift from Dr. H. Noteborn (RIKILT-DLO, Wageningen, The Netherlands). V79 Chinese hamster cell line (MZ or wild type) and G418-resistent V79 derived cell line expressing rat P450IA1 (XEM2) were provided by Dr. A. Goeptar (Dept. Pharmacochemistry, Free University, Amsterdam, The Netherlands) and originally came from Dr. J. Doehmer (Institute of Toxicology, University of Mainz, Germany). Stearic acid, Lucifer Yellow, indole-3-carbinol (I3C), and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) were purchased from Sigma

Chemical Co. (St.Louis, USA). 2,3,7,8-Tetrachloordibenzo-p-dioxine (TCDD) was purchased from Merck AG (Darmstadt, Germany).

Preparation of crude vegetables-fruit extracts

The composition and preparation of the vegetables-fruit mixture is described in detail by Rijnkels *et al.* (1997). In short, the mixture contained the following vegetables and fruit (% wet weight); potatoes (35.1), bananas (3.0), oranges (9.0), apples (19.10), lettuce (3.75), green pepper (1.25), tomatoes (3.75), cucumbers (3.75), cauliflower (3.75), spinach (2.50), leek (2.50), red cabbage (2.50), white cabbage (2.50), sauerkraut (2.5), carrots (1.25), Brussels sprouts (1.25), and beetroot (2.50). The vegetables and fruit were processed under household conditions (cooked and cut in pieces), freeze dried, mixed together and powdered.

Five gram portions of the powdered vegetables-fruit mixture were extracted (Tecator, Soxlet system HT, 1043 extraction unit, Sweden) in 50 ml hexane, followed by an extraction in 50 ml de-ionised water, for 90 minutes at 160°C. After filtration, hexane-extracts were dried at room temperature to remove hexane. Water-extracts were evaporated at 60°C. Both hexane and water residues were dissolved in 2 ml DMSO (pure fractions), and stored at -20°C until assayed.

Cell culture conditions

Undifferentiated Caco-2 cells were seeded at approximately $1x10^5$ cells/cm^2, in either 75 cm^2 plastic flasks, 35x10 mm petri-dishes, 6-well or 96-well plates in Dulbecco's Modified Eagle Medium, supplemented with 10% (v/v) foetal bovine serum, 1% (v/v) non-essential amino acids, and 0.5% (v/v) gentamycine. Under these conditions, cells reached confluency in 2 days. Cells routinely grown in 75 cm^2 plastic flasks were harvested two times a week, by rinsing with phosphate buffered saline containing 0.022% (wt/v) EDTA, followed by brief trypsinization, and re-cultured in new plastic flasks, petri-dishes or plates. Differentiated cells at 14-day post-confluency, were used for exposures. V79 cells were seeded under almost the same conditions as Caco-2 cells. XEM2 (V79-rIA1) cells were maintained in the same medium supplemented with 400 μg/ml Geneticin-G418. Furthermore, cells were harvested three times a week, by rinsing with PBS, followed by brief trypsinization. When cells reached confluency, in 2 days, they were used for exposures. Caco-2 and V79 cells were maintained and exposed at 37°C in an atmosphere of CO2/air (1:19) at 90-100% relative humidity. Cells exposed to stearic acid were maintained in the same medium as used for routine growth, supplemented with 10 mg/ml bovine serum albumin (BSA).

Cytotoxicity

Cells were exposed in 6-well plates in 2 ml medium. Cytotoxic concentrations of stearic acid (300 μM, V79-wild; 200 μM, V79-rIA1; 1000 μM, Caco-2) were combined with non-cytotoxic concentration of I3C (25 μg/ml, dissolved in ethanol). The order in which stearic acid and I3C were combined was as follows. A) 48-hours exposure to stearic acid, followed by a 24-hours co-exposure with stearic acid, b) 72-hours co-exposure with stearic acid, or c) 24-hours exposure to I3C, followed by a 72-hours co-exposure with stearic acid. Solvents, stearic acid, and I3C alone served as controls. 100 mM α-solanine was used as positive control. Each experiment was performed in triplicate.

Cytotoxicity was determined by measuring lactate dehydrogenase activity. After treatment, media were removed from each well and centrifuged. LDH activity of 200 μl supernatant was measured spectrofotometrically at 340 nm. The maximum release of LDH was obtained by scraping cells from the bottom after addition of 2 ml 0.5% Triton X-100 in 0.1 M phosphate buffer (pH 7.5) to each well. After sonification for 5 minutes and centrifugation, LDH activity was measured in 200 μl supernatant. Cytotoxicity is expressed as percentage LDH leakage.

Gap junctional intercellular communication

Cells were exposed in 35x10 mm petri-dishes in 2 ml medium. Concentrations of 10 μM stearic acid, 25 μg/ml I3C, and pure hexane and water extracts of vegetables-fruit mixture were used for combination studies in V79-rIA1 cells. The order of the combined treatments differed, as described for cytotoxicity measurements. Solvents, stearic acid, I3C, and vegetables-fruit extracts alone served as controls. 100 ng/ml TPA was used as positive control. Each experiment was performed in duplicate.

Gap junctional intercellular communication (GJIC) was determined in confluent cell cultures after microinjection of a 20% Lucifer Yellow solution (in 0.33 M lithium chloride) in a single cell. In each well, at least 20 individual cells were microinjected, using a vertical injection system (Olympus Injectoscope IMT-2-syf, Japan) with a dye-filled capillary glass tip (Clark, Pangbourne, UK). The glass-capillary tip, with a diameter of 1 μm, was prepared by an automatic magnetic puller (Narishige, Tokyo, Japan). Between 15 and 20 minutes after the first injection the number of communicating cells was determined using fluorescence microscopy.

Cytochrome P450-IA (EROD) activity

Caco-2 cells were exposed in 96-well plates in 200 µl medium. Concentrations of 500 µM stearic acid, 10, 25 and 50 µg/ml I3C, and pure, 10x, and 100x dilutions of vegetables-fruit extracts were used for combination studies in Caco-2 cells. The order of the combined treatments differed, as described for cytotoxicity measurements. Solvents, stearic acid, I3C, and vegetables-fruit extracts alone were used as controls. 1 nM TCDD served as positive control. Each experiment was performed once or twice.

Cytochrome P450-IA activity was determined by measuring 7-ethoxyresorufin-O-demethylase (EROD) activity. After cells were washed with PBS, they were lysed by adding 20 µl nanopure to the wells, followed by freeze-thawing of the cells at -80°C for at least 20 minutes. To the lysed cells, 50 µl 50 mM Tris(hydroxymethyl)-aminomethane-sucrose + 2 mM dicumarol (50:1) buffer (pH 8.0) and 25 µl 7-ethoxyresorufin-solution (20 µM) were added. The plates were covered with aluminum foil to protect light sensitive ethoxyresorufin against daylight, and placed at 37°C for 20 minutes. Enzyme reactions were started by adding 25 µl 1 mM NADPH to the cells at 37°C. After 1 hour EROD activity was fluorimetrically measured at 572 nm using a CytoFluor2350 autoanalyzer (Millipore, Isle of Man, British Islands). A 0-2000 nM resorufin standard was used as calibration. After measurement of enzyme activities, microsomal protein concentrations were determined as follows. To lysed cells 30 µl 0.075% sodiumdeoxycholate was added and, after 10 minutes, 50 µl 18% trichloroacetic acid. Plates were centrifuged (2000g) for 10 minutes at room temperature. Next, pellets were dissolved in 50 µl 5% sodiumdodecylsulphate and 200 µl bicinchoninic acid-reagent (reagent A:B=100:2). After 20 minutes (37°C), followed by 10 minutes (room temperature), protein concentrations were spectrofotometrically (Molecular Devices, Microplate Reader, USA) measured at 562 nm. BSA (0-2000 µg/ml) was used as standard.

Statistical analysis

Statistical significance between treated cells and controls and among treated cells was analyzed with the one-way analysis of variance and the student-t-test. A p value of $\leq$ 0.05 was considered to be indicative of significant differences.

Results

Control values

In control cells, LDH leakage ranged from 8.3±1.2 (standard deviation; V79 cells) to 20±1.2 % (Caco-2 cells), whereas 100 mM solanine resulted in 91.1±1.3% LDH leakage (72 hours; Caco-2 cells). Intercellular communication ranged from 7.5±0.9 (V79 cells) to 17.7±3.0 (Caco-2 cells) communicating cells per injected cell, whereas 100 ng/ml TPA lowered communication to 1.7±0.2 cells (V79 cells) or 5.5±0.7 cells (Caco-2 cells). EROD activity in controls (Caco-2 cells) averaged 1.53±0.28 nM/mg prot/min, whereas 1 nM TCDD enhanced it to 19.5±8.4 nM/mg prot/min.

Cytotoxicity

In V79-wild type cells, a non-cytotoxic concentration of 25 µg/ml I3C did not prevent cytotoxicity caused by 300 µM stearic acid (Figure 1a). In stead, I3C even enhanced stearic acid-induced cytotoxicity, irrespective whether it was given before, simultaneously or after stearic acid treatment. Furthermore, enhanced cytotoxicity was observed in V79-rIA1 cells, when I3C was given simultaneously with 200 µM stearic acid in comparison with 200 µM stearic acid alone (Figure 1b). On the other hand, I3C given before the cells were exposed with stearic acid resulted in a marked inhibition of cytotoxicity in these V79-rIA1 cells. In Caco-2 cells, I3C did not prevent cytotoxicity caused by 1000 µM stearic acid, independently of the order at which I3C and stearic acid were added to the cells (Figure 1c).

Gap junctional intercellular communication

25 µg/ml I3C markedly prevented a decrease of intercellular communication by 10 µM stearic acid, when given to V79-rIA1 cells before or simultaneously with the fatty acid (Figure 2a). However, when given to cells after stearic acid, I3C did not clearly prevent a decrease of intercellular communication by the fatty acid. Both the hexane and water extracts of the vegetables-fruit mixture did not suppress intercellular communication. These extracts prevented a decrease of GJIC caused by stearic acid, independently of the order at which they were added to the cells with stearic acid (Figure 2b,c).

Cytochrome P450-IA activity (EROD)

In Caco-2 cells, exposed to 10-50 µg/ml I3C, and to pure, 10x and 100x diluted hexane or water vegetables-fruit fractions, EROD activity was significantly higher in comparison with cells exposed to 500 µM stearic acid.

Figure 1. Combined cytotoxic effects between 25 μg/ml I3C and 300 μM stearic acid (C18:0) in V79-wild type (a), 200 μM stearic acid in V79-rIA1 (b), and 1000 μM stearic acid in Caco-2 (c) cells. Co-exposures were performed in different orders (F+I; co-exposed simultaneously (72 hours), F→I; co-exposed with stearic acid last 24 hours only, I→F; pre-exposure with I3C (24 hours), followed by co-exposure with stearic acid (72 hours)). Mean value ± standard deviation is expressed as percentage LDH leakage. * Indicates significant differences compared with exposures to stearic acid only ($p \leq 0.05$).

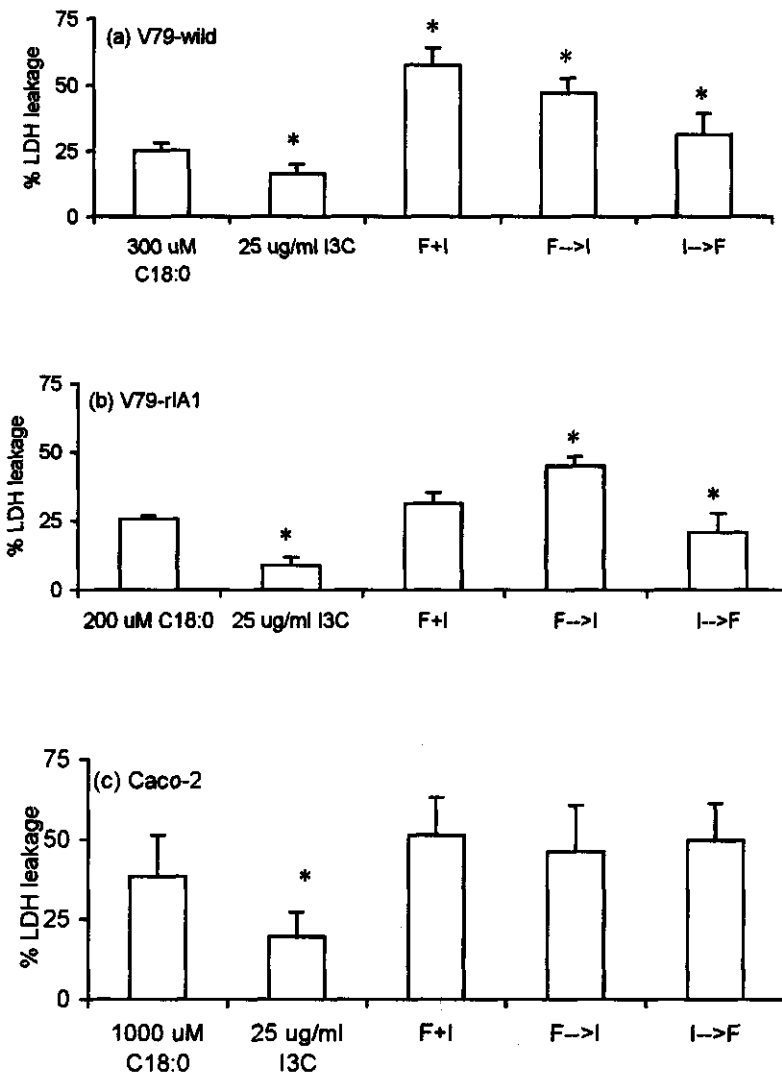
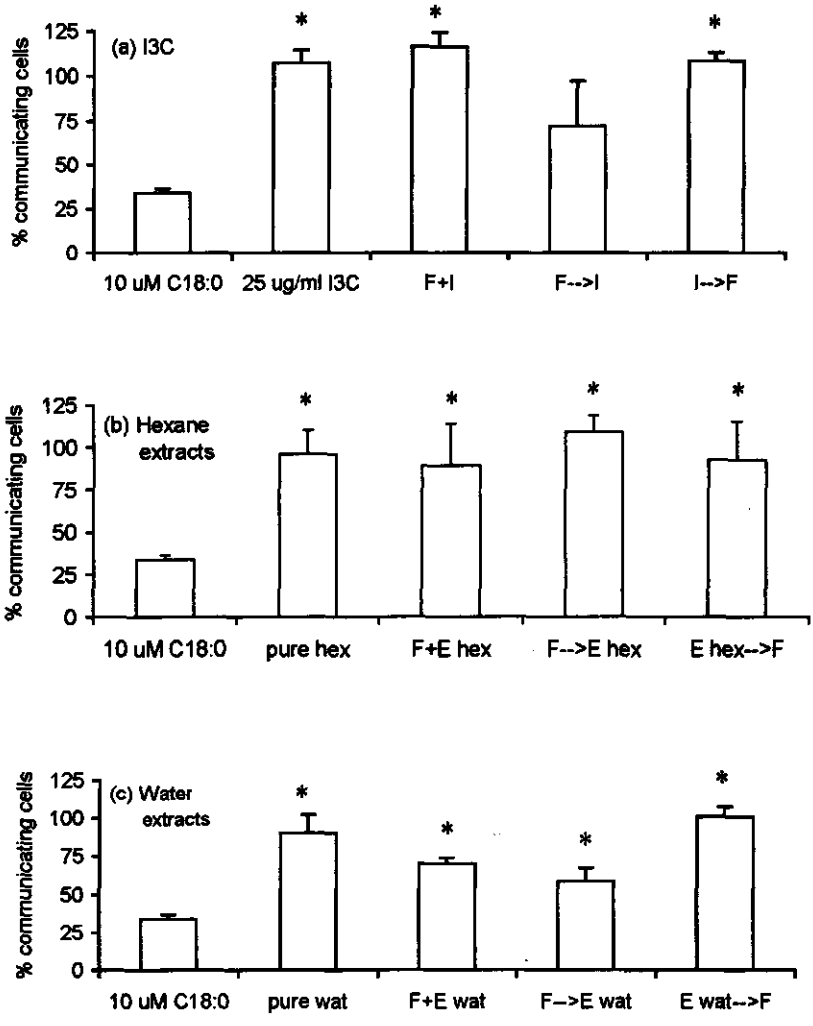


Figure 2. Combined effects on GJIC in V79-rIA1 cells between 10μM stearic acid (C18:0) and 25 μg/ml I3C (a), pure hexane (b) and water (c) extracts of a vegetables-fruit mixture. Co-exposures were performed in different orders (see legend Figure 1). Mean value ± standard deviation is expressed as percentage communicating cells. * Indicates significant differences compared with exposures to stearic acid only ($p \leq 0.05$).



Furthermore, 10-50 µg/ml I3C enhanced EROD activity in cells also exposed with stearic acid as compared to stearic acid only (Figure 3a). Significantly enhanced EROD activity was also observed in cells exposed to hexane or water extracts and stearic acid, although this was only observed when cells were first exposed to the extracts and thereafter together with stearic acid (Figure 3b,c).

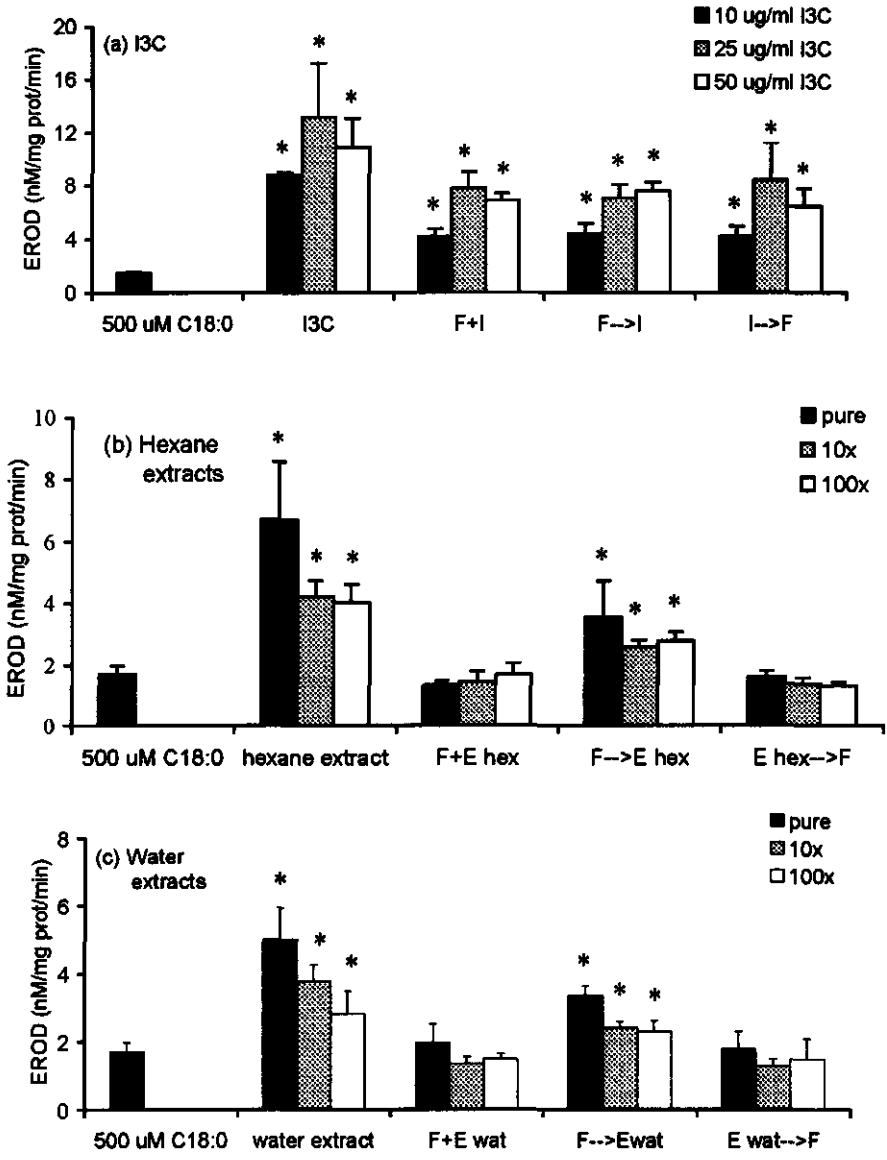
Discussion

Indole-3-carbinol

I3C did not protect against stearic acid-induced cytotoxicity. On the contrary, cytotoxicity was clearly enhanced in V79-wild type cells. In contrast, I3C prevented a decrease of intercellular communication by stearic acid, and induced cytochrome P450IA activity in the presence of stearic acid. Up to now, research has been focused on studying possible antagonistic interactions between I3C and known chemical carcinogens, rather than between I3C and other dietary components. The results of the present study provides additional support for observations made by those of others, indicating that the same levels of I3C are not cytotoxic, induce cytochrome P450-IA enzyme activities, and show modulating properties (Babich *et al.*, 1993; Birt *et al.*, 1986; Jongen, 1988; Shertzer *et al.*, 1988). However, in the present *in vitro* study I3C effects on intercellular communication points to protection against tumor promotion, whereas two *in vivo* studies revealed stimulation of tumor promotion by I3C (Bailey *et al.*, 1987; Birt *et al.*, 1986). Further studies into the role of I3C on tumor promotion are required to understand the present observation.

Another interesting observation made in the present study is that the order at which I3C and stearic acid were added to the cells (cytotoxicity and GJIC in V79-rIA1 cells) and the type of cells used (cytotoxicity) influenced the observed combined effects. The influence of the order at which I3C and stearic acid were added to the cells, has been described before (Bailey *et al.*, 1987; Nixon *et al.*, 1984). For example, in AFB_1-induced carcinogenesis in rainbow trout, I3C inhibited carcinogenesis when given simultaneously with AFB_1-treatments, but it enhanced carcinogenesis when given afterwards.

Figure 3. Combined effects on EROD activity in Caco-2 cells between 500 μM stearic acid (C18:0) and 10-50 μg/ml I3C (a), pure, 10x, or 100x dilutions of hexane (b) and water (c) extracts of a vegetables-fruit mixture. Co-exposures were performed in different orders (see legend Figure 1). Mean values ± standard deviation are expressed as EROD activity (nM/mg prot./min) in comparison with controls. * Indicates significant differences compared with exposures to stearic acid only ($p \leq 0.05$).



Concerning the cell types, the most striking differences among the three cell types used, which were likely to be responsible for the difference in effects, were their origin (Chinese hamster lung fibroblasts, human colon carcinoma cells), growth characteristics, and the presence of a functional xenobiotic metabolism system (V79-wild type, none; V79-rIA1, artificial; Caco-2, natural; Doehmer *et al.*, 1992; Meunier *et al.*, 1995).

Crude vegetables-fruit extracts

The present results indicate that crude hexane and water extracts of a vegetables-fruit mixture prevent a decrease in intercellular communication and, furthermore, enhance EROD activity in the presence of stearic acid. For EROD activity, this modulation was, however, only observed when extracts were added to cells that had been exposed with stearic acid, showing again an influence of the order at which components are added to the cells, as was observed for I3C. Comparison of the present data with data from literature is difficult, since mechanistic investigations of combined effects between whole vegetables and fruit and other dietary components are almost absent. Moreover, in the present study a unique mixture of several different vegetables and fruit was used, each composed of specific nutrient and non-nutrient constituents, whereas most studies published only investigated the effects of a single constituent. Concerning the presence of non-nutrients, chemical analysis of the mixture showed at least the presence of flavonoids (quercetin, catechin, naringenin, hesperetin), while total glucosinolate content was near detection level (Rijnkels *et al.*, 1997). Both flavonoids and glucosinolates are suggested to possess anti-carcinogenic properties (Birt *et al.*, 1986; Thorling, 1993). Quercetin may have played a role in the prevention of decreased intercellular communication, because it is reported to prevent TPA-lowered intercellular communication *in vitro* (Chaumontet *et al.*, 1996; Wärngård *et al.*, 1987). The presence of cauliflower, Brussels sprouts, cabbages and carrots in the mixture may be responsible for the observed induction of cytochrome P450-IA (EROD) (Alink *et al.*, 1987; Jongen, 1996; McDanell *et al.*, 1988; Steinmetz *et al.*, 1991b).

In conclusion, both I3C and vegetables-fruit extracts modulated the effects of stearic acid on cytotoxicity, intercellular communication and cytochrome P450-IA activity *in vitro*. This modulation was partly influenced by the different cell types used and the order at which the dietary components were added to the cells.

Acknowledgements

The authors gratefully acknowledge Anne Mensink for her useful comments, Laura de Haan for her technical support (Dept. of Toxicology, Agricultural University Wageningen), Ruud Woutersen (Dept. of General Toxicology, TNO Nutrition and Food Research, Zeist) and Jan Koeman (Dept. of Toxicology, Agricultural University Wageningen) for critical reading this manuscript. This study was financially supported by Grant LUW93-485 of the Dutch Cancer Society.

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Summary and concluding remarks

Summary

Several dietary compounds are associated with colorectal cancer risk. These include the amount of dietary fat, which is positively associated with colorectal cancer, and a variety of vegetables and fruit, which are suggested to possess anticarcinogenic potential. Because diet is complex and dietary factors most probably interact, it is likely that these interactive effects between dietary components, rather then the effects of individual components, may account for a large part in the risk for developing colorectal cancer. The results of the studies performed by Alink *et al.* (1993) demonstrated that it is of paramount importance to study interactive effects between dietary components in evaluating the effects of total diet in colorectal carcinogenesis. They showed that the effects of a vegetables-fruit mixture on DMH-induced colorectal carcinogenesis in rats maintained on complete human diets, was modulated by the presence of other dietary components, such as the amount of dietary fat or fried meat.

To investigate these observations by Alink *et al.* in more detail, we performed three long-term animal studies, which have been described in detail in Part I of the present thesis. In these studies, interactive effects between dietary fat (20 and 40 energy%) and a vegetables-fruit mixture (19.5% wt/wt) were studied on 1,2-dimethylhydrazine (DMH)- and *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine (MNNG)-induced colorectal carcinogenesis in rats, and in Apc^{Min} mice, which are genetic susceptible for developing multiple intestinal neoplasia (Chapters 1, 2, and 3). The composition and amount of fat (40e%) and of the vegetables-fruit mixture were based on regular amounts consumed in The Netherlands. The animal diets used for these studies were balanced for protein and micronutrient content. The diets differed only in the amount of fat/carbohydrate and the presence of specific plant constituents when a vegetables-fruit mixture was included in the diets. Notwithstanding the use of the same experimental diets in all three studies, the results were remarkably different. A vegetables-fruit mixture added to high-fat (40e%) diets resulted in a distinct protection of colorectal carcinogenesis in the MNNG-study, whereas in the DMH study no clear effect of the vegetables-fruit mixture was observed.

In *Apc*^{Min} mice the vegetables-fruit mixture enhanced rather then inhibited tumor development. Furthermore, a diet high in fat (40e%) enhanced colorectal carcinogenesis in the DMH-study and, although less pronounced, in the MNNG-study, whereas in the *Apc*^{Min} mice no effect was observed. Finally, a vegetables-fruit mixture added to low-fat diets did not result in protection against colorectal cancer development in the MNNG-study, whereas when added to a high-fat diet an inhibitory effect was found. In male *Apc*^{Min} mice, the same mixture added to low-fat diets decreased the number of small intestinal tumors, whereas it increased the number of small intestinal neoplasia when added to high-fat diets.

To examine the differences observed between the effects of the vegetables-fruit mixture observed in the DMH-model and those in the MNNG-model in more detail, an experiment was designed, in which the inhibitory potency of a vegetables-fruit mixture was investigated on either the initiation or the promotion phase, using azoxymethane (AOM) to induce colorectal carcinogenesis and using both low- and high-fat diets (Chapter 4). In this study no protection of this mixture on colorectal carcinogenesis was observed either when present during the initiation or during the promotion phase, irrespective of the fat content.

In conclusion, the variation in the results of the aforementioned studies can most probably be ascribed to methodological differences, such as differences in DMH and MNNG metabolism, route of administration and type of DNA damage. Apart from these differences, genetic susceptibility may play a role. Taken these methodological differences into account the present results do not consistently show that dietary fat has modulated the tumor preventive properties of a vegetables-fruit mixture in colorectal carcinogenesis (Alink *et al.*, 1993).

The second part of the thesis describes the results of studies into the interactive effects between dietary fat and the vegetables-fruit mixture on (anti)carcinogenic mechanisms. Hepatic xenobiotic enzyme activities (ethoxyresorufine-*O*-deethylation (EROD), pentoxyresorufine-*O*-deethylation (PROD), *N*-nitrosodimethylamine-demethylase (NDMA-d), cytosolic glutathion-S-transferase (GST), UDP-glucuronyl transferase (UDP-GT)) and immune parameters in spleen and mesenteric lymph nodes (NK cell activity, lymphocyte stimulation test, mixed lymphocyte reaction) were measured in rats of the long-term DMH-study (Chapter 5). Overall, it was shown that both a vegetables-fruit mixture and dietary fat had no effect on the enzyme activities and immune parameters. In a short-term (seven weeks) animal study, DMH-treatment appeared to influence hepatic and colonic xenobiotic enzyme activities (EROD, PROD, NDMA-d, UDP-GT and GST) rather strongly (Chapter 6). Furthermore, interaction between dietary fat and the vegetables-fruit mixture was observed on colonic NDMA-d

activity. Finally, interactive effects between stearic acid, indole-3-carbinol and crude extracts of the vegetables-fruit mixture were studied *in vitro* (Chapter 7). Both indole-3-carbinol, an isolated plant constituent, and vegetables-fruit extracts, a complex mixtures of plant constituents, modulated the effects of stearic acid on cytotoxicity, gap junctional intercellular communication, and cytochrome P450-IA (EROD) activity. The effects of indole-3-carbinol and extracts of the vegetables-fruit mixture were partly influenced by the order at which these components with stearic acid were added to the cells as well as by the type of cells used.

In general, the results of the studies described in Part II of this thesis supports the hypothesis that interaction between dietary constituents may influence their modulating effect on colorectal carcinogenesis.

Concluding remarks

The results of the long-term animal studies, described in this thesis, indicate that a vegetables-fruit mixture, in which regular amounts of specific plant constituents are present, added to either a low- or a high-fat diet, has a dual role regarding colorectal carcinogenesis. Apart from an absence of meaningful effects, the vegetables-fruit mixture showed both tumor inhibitory as well as tumor enhancing effects. This finding is in contrast with most epidemiological studies, which point to a strong inverse association between vegetables and fruit and colorectal cancer risk. A large part of the research with animal models for colorectal cancer has been focussed on elucidating the mechanisms of action of isolated potential anticarcinogenic constituents of vegetables and fruit. Inherent to these animal experiments is the use of high amounts of the isolated dietary constituents and the relative short duration of the experiments. The experimental design of such studies do not easily allow extrapolation of the results to the human situation, since in real life human diet contains low concentrations of hundreds of different constituents. Because it is not illogical to assume that these different dietary constituents interact, it can be expected that interactive effects between the various dietary constituents, rather than the effects of the individual constituents, play an important role in modulating colorectal carcinogenesis. The studies described in this thesis were performed with the aim to investigate the effects of whole diets on colorectal carcinogenesis, instead of isolated constituents, and to take interactive effects into account. To reach this aim, diets were used in which the choice of the vegetables and fruit, the amount of fat and its fatty acid composition were based on the mean

consumption values in The Netherlands. The results of the MNNG- and *Apc*^{Min} mice-study, the short-term study, and of the *in vitro*-study indicate that interactive effects between vegetables and fruit and dietary fat may take place.

These interactive effects and the magnitude of these effects were influenced by the duration of the experiments and by the study designs used. Regarding the duration of the experiments, effects of dietary fat and a vegetables-fruit mixture on xenobiotic enzyme activities were diminished or altered as the experiment lasted longer. The observation that effects observed in short-term studies are different from the effects observed in long-term studies indicates that apart from short-term studies, long-term animal experiments are warranted in order to establish causal relationships between dietary factors and colorectal cancer.

Concerning the study designs used, the results of the present investigations with *Apc* deficient mice demonstrate that constituents present in relevant amounts in vegetables and fruit may stimulate colorectal carcinogenesis in persons having genetic defects in the *Apc* gene and consuming amounts of fat representative to the mean consumption values. This *Apc* gene is involved in the onset of colorectal cancer in humans and is considered to be present in the latent period of the cancer process. Therefore, it is logical to assume that once an initiated gene is present, the supplementation of vegetables and fruit may fail to protect against cancer progression or may even enhance this process, whereas healthy persons may be protected. This finding supports observations made by other investigators, such as Bailey *et al.* (1987) who revealed enhanced colorectal carcinogenesis in rats which were fed indole-3-carbinol after colorectal cancer had developed, and Albanes *et al.* (1996) who found that β-carotene enhanced progression of lung cancer in heavy smokers. These remarkable findings lead to the recommendation that one should be cautious with a dietary advice, because dietary constituents may have both tumor inhibitory as well as tumor promoting potencies depending on the stage of the disease. Further research is warranted to elucidate the relationship between genetic defects, such as on the *Apc* gene, and dietary habits.

The studies described in the present thesis demonstrate that chronic (long-term) animal studies with regular whole diets need to be performed for an appropriate analysis of possible effects of life style factors on carcinogenesis. Long-term animal studies provide, therefore, valuable data to fill the gap between epidemiological studies and short-term studies.

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Samenvatting en slotopmerkingen

Samenvatting

Het is onmiskenbaar dat verschillende voedingsmiddelen een rol spelen bij het ontstaan van dikke darmkanker. Zo is er bijvoorbeeld een relatie gelegd tussen het hoge vetgehalte van onze voeding, waardoor men een hoger risico op het ontstaan van darmkanker zou hebben, en tussen de hoge consumptie van groenten en fruit, waardoor men juist minder risico zou lopen. Omdat de samenstelling van onze voeding zeer complex is, is de kans groot dat er een wisselwerking (interactie) tussen de verschillende voedingsstoffen plaatsvindt. Daarom is het aannemelijk dat juist deze wisselwerking voor een belangrijk deel het uiteindelijke risico op het ontstaan van darmkanker bepalen. Hoe belangrijk die wisselwerking tussen voedingsmiddelen kan zijn in relatie tot dikke darmkanker is aangetoond door Alink en medewerkers (1993). Zij vonden in een chronische dierexperimentele studie dat de remmende werking van een groenten-fruit mengsel op de dikke darm carcinogenese sterk werd verminderd door de aanwezigheid van andere voedingsfactoren, zoals een hoog vetgehalte of gebakken vlees.

Deze waarneming van Alink en medewerkers is specifieker onderzocht met behulp van drie chronische dierstudies, die uitgebreid beschreven zijn in Deel I van dit proefschrift. Doel van deze studies was mogelijke interactieve effecten tussen voedingsvet (20 en 40 procent van de dagelijkse energie inname) en een normale hoeveelheid groenten-fruit mengsel (19,5% van het totale rantsoen) vast te stellen op de darm carcinogenese. Daarvoor zijn twee chemisch-geïnduceerde darmkanker modellen voor de rat gebruikt (1,2-dimethylhydrazine (DMH) en *N*-methyl-*N'*-nitro-*N*-nitroso-guanidine (MNNG)) en Apc^{Min} muizen (Hoofdstuk 1, 2 en 3). Apc^{Min} muizen hebben een genetische afwijking in het *Apc* gen, waardoor zij spontaan meerdere darmtumoren ontwikkelen op relatief jonge leeftijd. De samenstelling en hoeveelheid van het voedingsvet (40 energie%) en van het groenten-fruit mengsel zijn gelijkgesteld aan de gemiddelde consumptie in Nederland. Het gehalte aan eiwitten, vitaminen, mineralen en andere spoorelementen van de rantsoenen zijn onderling gelijkgesteld, zodat de

dierrantsoenen onderling alleen verschilden in het vet- en koolhydraatgehalte en in de aanwezigheid van specifieke plantbestanddelen, ook wel non-nutriënten genoemd.

Ondanks het gebruik van dezelfde rantsoenen in deze drie chronische dierstudies, verschilden de resultaten ervan opmerkelijk. Een groenten-fruit mengsel in rantsoenen met een hoog vetgehalte (40e%) verminderde duidelijk de incidentie van MNNG-geïnduceerde darmtumoren, terwijl de ontwikkeling van DMH-geïnduceerde darmtumoren niet duidelijk werd afgeremd. In Apc^{Min} muizen werd zelfs een verhoogde tumorontwikkeling vastgesteld in de groepen die een hoog vet rantsoen in combinatie met een groenten-fruit mengsel gevoerd kregen. Het hoge vetgehalte zelf bevorderde de ontwikkeling van DMH-geïnduceerde darmtumoren en, hoewel iets minder duidelijk, de incidentie van MNNG-geïnduceerde tumoren, maar veroorzaakte geen effecten in de Apc^{Min} muizen. Tenslotte bleek dat een groenten-fruit mengsel toegevoegd aan laag vet rantsoenen niet noemenswaardig beschermde in de MNNG-geïnduceerde colon carcinogenese, hoewel het mengsel wel beschermde toegevoegd aan hoog vet rantsoenen. Mannelijke Apc^{Min} muizen gevoerd met dezelfde groenten-fruit rantsoenen ontwikkelden daarentegen minder darmtumoren wanneer het mengsel toegevoegd was aan laag vet rantsoenen en ontwikkelden juist meer darmtumoren wanneer het mengsel toegevoegd was aan hoog vet rantsoenen.

De verschillende effecten van het groenten-fruit mengsel zijn nader onderzocht door middel van een vierde chronisch dierexperiment (Hoofdstuk 4). In dat experiment is de beschermende potentie van het groenten-fruit mengsel, toegevoegd aan laag of hoog vet rantsoenen, bestudeerd op de initiatie en promotie fase van het colon carcinogenese proces. Hiervoor werd het azoxymethaan (AOM)-geïnduceerde colon carcinogenese rattenmodel gebruikt. Azoxymethaan is een metaboliet van DMH. Een beschermende werking van het groenten-fruit mengsel tijdens de initiatie en tijdens de promotie fase bleef uit, zowel in ratten gevoerd met een laag als met een hoog vet rantsoen.

Het verschil in effecten van voedingsvet en van het groenten-fruit mengsel in de vier chronische studies op de colon carcinogenese wordt voornamelijk verklaard door de verschillende methoden die gebruikt zijn om darmkanker te induceren. DMH/AOM en MNNG verschillen in metabolisme, de manier waarop ze toegediend worden en in het type DNA schade dat zij veroorzaken. Verder lijkt de genetische gevoeligheid een rol te spelen in de variabele voedingseffecten. Rekening houdend met deze methodologische verschillen geven de dierexperimentele onderzoeken niet duidelijk aan dat voedingsvet op een consistente wijze het beschermend vermogen van een groenten-fruit mengsel op de colon carcinogenese beïnvloed heeft, zoals werd aangenomen naar aanleiding van de resultaten verkregen uit het onderzoek van Alink en medewerkers (1993).

In het tweede deel van dit proefschrift zijn de experimenten beschreven over interactieve effecten van voedingsvet en een groenten-fruit mengsel op de mechanismen van de carcinogenese. Het hoge vet rantsoen en het voeren van met groenten-fruit mengsel verrijkte rantsoenen veroorzaakte geen duidelijke effecten op de activiteiten van verschillende metabolisme enzymen (ethoxy- en pentoxyresorufine-*O*-deethylatie, *N*-nitrosodimethylamine-demethylase (NDMA-d), glutathion-S-transferase, UDP-Glucuronyl transferase) in de levers van ratten uit de chronische DMH-geïnduceerde colon carcinogenese studie (Hoofdstuk 5). In deze dieren werd ook geen verschil geconstateerd in functioneren van het immuunsysteem in de milt en mesenteriale lymfeklieren, gemeten door middel van de natural killer-cel test, lymfocyten-stimulatietest en gemengde lymfocytenkweektest. De resultaten van een kort durend dierexperiment wijst uit dat de activiteiten van dezelfde metabolisme enzymen in zowel de lever als de dikke darm onder invloed van de dieetsamenstelling sterk worden beïnvloed door DMH (Hoofdstuk 6). Daarnaast blijkt dat het effect van het groenten-fruit mengsel op de NDMA-d activiteit in de dikke darm is beïnvloed door voedingsvet. Tenslotte is onderzocht of interacties plaatsvinden tussen stearinezuur, een verzadigd vetzuur dat veel in dierlijke vetten voorkomt, en indole-3-carbinol (I3C) en extracten van het groenten-fruit mengsel, door middel van *in vitro* testen met verschillende cellen (Hoofdstuk 7). I3C is een non-nutriënt met een mogelijke kankerbeschermende werking. Zowel I3C als extracten van het groenten-fruit mengsel beïnvloeden het effect van stearinezuur op de cytotoxiciteit, intercellulaire communicatie en op de activiteit van het cytochroom P450-IA enzym. De effecten van I3C en de groenten-fruit extracten werden deels beïnvloed door de volgorde waarin deze stoffen met stearinezuur aan de cellen werd gegeven en door de verschillende typen cellen die gebruikt zijn voor deze *in vitro* studie.

De resultaten van het onderzoek beschreven in het tweede deel van het proefschrift tonen dat interactie tussen voedingscomponenten zeer waarschijnlijk is en dat deze interacties de carcinogenese beïnvloeden.

Slotopmerkingen

De resultaten van de chronische dierstudies beschreven in dit proefschrift duiden erop dat een groenten-fruit mengsel, dat qua samenstelling en hoeveelheid overeenkomt met de gemiddelde samenstelling en consumptie in Nederland, toegevoegd aan een laag of hoog vet rantsoen uiteenlopende effecten kan veroorzaken op de dikke darm

carcinogenese. Deze effecten kunnen zowel beschermend als stimulerend zijn, maar de effecten kunnen ook uitblijven. Dit komt niet overeen met de resultaten van epidemiologische onderzoeken, die aangeven dat groenten en fruit beschermend werken tegen het ontstaan van darmkanker. Het overgrote deel van het dierexperimentele onderzoek is dan ook gefixeerd op het ophelderen van de mechanismen waarmee bestanddelen in groenten en fruit, waaronder de non-nutriënten, hun kankerbeschermende werking uitoefenen. Inherent aan dit soort dierexperimenten is het gebruik van grote hoeveelheden van een bepaalde (non-)nutriënt en de relatieve korte tijdsduur van deze experimenten. Deze opzet maakt het echter moeilijk de resultaten te extrapoleren naar de menselijke situatie, omdat in werkelijkheid mensen voedsel consumeren dat bestaat uit wel honderden verschillende voedingscomponenten, waaronder non-nutriënten, die bovendien vaak in relatief lage hoeveelheden in het voedsel aanwezig zijn. Omdat daarnaast een reële kans bestaat dat de effecten van de verschillende voedingscomponenten door elkaar worden beïnvloed, is het logisch te veronderstellen dat het resultaat van die interactieve effecten het risico bepalen op het krijgen van darmkanker en niet het enkele effect van dat ene voedingscomponent. Het dierexperimenteel onderzoek beschreven in dit proefschrift had tot doel effecten te onderzoeken van een meer completere voeding op de ontwikkeling van darmkanker, namelijk het toevoegen van een mengsel van groenten en fruit in rantsoenen met een vetgehalte en samenstelling afgeleid van het Nederlandse consumptiepatroon. Daarmee is bewust gekozen voor een meer complexere samenstelling van de rantsoenen en is rekening gehouden met het optreden van mogelijke interactieve effecten tussen voedingscomponenten. De resultaten van de MNNG-studie, de Apc^{Min} muizen studie, de korte dierstudie en zelfs de *in vitro* studie geven duidelijk aan dat interactieve effecten tussen het groenten-fruit mengsel en vet kunnen plaatsvinden.

Het optreden van interactie tussen voedingsvet en een groenten-fruit mengsel werd echter voor een deel beïnvloed door de duur van de experimenten en door de onderzoeksopzet. Ten aanzien van de duur van de experimenten bleken de effecten van het voedingsvet en het groenten-fruit mengsel op de activiteiten van de metabolisme enzymen te veranderen en zelfs af te nemen naarmate het experiment langer duurde, waarschijnlijk door het optreden van tolerantie. Dat de duur van de dierexperimenten van invloed is op het te meten effect impliceert dat behalve kortdurende dierexperimenten ook langdurige dierexperimenten nodig zijn om inzicht te kunnen krijgen in de relatie tussen voedingsgewoonten en darmkanker.

Een opmerkelijk gegeven ten aanzien van de verschillen in gekozen onderzoeksopzet is dat normale hoeveelheden groenten en fruit in hoog vet rantsoenen, gevoerd aan muizen met een deficiënt *Apc* gen, meer darmtumoren lijkt te geven. Volgens de huidige

opvatting treden afwijkingen van het *Apc* gen al op in de vroege fase van de ontwikkeling van darmkanker, wanneer de aanwezigheid van een darmtumor nog niet duidelijk is. Dit zou dus kunnen betekenen dat iemand met een dergelijke genetische afwijking die zich nog 'gezond' voelt en zijn typisch westers dieet (hoog vet) aanvult met groenten en fruit een hoger risico loopt daadwerkelijk darmkanker te krijgen. Dit in tegenstelling tot gezonde mensen, die niet een extra risico lopen en mogelijk bescherming ondervinden van het consumeren van groenten en fruit. Het verschil in effecten van voedingscomponenten op gezonde en niet gezonde mensen is ook gevonden door Bailey en medewerkers (1987), die een toename in darmtumoren constateerden in ratten die met indole-3-carbinol gevoerd werden, nadat de carcinogenese was geïnduceerd en door Albanes en medewerkers (1996), die constateerden dat β-caroteen het ontstaan van longkanker in zware rokers versnelde. Deze opmerkelijke bevindingen geven aanleiding om voorzichtig te zijn met voedingsadviezen: sommige voedingscomponenten lijken zowel een tumor beschermende als een tumor stimulerende werking te bezitten, afhankelijk van het bezitten van een genetische aanleg en het stadium van de ziekte. Verder onderzoek naar de relatie tussen voedingsfactoren en genetische defecten is noodzakelijk, evenals onderzoek naar de effecten van voedingsfactoren op (pre)neoplastische laesies.

Het onderzoek beschreven in dit proefschrift toont duidelijk aan dat chronische dierstudies, waarin complete voeding wordt bestudeerd, uitgevoerd moet blijven worden om effecten van levensstijl op de colon carcinogenese te kunnen vaststellen. De resultaten van dit type studies geven een waardevolle aanvulling op de gegevens verkregen uit epidemiologisch onderzoek en de gegevens verkregen met kortdurende dierexperimenten.

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About the author

Jolanda Rijnkels was born on 11 October 1964 in Roosendaal, The Netherlands. In 1984 she completed secondary school (HAVO) at the Norbertus College in Roosendaal and started the study of Medical Analyst at the Dr. Struycken Institute in Etten-Leur. In 1988 she completed this study and started the study Biomedical Sciences at the Faculty of Medicine of the State University of Leiden (RUL). As part of this study, she conducted research projects in human drug research (Center of Human Drug Research, Leiden), molecular carcinogenesis (Department of Radiation Genetics & Chemical Mutagenesis, RUL), and in metal toxicology (Department of Human Toxicology, RUL). Furthermore, she was co-organizer of the symposium "Morgen nog ouder...?" of the Society for Medical Students at the RUL.

In December 1992 she received the MSc degree and started to work as scientific assistant in the field of metal toxicokinetics (Department of Human Toxicology, RUL) till August 1993. From August 1993 till November 1997 she was appointed as Ph.D. fellow at the Department of Toxicology, Wageningen Agricultural University. During this appointment she conducted the research described in this thesis, in association with the Department of General Toxicology (TNO Nutrition and Food Research, Zeist) and the Laboratory of Pathology and Immunobiology (RIVM, Bilthoven). The research was financial supported by the Dutch Cancer Society. During this appointment she followed the post-doctoral training in Toxicology. Furthermore, she was chairman of the Ph.D.-council of the research school Environmental Chemistry & Toxicology, secretary of the daily board of the National Ph.D. Organization (LAIOO), and a board member of the Wageningen Ph.D. Organization (WAIOO).

From November 1997 she is appointed as scientific researcher at the Department of Medicinal Photochemistry, Section Biopharmaceutical Sciences (RUL).

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