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Effects of flavonoids on anti-carcinogenesis and immunosuppression

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Preface

This report summarizes all the published and non-published results of experiments in animal tumor models performed in project 313700:"Anticarcinogenen en Biomarkers". It shows a number of experiments from which it can be concluded that flavonoids in diets prevent immunosuppression induced by ultraviolet light. However, no strong evidence is found that this protection is associated with tumor growth prevention. Most of the results have been published in: Photochem. and Photobiol. 65, 150-154, 1997. Photochem. and Photobiol. 65, 342-346, 1997. Photochem. and Photobiol. 65, 736-744, 1997. Cancer Letters 114, 187-189, 1997, Photochem. and Photobiol. 67, 456-461, 1998.

Contents

Samenvatting

Epidemiologische en dierstudies hebben aangetoond dat voedingscomponenten de gevoeligheid voor het ontstaan en de groei van kanker in de mens beinvloeden. Het meest bestendig zijn de epidemiologische studies, waarin een inverse relatie is gevonden tussen hoge inname van groente en fruit en het risico voor kanker. Dit heeft geleid tot een aantal studies in tumormodellen in het proefdier naar componenten in groente en fruit die mogelijk verantwoordelijk zijn voor het waargenomen anticarcinogene effect.
Er is een aantal voedingscomponenten waarvan een beschermende werking is verondersteld. In het voorliggende rapport hebben wij tumormodellen in laboratoriumdieren gebruikt om voedingscomponenten te testen die een beschermende werking hebben. Bepaalde tumoren staan onder controle van het immuunsysteem en daarnaast kunnen voedingscomponenten het immuunsysteen moduleren. Daarom is tevens onderzocht of modulaties in de tumorgroei verklaard kunnen worden vanuit beinvloeding van de immuunresponse door deze voedingscomponenten. Door het immuunsysteem te bestuderen is het wellicht mogelijk biomerkers te identificeren die gebruikt kunnen worden in epidemiologische studies.

In het onderzoek zijn bestudeerd: diëten rijk aan flavonoïden afkomstig van groente en fruit, groene en zwarte thee extracten, en specifieke flavonoide componenten zoals quercetine en chrysine. De diëten werden onderzocht op hun beschermende werking voor UV-geinduceerde huidtumoren. Deze huidtumoren zijn zeer immunogeen. Tevens zijn deze dieten onderzocht op hun beschermende werking tegen UV-geinduceerde immunosuppressie van contact overgevoeligheid (CHS) voor picrylchloride (PCL) in de haarloze SKH muis. Om de bruikbaarheid van andere tumormodellen te onderzoeken, is de anticarcinogene potentie van groene thee bestudeerd in een model met chemisch geinduceerde tumoren en in een model waarin zich adenomen ontwikkelen in de dunne en dikke darm als gevolg van een mutatie in het Apc gen. Het Apc gen is een tumor suppressor gen, dat bij verlies van functie leidt tot het ontstaan van familiaire dikke darmkanker bij de mens en de sporadische vorm van darmkanker.

Resultaten van studies in tumormodellen in laboratorium dieren met de bovengenoemde diëten tonen aan dat er geen consistente preventie is tegen UV geinduceerde huidtumoren. Ook de studies met groene en zwarte thee in chemisch geinduceerde dunne darm en colontumoren laat geen bescherming zien. Alleen in de dunne darm en colon van mannelijke Apc^{Min} muizen, was onder invloed van groene thee de spontane tumor incidentie gehalveerd. In vervolg studies zal onderzocht worden of deze resultaten gereproduceerd kunnen worden.
Betreffende de invloed op het immuunsysteem kon vastgesteld kon worden dat groente en fruit (19%), groene thee (0,8%--0,01% polyfenol extract in drinkwater) en zwarte thee (0,8% poeder in drinkwater), quercetine (5%-1% in dieet), en chrysine (1%-0,01% in dieet), muizen een statistisch significante ($p<0,05$-0,01) bescherming bieden tegen UV-geinduceerde onderdrukking van de CHS met PCL. Mogelijk spelen de antigeen presenterende Langerhans cellen (LC) in de epidermis van de huid een rol bij de preventie van de UV-geinduceerde immunosuppressie. In de huid van muizen gevoerd met fruit en groente of met quercetine gecombineerd met UV bestraling, bleek het aantal LC in de epidermis vergelijkbaar met de controle muizen, terwijl het aantal LC significant verminderd was in muizen die alleen met UV waren bestraald. Naast bescherming van CHS, kon ook worden aangetoond dat toevoegen van groene thee aan het drinkwater (0.1% -0.01%) beschermend was tegen UV geinduceerde suppressie van het vertraagde type van overgevoeligheid (VTO) voor hitte gedode *Listeria monocytogenes.*

Conclusie: het is zeer opmerkelijk dat flavonoiden geen bescherming bieden tegen relatief sterk immunogene huidtumoren, terwijl er wel een bescherming wordt verkregen tegen UV-geinduceerde immunosuppressie op CHS en VTO. Dit wijst er op dat de de mogelijke relatie tussen modulatie van UV-geinduceerde immunosuppressie en modulatie van de tumorgroei nog verre van duidelijk is.

Summary

Epidemiological and animal studies have shown that dietary components influence the susceptibility for the induction and growth of cancer in humans. Most consistent are the epidemiological studies in which an inverse relation is found between high intake of fruit and vegetables and risk of cancer. This has initiated a number of studies in animal tumor models to investigate which components in fruit and vegetables are responsible for the observed anticarcinogenic effect. There has been a number of dietary component for which a protective effect is postulated. In this study, we investigated tumor models in laboratory animals to identify dietary components with an anticarcinogenic potential. As the growth of certain tumors are under control of the immune system and dietary components can modulate the immune system, we investigated the association between the protective effect of dietary components and the immune response. Studying the immune response may identify potential biomarkes for application in epidemiological studies.

We have tested flavonoid-rich diets of fruit and vegetables, green-and black tea extracts and specific flavonoid components such as quercetin and chrysin. These diets were studied for their putative protective effect on UV-induced skin tumors in the SKH mouse. These tumors are very immunogenic. Moreover, the same diets were studied in UV-induced immunosuppression of contact hypersensitivity (CHS) to picryl chloride (Pcl) in the same mouse. In order to study the usefulness of other tumor models, we investigated the anticarcinogenic potential of green tea in carcinogen induced tumor models and in a model in which adenomas develop spontaneously in the small intestine and colon due to a mutation in the *Apc* gene. This Apc gene is a tumor suppressor gene, for which loss of function is associated with familial adenomatous polyposis and sporadic colorectal cancer in human.

Results of animal tumor studies show that diets mentioned above did not consistently prevent UV induced tumors in skin. Studies with green and black tea in chemically induced small intestine and colon tumors showed no protection. Only in the spontaneously arising tumors in the small intestine in Apc^{Min} male mice, the tumor multiplicity was reduced with 50% after administration of green tea. These results will be verified in further studies.

It was found that fruit and vegetable (19% in the diet), green tea (0.8%-0.01% in the drinking water), and black tea (0.8% solids in drinking water) quercetin (5%-1% in the diet), and chrysin (1% and 0.1% in the diet), protected mice from UV-induced suppression of CHS to PCl. We postulate that the antigen-presenting Langerhans cells (LC), present in the epidermis of the skin, may play a role in the prevention of UV induced immunosuppression by the flavonoids tested. In the skin of mice fed with fruit and vegetables or quercetin combined with UV irradiation the number of LC were comparable to the control mice, whereas the number of LC was significantly diminished in mice treated with UV only. Besides the protection of CHS, it was also shown that adding green tea to drinking water resulted in protection to UV-induced immunosuppression of the delayed type of hypersensitivity (DTH) to heat-killed *Listeria monocytogenes.*

In conclusion, it is remarkable that flavonoids induce a very limited protection against relatively strong immunogenic skin tumors, while a protection was induced against UV-induced suppression on CHS and DTH. This shows that causality between modulation of the UV-induced immune suppression and modulation of tumor growth is far from clear.

1 Introduction

Nearly 200 epidemiological studies have been reviewed and relate, with great consistency, adequate consumption of fruit and vegetables to lower cancer incidence (1). Compared to the highest intake, the population with the lowest dietary intake of fruit and vegetables has roughly twice the cancer rate for most types of cancer (e.g. lung, larynx, oral cavity, stomach, colon, bladder, pancreas, cervix and ovary). It is proposed that micronutrients, non-nutrients and fibers contribute to the prevention of cancers (2,3). In animal studies, an inverse relationship is found between the induction of certain tumors and the intake of micronutrients, such as carotenoids, vitamins A,C,E and probably fibers (4). Results from animal studies indicate that non-nutrients including flavonoids may prevent the development of certain tumors (5).The mechanism of the anti-carcinogenic effect is still obscure and many possibilities have been proposed (4).

Flavonoids, which are low molecular weight polyphenolic secondary plant metabolites, represent the major group of the non-nutrients. It has been shown that flavonoids may alter both tumor growth and certain immune responses (6). The dietary exposure to flavonoids is significant, as the content of the average European Western diet varies from 2.6 to 58.2 mg flavonoids/day (7). Of the mixture of dietary flavonoids, the intake of quercetin is 16 mg/day (7).

For certain tumors, including chemically and UV induced tumors, the development of the final tumor burden is believed to be partly under the control of the immune system. In addition to altering the oncogenes and tumor suppressor genes target cells, carcinogenic compounds may cause immunosuppression (8,9). It is in generally accepted that UV induces immunosuppressive effects in animals and man and has a strong carcinogenic potency, especially as an inducer of non-melanomic skin tumors (10). Tumors arising after UV exposure are highly antigenic (10). However, during the induction of tumors by UV radiation, immune mediated control of tumor growth may be deficient since UV also suppresses the immune system. Therefore, if flavonoids counteract UV-induced immunosuppression, consequent prevention of the development of UV-induced tumors may be expected.

Since epidemiological studies have consistently shown that high consumption of fruit and vegetables reduces the risk of cancer, we investigated the existence of a causal link between diet and cancer in animal tumor models. Moreover, we studied the association between the anticarcinogenic action and the immune response. Therefore we tested whether flavonoid-rich diets of fruit and vegetables, green tea extracts or the specific flavonoid components quercetin (a flavonoid with a high number of hydroxyl groups) and chrysin (a flavonoid without hydroxyl groups), prevent the development of UV-induced tumors, and prevent the UV-induced immunosuppression of contact hypersensitivity (CHS) to picryl chloride (PCl) in the SKH hairless mouse. This strain of mice is widely used to study UV induced carcinogenesis and immunosuppression, as well as effects of dietary components (5). In addition, the anticarcinogenic potential of diet components was investigated in chemical induced - and genetically predisposed animal models for intestinal carcinogenesis.

2 Materials and Methods

2.1 Animals

Female SKH-1 hairless albino mice (8-10 weeks of age) and CF-1 male and female mice were purchased from Charles River Laboratories, France. For tumor transplantation experiments the HRA/SKH inbred mice were used, bred and housed in the central animal facility of Utrecht University, the Netherlands. To test intestinal tumors, Apc^{Min} (C57BL/6J-$Apc^{Min/+Apc}$) mutant heterozygote mice were obtained from a colony at Leiden University (R. Fodde, MGC-Dept. Human Genetics). This colony was established with *Min* mice obtained from the original colony of the McArdle Laboratory (W.F. Dove, Mc Ardle labs, University of Wisconsin, Madison). The animals were kept at standard conditions: i.e. 12 h light/12 h dark cycle, 21 ± 2^{0}C temperature, and 50 ± 10% relative humidity in controlled rooms in the animal facility 2-4 weeks prior to and during the experiment. They had free access to water and chow.

2.2 Diets

Fruit and vegetable mixture

The choice of fruit and vegetables used approached the average consumption pattern in The Netherlands. Fruit and vegetables were prepared under household conditions. The following products were used: bananas, oranges, apples, lettuce, green pepper, tomatoes, cucumber, cooked potatoes, cauliflower, spinach, leek, red and white cabbage, sauerkraut, carrots, Brussels sprouts, and beet. Mice are daily fed with about 5 g SRM-A chow(HopeFarms, Woerden, The Netherlands), including 19.5% fruit and vegetable (11).

Green tea polyphenol extract

Green tea polyphenol extract (GTP) was a gift from Dr. L. Tijburg, (Unilever Research Laboratorium Vlaardingen, The Netherlands) and prepared as described by Wang et al., 1992 (12). In short: One hundred g of green tea leaves were extracted 3 times with 300 ml of methanol at 50° C for 3 h, and the samples were filtrated after each extraction. Solvent was removed from the combined extract with a vacuum rotary evaporator. The residue was dissolved in 500 ml water (50°) and extracted 3 times with 200 ml hexane and 3 times with 200 ml chloroform. The aqueous phase was extracted 3 times with 180 ml ethyl acetate, and the ethyl acetate was evaporated under reduced pressure. The residue was redissolved in 300 ml water and lyophilized to 8-9 g of dried green tea polyphenol fraction. 0.1% or 0.01% GTP was added to drinking water and administered to the mice *ad libitum* as their sole source of drinking water throughout the experiment (daily intake was about 4 and 0.4 mg flavonoids, respectively). Mice were supplied with SRM-A chow (HopeFarms, Woerden, The Netherlands).

In the colon tumor studies 0.2 and 0.8 % tea solids were studied (0.8% tea ~ 0.1% GTP). Tea solids were obtained as followed: (in short) green tea leaves (12.5 g, special gunpowder type) were added to 500 ml of freshly boiled water and were steeped for 15 min (13). After cooling end filtration this resulted in 1.25% green tea water extract. The amount of solids present in 1.25% green tea extract was determined to be 4.0 mg/ml by drying the samples. Mice were supplied with a semi-synthetic high fat (40e%) /low Ca (1.1g/kg) Muracon SSP/tox diet (K4121.11, HopeFarms, Woerden, the Netherlands).

Quercetin and chrysin
Quercetin and chrysin (Aldrich Chemie, Bornum, Belgium) were blended into the powdered normal SRM-A diet at a concentration of 1 g or 0.1 g dry weight/100 g dry weight and stored in sealed containers at 4^0C. Animals were fed with normal SRM-A or quercetin and chrysin blended in SRM-A throughout the experiment (daily intake of quercetin 400, 50 and 5 mg; chrysin 50 and 5 mg).

2.3 Tumorigenesis Protocols

UV induced tumors
Two or four weeks after the beginning of feeding with the diets, mice were exposed to UV. For UV exposure, Westinghouse FS40 lamps were used. They emit the total spectrum of UVR of which 0.6% is UV-C (250-280 nm), 53% UV-B (280-315 nm) and 46% UV-A (315-400 nm). Using an Optronics OL-752-O-PMT spectrophotometer (Florida, USA), the energy load at 25 cm beneath the two lamps was approximately 3.26 W/m^2 (100% maximal intensity). Every day the animals were irradiated for 24 min, using a decreased intensity without affecting the energetic spectrum, which was equal to approximately 95 mJ/cm^2 UV or 0.5 of the minimal erythema dose (MED) for this strain of mouse.

UV and DMBA induced tumors
For the induction of UV tumors at lower UV dose, mice were initiated by topical pretreatment with 200 nmoles 7,12-dimethylbenz[a]anthracene (DMBA, Sigma-AldrichChemie, Zwijndrecht, the Netherlands) in 200 microliters of acetone on the back of the mouse. Three weeks later, this initiation was followed by UV irradiation twice a week 30 mJ/cm^2 (13).

1,2-dimethylhydrazine (DMH) induced colon tumors
CF-1 mice were randomly distributed by weight and were given subcutaneous injections for 7 weeks of DMH at an increasing dose (20.5, 24.5, 27, 31, 31, 31, and 31 mg/kg) The dose was gradually increased since mice develop tolerance to DMH (14)

Intestinal tumors.
C57BL/*6J-Apc*$^{Min/+Apc}$ were bred with C57BL/6JIco (+/+) females purchased from Broekman Institute B.V. in the Netherlands. Using DNA isolated from mouse tails, progeny was genotyped before weaning by an allele-specific PCR for the nonsense mutation at codon 850 (15) as described by Jacoby et al. (16). The total number of mice (30 males and 30 females) for this experiment were obtained from breeding couples, each containing one male (C57BL/*6J-Apc* $^{Min/+Apc}$) mouse and two female (C57BL/*6J-*$^{+Apc/+Apc}$) mice. Already from the time of mating, breeding couples were randomly divided in 3 dietary groups to which the different concentrations of green tea (0, 0.2% and 0.8% tea solids in drinking water) were provided *ad libitum*. Mice were sacrificed around day 90 after birth. This point in time was chosen to minimize the risk of intercurrent mortality due to intestinal bleeding resulting in severe progressive anemia or rectal prolaps typically for Min mice around day 120 (17)

2.4 Examination of tumors

UV-induced skin tumors
From week 10 after the start of the UV exposure mice were carefully examined weekly for presence of skin tumors. The tumors were followed in their growth pattern by recording diameters (<1 mm, 1-2 mm, 2-4 mm, >4 mm).

Small intestinal tumors and colon tumors
Mice were killed and complete necropsy was performed on all mice. The gastro-intestinal track was removed from the abdomen, spread onto filter paper, dissected longitudinally with fine scissors and mucus and faeces were removed gently. Kept on filter paper, the small intestin, divided in 6 parts, and the colon were immediately fixed in neutral buffered formaldehyde (4%) overnight. Thereafter the intestinal parts were stained with methylene blue (0.2 %) for 0.5 min. Tumors were count using a microscope (objective 2.5x).

2.5 Hypersensitivity reactions

Contact hypersensitivity (CHS)
The CHS test was performed between 9 and 10 weeks after the start of the UV exposure. Before use picryl chloride (PCl) (Chemotronix, Swannanoa NC) was recrystallized three times from methanol/H_2O and was protected from light during storage. For active contact sensitization, mice (4-5 of each group) were immunized with PCl by epicutaneous application of 150 µl of a 5% solution diluted in ethanol/acetone (3:1) on non-irradiated sites (abdomen, thorax, and 4 feet). Four days after sensitization, these mice were challenged on both sides of each ear by topical application of one drop (27-gauge needle) of 0.8% PCl in olive oil. The ears of non-sensitized controls (4-5 mice) were also treated with one drop of 0.8% PCL in olive oil. Duplicate measurements of ear thickness were made with an engineers micrometer (Mitutoyo model 193-10, Tokyo, Japan) before ear-challenge, and 24 hr after challenge. The increment in ear thickness was expressed as the mean ± SEM in units of 10^{-2} mm. Control or background ear swelling responses, approximately 20 units at 24 hr, were subtracted from the reactions of the related experimental groups to provide the net ear swelling responses.

Delayed type hypersensitivity (DTH) assay.
The DTH assay was performed at week 12 after the start of the UV exposure in 10 mice per group. At week 12 mice were immunized subcutaneously in the neck region with $2x10^8$ heat killed *L. monocytogenes* and were afterwards challenged with heat inactivated *Listeria monocytogenes* (0.01 ml/ear of $2x10^8$ particles/ml) in both ears. After 24 hour, ear thickness was determined in duplicate in both ears as described above (18).

Tumor cell line.
The T51/6.53 tumor cell was a kind gift of Dr V.E. Reeve, Dept. of Veterinary Pathology, University of Sydney, Australia. This cell line was cloned from the T51 cell line that was previously established from a well-differentiated squamous cell carcinoma that was induced by UV in an HRA/SKH mouse. Approximately 4x 10^6 T51/6.53 cells in 0.1 ml Dulbecco's modified Eagle's medium without fetal calf serum was injected intradermally into the unirradiated ventral sites of the mice in a midline position at the level of the forelegs (19).

2.6 Immunohistochemistry of Langerhans cells

Six µm cryostat sections of the skin were fixed in ethanol with 0.02% H_2O_2 for 10 minutes. The first step of the immunoperoxidase technique included the monoclonal rat antibody of nonlymphoid dendritic positive cells (NLDC, 40, 1:10 diluted, donated by Dr G. Kraal, Free University, Amsterdam, The Netherlands) and the second step consisted of PO-labeled rabbit anti rat (1:50 diluted, Jackson ImmunoResearch Laboratories Inc., Pensylvania USA), The second step was enhanced by using a PO-labelled swine anti rabbit (SwAR/PO 1:100 diluted, Dako A/S, Glostrup, Denmark. The peroxidase activity was visualized by di-amino-benzidine tetrahydrochloride and H_2O_2 as substrates with signal enhancement by adding nickel-

ammonium sulfate-6-hydrate during development. The counter staining was performed with Nuclear Fast Red. Negative controls were prepared by using a non-relevant antibody in the first step. The NLDC-positive cells in the epidermis were counted on the basis of 3 to 4 epidermal high power fields (40x) per animal. The number of NLDC positive cells in mice (10 per group) were investigated in the experiment in which the effect of fruit and vegetable consumption was studied.

2.7 Experimental design

Ten mice per group were fed a fruit and vegetable mixture (19.5%), quercetin (5%, 1.0 and 0.1%), chrysin (1.0 and 0.1%), and twenty mice per group were given green tea polyphenol extract (GTP, 0.1 and 0.01%) into the drinking water. After two weeks (four weeks for GTP), mice were exposed to UV irradiation during 9-10 weeks. This time-point was chosen because tumors started to develop and immunosuppression induced by skin tumors was not yet expected. Then mice were immunized with PCL on non-irradiated sites and four days later mice were challenged with PCL on both sides of each ear. The CHS reaction was measured 24 hours after challenge. In addition, after drinking green tea extracts, mice were immunized subcutaneously with heat killed *Listeria monocytogenes* and the DTH skin test was performed. During the entire studies UV induced tumors were enumerated till week 19.

In an additional study, mice fed with a mixture of fruit and vegetables and irradiated with UV, were inoculated with tumor cells at week 13 after the start of UV irradiation to study the effect of diet on the suppression of tumor rejection.

As the poor antitumor activity observed in the UV induced tumors might be caused by the high dose of UV, low dose UV irradiation was combined with topical administration of DMBA on the back of mice. These mice were fed with green and black tea (0.2 and 0.8 % solids).

To study the mechanisms of flavonoid protection in CHS, Langerhans cells in skin were immunohistologically stained and the presence of the cells studied.

Finally, two gastric-intestinal tumor models were employed. Green and black tea (0.8% solids) were tested on the DMH induced colon tumor in the CF-1 mouse, and green tea (0.8% and 0.2% solids) was tested in the Apc^{min} mouse (20 mice/group).

2.8 Statistics

For the statistical analysis of the data from the CHS and DTH tests one way ANOVA was used, and statistical differences between groups were determined using the Student t-test based on the ANOVA. The immunohistochemistry data were calculated using Lotus 2.4 (Lotus development European Corporation, Staines, England) and transferred to the statistical program Minitab (Minitab Inc., State College PA, USA).

3 Results

3.1 Effect of flavonoids on tumor induction and growth

UV induced skin tumors

The effect of fruit and vegetables, GTP and quercetin on UV-induced skin tumors in SKH-1 hairless mice is illustrated in Figure 1. In mice exposed to UV, visible skin tumors appeared from week 10 onwards. During the first 13 weeks of UV exposure few and very small (1 mm) tumors were present. The size and the number of tumors per mouse which were clinically visible increased nearly to 100% by week 16/17. No skin tumors were observed in non-UV-exposed mice. Neither the intake of fruit and vegetables (Figure 1A), nor different concentrations of quercetin (Figure 1C), nor chrysin (data not shown) influenced the appearance or growth of the UV-induced skin tumors. Only GTP added to the drinking water induced a two week delay of the onset of the tumors. However, at week 14 no differences were present (Figure 1B).

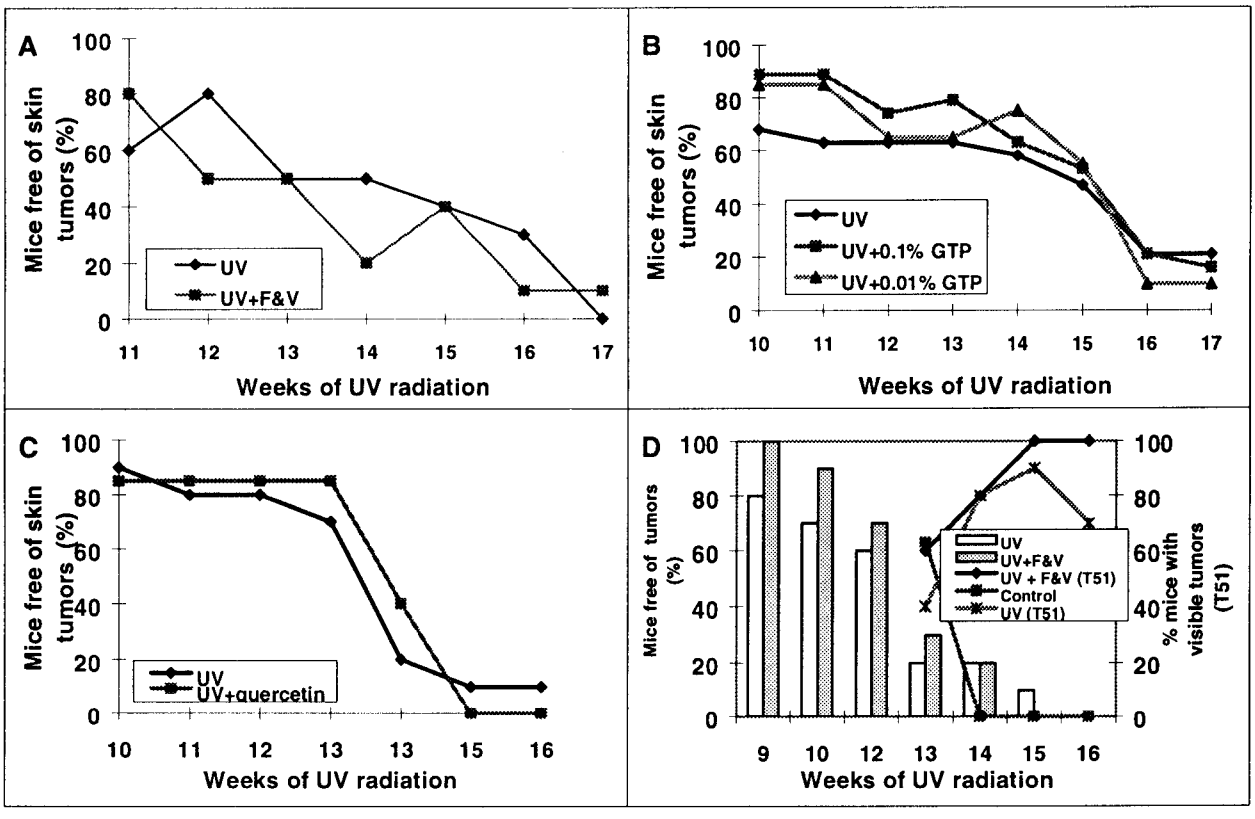

Figure 1. Anticarcinogenicity of fruit and vegetables, green tea and quercetin on UV-induced skin tumors. A) effect of fruit and vegetables (F&V), B) effect of 0.1% and 0.01% green tea polyphenol extract (GTP), C) effect of 4% dietary quercetin on UV-induced tumors, D) effect of F&V on UV induced tumors. At week 12 mice were also challenged with T51 tumor cells intradermally, the % of tumor take is presented. Number of mice per group is 10.

We repeated the fruit and vegetable experiment to test the involvement of the immune system in the anticarcinogenesis process (Figure 1D). For this purpose, we irradiated chronically syngeneic SKH/HRA mice and injected intradermally T51/6.53 tumor cells at week 13 after the start of UV radiation. During UV irradiation the tumor appearance showed some delay (see open and solid bars, figure 1D). However at week 14 no difference was observed. As expected tumor cells were rejected in the control animals, whereas tumor growth was observed

in 80 % of the UV treated mice (see solid lines). All mice UV-exposed and fed with fruit and vegetable diet also showed tumor growth. This indicates that the local immunity observed in the CHS differs from tumor rejection immunity.

The high dose of UV might have been the reason that anticarcinogenic action of the used flavonoids were poor. Combining UV and chemical carcinogenic agents allows reduction of the UV dose needed to induce skin tumors. Therefore, we reduced the dose of UV from 95mJ/cm^2 (daily) to 30 mJ/cm^2 (twice a week) and extended the tumor induction protocol with one topical application of 200 nmol DMBA in 100µl acetone on the back of the mice. Moreover, we tested green tea as well as black tea (0.8% and 0.2% solids). Again no statistical significant effect on tumor induction or on tumor growth was observed (data not shown).

3.2 Colon tumors

DMH-induced

Since the anticarcinogenic activity of green tea has been reported to be very effective on alimentary tract tumors, green and black tea were studied in two intestinal tumor models. Green and black tea (0.2 and 0.8% solids) were added in the drinking water of mice previous treated with DMH. Mice were sacrificed after 32 weeks and tumors of the colon were counted. From Table 1 it appears that the number of colon tumors did not significantly differ in the tea groups compared to the control group.

Table 1

Diet groups	number of mice	number of colon tumors (mean and ± sd)
Control	18	7.6 ± 2.8
green tea (0.8%)	16	7.7 ± 3.8
green tea (0.2%)	13	8.3 ± 3.2
black tea (0.8%)	15	6.6 ± 4.1
black tea (0.2%)	15	5.9 ± 3.2

Spontaneously arising tumors

From Figure 2 it is shown that green tea induced a statistically significant reduction ($p < 0.05$) of the number of small intestinal tumors in male *Apc*^{Min} mice in both dose groups. In contrast, in female mice the number of small intestinal tumors was relatively high in the 0.8% group, although the difference with the controls was not statistically significant. Although a trend was found in the reduction of tumors in males and an increase in female, no statistical significant difference were found in colon tumors (Figure 3).

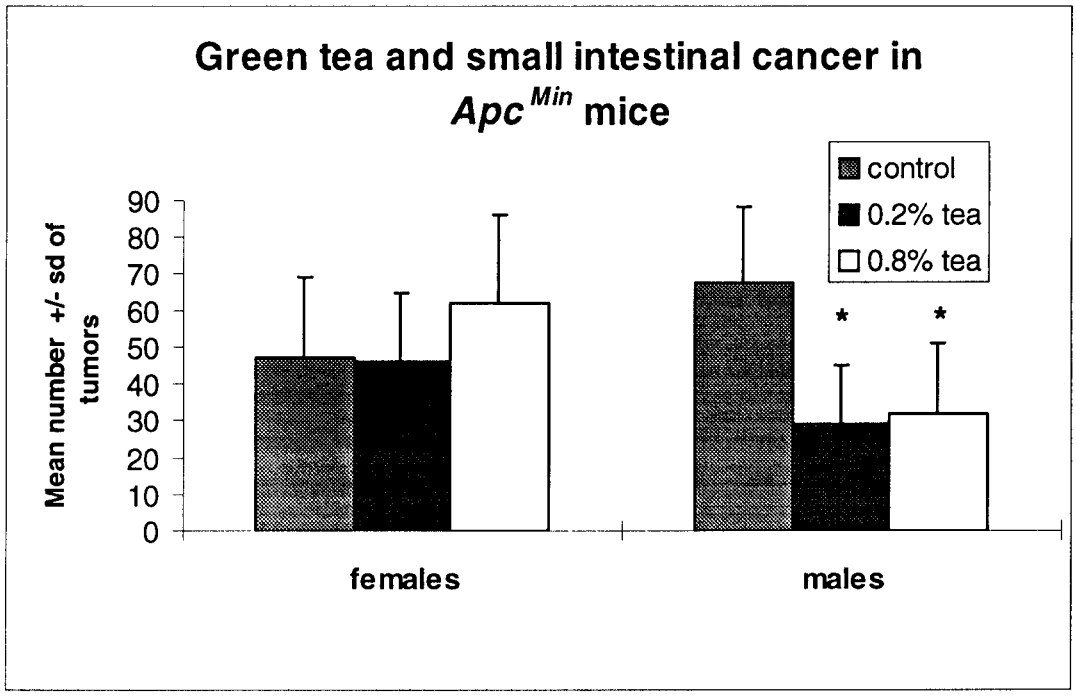

Figure 2 Tea (0.2 and 0.8%) was administered in drinking water during the entire life of the mice (including the prenatal period). Mice were sacrificed around day 90 after birth
*) $p < 0.05$ compared to the control mice.

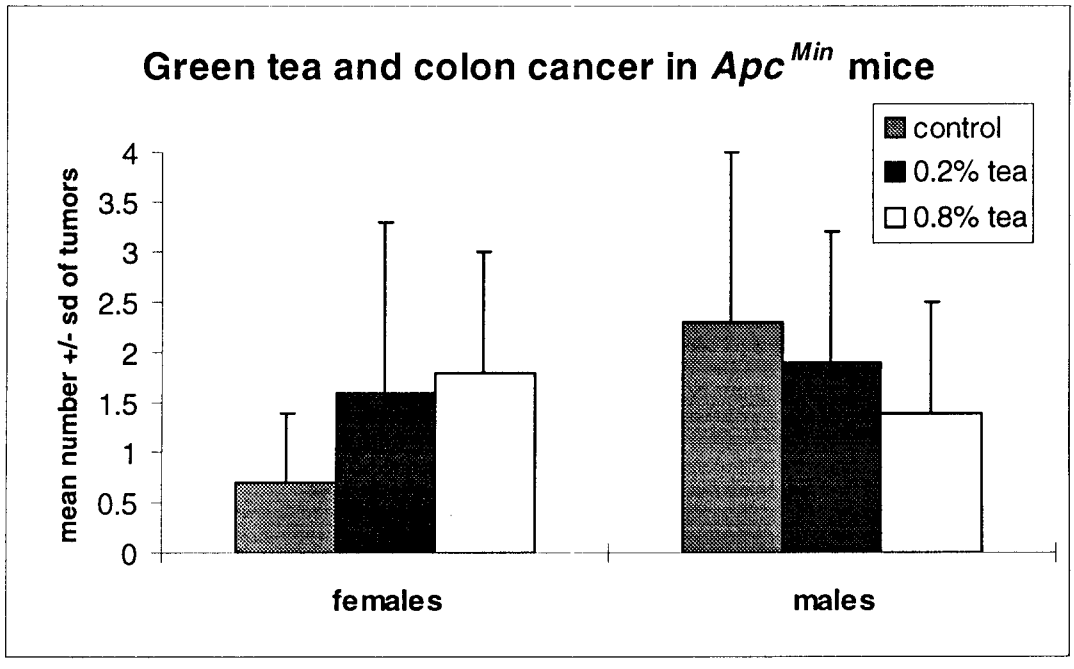

Figure 3 Tea (0.2 and 0.8%) was administered in drinking water during the entire life of the mice (including during the prenatal period). Mice were sacrificed around day 90 after birth.

3.3 Effect of diets on UV-induced immunosuppression of PCl CHS

Nine to ten weeks after starting the UV exposure, animals were sensitized by topical application of PCl to the abdomen, thorax, and four feet. Four days later all mice were challenged on the ears with PCl. Twenty four hours later the ear swelling reactions were measured. In Figure 4 the results of 3 different experiments are summarized. Mice exposed to UV showed a significant suppression of the PCl induced CHS ($p< 0.01$) compared with the control group. Mice fed with fruit and vegetables or chrysin showed no significant alteration of the CHS response. In contrast, dietary quercetin significantly suppressed the CHS. Mice fed with fruit and vegetables, GTP (0.1% and 0.01%), quercetin (1%) and chrysin (1% and 0.1%) and exposed to UV irradiation, were protected against UV-induced suppression of the CHS response: the ear swelling response was significantly different ($p<0.05$-0.01) from UV exposed mice. Concentration of quercetin of 0.1% failed to prevent the UV induced immunosuppression.

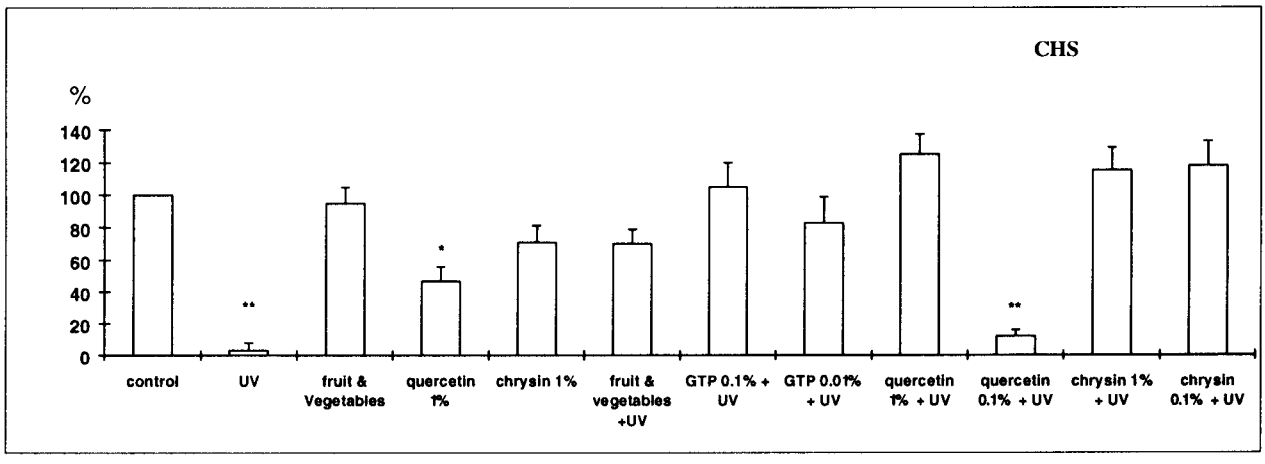

Figure 4. Effect of diets with increased content of flavonoids on UV induced suppression of contact hypersensitivity (CHS). Animals received normal diet or a diet containing a mixture of fruit and vegetable juice (19%) green ttea phenol extract (GTP, 0.1 and 0.01% in drinking water), quercetin (1% and 0.1% in diet), chrysin (1% and 0.1% in diet), throughout the experiment. At week 9, four mice of each group were sensitized by painting the abdomen, thorax and four feet with picryl chloride (PCl). Four days later all sensitized mice and non-sensitized mice (n=4) were challenged on both ears with PCl. Twenty four hours later the ear swelling reactions were measured. Summary of different experiments and presented as percentage of the mean control. Versus control: *) $p<0.05$; **) $p<0.01$.

Finally we studied whether black tea has potential to protect UV induced suppression of CHS. The CHS was performed in the skin tumor study in which low UV dose was combined with DMBA to initiate skin tumors. Figure 5 shows that low dose of UV completely suppresses the CHS to PCl and that the dose of 0.8% of black or green tea solids prevented the UV-induced suppression ($p<0.01$).

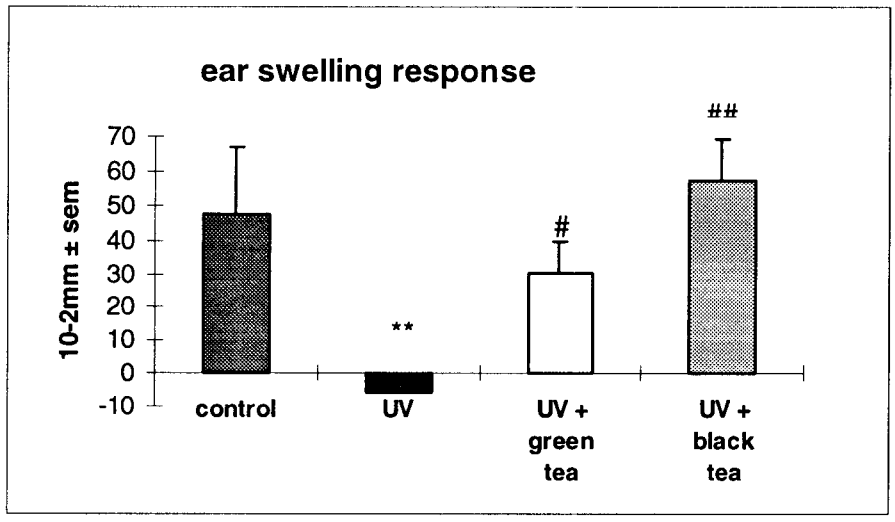

Figure 5. Effect of green and black tea (0.8% solid tea) on UV induced suppression of contact hypersensitivity (CHS)

Animals were fed with normal diet and received green and black tea via drinking water (0.8% solid tea) throughout the experiment. At week 9, four mice of each group were sensitized by painting the abdomen, thorax and four feet with picryl chloride (PCl). Four days later all sensitized mice and non-sensitized mice (n=4) were challenged on both ears with PCl. Twenty four hours later the ear swelling reactions were measured. Summary of different experiments and presented as percentage of the mean control. Versus control: **) $p<0.01$; Versus mice irradiated with UV: #) $p<0.05$; ###) $p<0.001$

3.4 UV-induced immunosuppression of *Listeria monocytogenes* induced DTH reaction

Mice immunized subcutanously with killed *Listeria monocytogenes* followed by intradermal injection of *Listeria monocytogenes*, showed a strong DTH response (measured at 24 hours), which was suppressed significantly ($p<0.001$) after prior UV exposure for 12-14 weeks (Figure 6). UV exposure in combination with GTP (0.1%) showed also a significant ($p<0.001$) reduction of the DTH response. In mice with receiving 0.01% GTP in drinking water the DTH reaction was fully present. Comparing the DTH response of UV irradiated mice to mice receiving tea, statistically significant protection against the UV-induced suppression was observed by 0.01% GTP ($p<0.001$) and a marginal protection was found at 0.1% GTP ($p<0.05$).

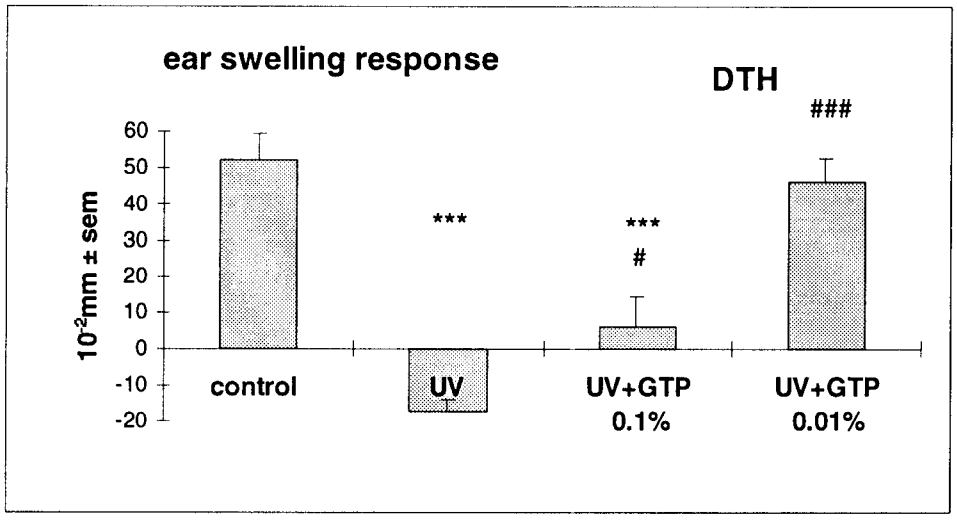

Figure 6. Effect of green tea polyphenol extract on UV induced suppression of delayed type hypersensitivity (DTH) to heat-killed *Listeria monocytogenes*.
Animals were fed with normal diet and received GTP via drinking water (0.1 and 0.01%) throughout the experiment. At week 12 after the start of UV irradiation a total of 10 animals (4-5 of each group) were sensitized and challenged with heat-killed *Listeria monocytogenes* intradermally.
Versus control:***) $p<0.001$; Vs UV irradiation: #) $p<0.05$, ###) $p<0.001$.

3.5 The number of Langerhans cells (LC)

The number of LC's were scored in the experiment in which mice were consuming fruit and vegetables or receiving GPT in drinking water. It was found that UV irradiation reduced the number of LC in the skin significantly. Mice receiving UV irradiation in combination with the consumption of fruit or drinking tea showed a significant ($p<0.01$) higher number of LC compared to the UV treated group (Table 2).

Table 2. Effect of fruit and vegetables on number of NLDC positive cells

Group	Quercetin (5%)	Vegetables & fruit†
control	8.6 ± 0.97	9.1 ± 1.0
UV	6.1 ± 1.6 **	6.6 ± 1.8**
Diet	8.9 ± 0.93	9.6 ± 1.2
UV+Diet	8.6 ± 1.5 ##	8.3 ± 1.1#

† Mean number of $NLDC^+$ cells (± sd) per high powerfield (3 or 4 fields per animal)
**$p<0.01$ UV *versus* control
# $p<0.05$: UV+ vegetables & fruit *versus* UV
##<0.01 UV + quercetin *versus* UV

Mice (n=8-10/ group) were fed with normal diet (control and UV) or normal diet blended with fruit and vegetables juice (19%) or blended with quercetin (5%) *ad libitum* throughout the experiment. Two weeks of the start after the diet mice were irradiated daily for 24 min. during 10 weeks with approximately 95 mJ/cm^2 UV or 0.5 minimal erythemal dose (MED) for this strain of mouse.

4 Discussion

In the present study, we demonstrated that the daily intake of flavonoids whether from the consumption of fruit and vegetables (6.4 mg flavonoids), GTP (4 and 0.4 mg flavonoids), quercetin (50 and 5 mg) or chrysin (50 and 5 mg) being of similar order of magnitude, result in an inconsistent protection against tumor growth, whereas a consistent prevention of UV-induced immunosuppression of the CHS is seen.

Effects of flavonoid enriched diets on tumor growth

None of our studies, in which the SKH-1 mouse was used to test our fruit and vegetable mixture, green tea (polyphenol) extract or single flavonoids such as quercetin or chrysin, showed any distinct and convincing anticarcinogenic activity. The only exception may be a reduced number of small intestinal tumors in male Apc^{Min} mice fed with green tea extract. This is in contrast with most of the published studies in tumor models in laboratorium animals and especially for green tea. The anti-carcinogenic effect of flavonoids has been investigated in e.g. SKH-1 hairless mouse, which is a widely used animal model to study the effects of dietary components on UV induced carcinogenesis and immunosuppression. In this model, the anti-carcinogenic effect of various substances including green tea polyphenols (12, 13, 20-25), the protective effect of fatty acids on immune responses and on UV-induced carcinogenesis (26, 27), and tumor growth inhibition by the consumption of fresh fruit and vegetables was investigated (28). In the same model garlic extract (containing relative high concentrations of quercetin, ca. 7 mg/100g fresh weight, personal communication Dr. T. Leth, Denmark) and green tea polyphenols (29,30) were found to protect against UV-induced suppression of contact hypersensitivity (CHS). In a number of studies, UV irradiation was preceded by a initiation step with a carcinogen such as DMBA or UV irradiation was followed by administration of 12-O-tetradecanoylphorbol-13-acetate (TPA) for tumor growth promotion. Our tumor model closely resembles the model used by Robinson et al. (28) using UV-radiation to induce skin tumors. In their study only cancer growth differed substantially with various diets. Raw fruits and vegetables were found to markedly delay the onset of tumor growth. This effect was not caused by carrots, tomatoes or wheat grass components, but was dependent upon the inclusion of apples and pears. These data may suggest that raw fruit may be essential for the effect on the tumor growth, whereas in our study in which freezed dried homogenized fruit and cooked vegetables were used no effects were found.

A number of studies has been carried out using SKH-1 mice in order to test the effect of green tea compounds on the induction of skin tumors by UV light. A possible explanation for the relatively small effect of in our study GTP on the prevention of skin tumor induction, as compared to published data (12, 13, 20-25), is the difference in treatment protocols: in our first studies we did not combine UV exposure with additional carcinogenic chemicals, such as DMBA or TPA, as was done in the studies published (12, 13, 20-25), but used only a daily UV exposure protocol to induce non-melanoma skin tumors. Combining UV and chemical carcinogenic agents allows reduction of the UV dose to induce skin tumors. Therefore, we reduced the dose of UV from 95mJ/cm^2 (daily) to 30 mJ/cm^2 (twice a week) and extended the tumor induction protocol with one topical application of 200 nmol DMBA in 100µl acetone on the back of the mice. Moreover, we tested green as well as black tea (0.8% solids). Again no statistical significant effect on tumor induction or on tumor growth was observed (data not shown). From this study, we can conclude that the high dose of UV is not the cause of the lack of clear effects of the

flavonoids. Therefore, we have to conclude that the results of our studies on the effects of green tea and its effect on UV induced skin tumors, are not in agreement with the literature.
Systemic administration of the procarcinogen DMH to CF-1 mice is a well-established laboratory model of colon carcinogenesis (14, 31, 32). DMH selectively induces colonic adenomas and adenocarcinomas, while only rarely causing liver or small intestine tumors (33). One injection of DMH causes the development of aberrant crypts within 2 weeks, while 6 injections of DMH, one per week for 6 weeks, typically causes grossly visible adenomas and carcinomas of the colon within 4-6 months. Although there is some controversy (34), there is evidence that DMH-induced colon tumors resemble human tumors in that both are able to progress from an adenoma-to-carcinoma. Moreover, the DMH-induced carcinogenesis might also be under control of local immune responses (35) In our study, we combined the diet with high fat (40energy%) to increase the number of colon tumors. The number of observed colon tumors was far above the mean number of colon tumors found in other studies (31, 32). However, our study with green and black tea solids did not reveal protective effects of the tea consumption. This is in contrast with the results of a study in which green tea showed a marked reduction in tumor incidence (31).

Only in male Apc^{Min} mice, green tea induces a reduction in the number of small intestinal polyps. In humans, an inherited heterozygous mutation of the *Apc* gene is found in patients having familial adenomatous polyposis (FAP) and in tumors of most patients having sporadic colorectal cancer. Various studies have demonstrated that *Apc* deficient mice are an appropriate animal model for intestinal neoplasia, in particular FAP (36, 37) . So far, no explanation can be given for the observed opposite effects of green tea for both sexes. In a previous study (38) in which a mixture of fruit and vegetables was incorporated in a high fat (40e%) diet a significant increase in intestinal and colon tumors for both male and female was observed. However, in male mice consuming a low fat diet (20e%) a reduction of only small intestine tumors was found, while in female mice the number was significantly increased.

These studies in tumor models in laboratory animals show that diets containing high concentrations of flavonoids do not consistently prevent tumor induction in skin, small or large intestines. The variability of the results of our studies and those of others may point to yet undiscovered nutritional or methodological factors that determine the effect on the tumor respons.

Effects of flavonoid enriched diets on UV-induced immunosuppression

In the mixture of fruit and vegetables used in this study only traces of quercetin and chrysin were present. The protection against UV-induced immunosuppression of CHS by fruit and vegetable suggests that in addition to the tested flavonoids other flavonoids or other components play a role in the prevention against immunosuppression.

Comparing quercetin and chrysin, it was found that chrysin was more potent in protection of the CHS ear swelling response than quercetin, as at a concentration of 0.1% chrysin was still protective, whereas quercetin (0.1%) failed to affect the UV-induced immunosuppression. In a previous study no differences in the antimutagenic potency, using an Ames test, was found for quercetin and chrysin, being components with a high and a low number of hydroxyl groups, respectively (39). A puzzling observation is that flavonoids such as quercetin itself show a tendency to induce immunosuppression. This might be explained by quercetin having an anti-inflammatory activity by the arachidonic acid pathway (20), reducing the CHS response. Immunosuppression (without UV radiation) is also observed in experiments using high dietary fat prior to the dinitrochlorobenzene induced -DTH (40).

Green tea contains 35-52% (measured in weight % of extract of solids) catechins and flavonoids. Oral intake of 0.05% (w/v) GTP in drinking water is a dose that approximates the intake of four cups of tea per day by an adult human (20). The protection of the CHS reaction by green tea is in accordance with the observation of Katiyar et al. (29). In stead of topical application of GTP on the skin, we have administered 0.1 % and 0.01 % GTP orally in the drinking water. Katiyar et al. (29), exposed mice with a single dose of UV (2-32 kJ/m^2). In our study, mice are exposed to 95 mJ/cm^2 /daily for 10 weeks (total irradiation ca. 63 kJ/m^2). In the study of Katiyar et al (29), among the four major epicatechin derivatives present in GTP, (-)-epigallo catechon-3 gallate were been most effective.

In addition to the results of green tea, the effect of black tea on the suppression of CHS is presented. The composition of back tea solids differs from green tea by containing 4 times less catechins, whereas, 2.5 times more unindentified polyphenols are present. In our study we found that black tea solids prevented the UV induced suppression of CHS to PCl, which was in line with the expectation, as black tea also prevented UV induced tumor growth (13).

We observed that flavonoid induced protection is not restricted to the CHS response. The DTH responses to L. *monocytogenes* is also protected after oral administration of GTP. In this experiment we do not immunize and challenge on the epidermis, but inject the heat-killed bacteria subcutaneously and for the challenge we use the intradermal route of injection. So, we suggest that UV interferes not only with the superficial LC, but also with antigen presenting cells in the deeper layers of the epidermis. The prevention of immunosuppression was, however, only observed on the local immune response in the skin. In previous studies (5, 41) it was shown that the suppression found in the spleen was not affected by the used diets.

To study whether this local prevention of immunosuppresion was also directed at tumor rejection responses, we inoculated the skin with syngeneic tumor cells, which are highly antigenic and will only be rejected in a UV induced immunocompromized SKH mouse. The observation that the tumor take in the fruit and vegetable diet group combined with UV was nearly 100, indicates that the protection against UV induced immunosuppression was only

restricted to CHS or DTH and not to tumor rejection. Adding our data to previous data of anticarcinogenic diets in UV induced immunosuppression and tumor growth in the SKH mouse, no strong evidence is present that prevention of immunosuppression is associated with tumor growth prevention (Table 3).

Table 3. Protection against local UVB induced immunosuppression and tumor growth by fruit & vegetables, single flavonoid components and fatty acids in SKH mouse

Diet component (oral administration)	Concentration in diet or drinking water	Prevention immunosuppression CHS + or -*	Referen-ces	Antitumor activity** + or -	References
Quercetin	1-5%	+	5, 42	-	5
Chrysin	0.1-1%	+	42	-	present study
Garlic	0.1-4%	+	30	?	-
Green tea	1-6 mg/ml***) 0.1-0.01%	+	23, 42	+	20 22, 23, 24
Black tea	0.8%	+	5	+	13, present study
Omega-3 Fatty acids****)	12%	+	27, 40	+	26, 27
Omega-6 Fatty acids*****)	1-12%	-	27, 40	-	26, 27
Fruit & vegetables	20%	+	5	-	present study

) UVB induced suppression of contact hypersensitivity (CHS) was prevented (+) ; **) prevention of tumor growth; ***) Topical administration; *) Menhaden oil; *****) Corn oil**

We studied the number of LC in the dermis upon UV radiation after consumption of fruit and vegetables and after the intake of quercetin. In both studies we found that the number of LC were preserved in the dermis after irradiation combined with the diet (5, 42). Protection of LC in the dermis may be one of the mechanisms flavonoids protect against UV induced immunosuppression of the CHS.

The mechanism of the prevention of the UV-induced suppression of the CHS response is far from clear. Flavonoids may act as a sunscreen and prevent DNA damage of dermal cells or cells of the immune system (43), or prevent the subsequent initiation of conversion of *trans* to *cis*-urocanic acid (UCA) (44,45), and/or prevent production of IL1β and TNFα by keratinocytes leading to the altered migration pattern and functional activity of LC cells after UV irradiation (10, 42, 46-48). From our previous and present studies we are able to exclude some of the above mentioned options. Concerning the sunscreen option, it was previously found that the transmission spectra of quercetin showed absorption of UV (5), whereas GTP does not (8). In a mouse model, it was shown that increasing the repair of UV-specific DNA lesions abrogated the suppression of CHS and DTH reactions (42). Results from the Ames test (39) show that bioflavonoids are potent antimutagens, which pleads for the role of flavonoids in preventing DNA damage resulting in an altered production of immunostimulatory cytokines in the skin. In a previous study (5) the protection of UV-induced CHS suppression by quercetin is not associated with a reduction of the *cis*-urocanic acid (UCA) concentration compared to the UV irradiated mice, indicating that UCA does not play a role in the protection. Therefore, it is not plausible that flavonoids act as a sunscreen or prevent conversion of *trans* to *cis* UCA. Finally, one may speculate that flavonoids may interfere with the activity of TNF α inducing migration of LC (49). However, it is still under debate whether the disappearance of LC from the epidermis is caused by an altered migration pattern (LC cells are forced to migrate, 44, 50), by an altered recruitment of LC (51) or by lethal cell damage of LC (52). Alcalay and Kripke (49) showed a decreased activity of LC in the draining lymph nodes before the start of the tumor growth while LC function was recovered during tumor growth. A similar trend was seen for the number of LC in the dorsal skin (49). In a previous study (5), in which quercetin (5%) prevented the UV induced suppression of CHS to PCl, it was shown that the number of LC

were not affected by UV in combination with orally administration of quercetin. The present results also show protective effect of fruit and vegetables on the number of LC before the start of the tumor growth

In conclusion, in our study orally administered fruit and vegetables, tea extracts, and single flavonoids, show no protection to UV-induced carcinogenesis. Moreover, oral intake of tea extracts did not prevent tumor growth in intestine and colon, except in the *Apc*^{Min} male mice. However, all diets show protection against UV induced suppression of the cellular immune response to contact sensitizers. This may indicate that flavonoids, natural ingredients in fruit and vegetables, have a common feature, i.e. protection of UV induced immunosuppression (53, see also Table 3). Although the growth of UV induced skin tumors are under control of the immune system, our data shows that this prevention did not include suppression of tumor rejection.

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