Effects of a flaxseed mixture and plant oils rich in α-linolenic acid on the adenoma formation in multiple intestinal neoplasia (Min) mice

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Flaxseed is a dietary source of possible chemopreventive compounds such as lignans and α-linolenic acid (ALA). To study the effects of a flaxseed mixture on adenoma formation in multiple intestinal neoplasia mice, the mice were fed a diet containing 2·7 % flaxseed, 4·5 % fibre and 3·7 % ALA. To elucidate the effect of oils of the mixture we also composed a diet without flaxseed but with the same oil composition. The median number of adenomas in the small intestine was fifty-four for the control group, and thirty-seven ($P=0·023$) and forty-two ($P=0·095$) for flaxseed and oil groups, respectively. Compared with controls (1·2 mm), the adenoma size was smaller in the flaxseed (0·9 mm; $P=0·002$) and oil (1·0 mm; $P=0·012$) groups. Both diets changed the proportions of n-3 and n-6 fatty acids in the colonic mucosa. Membrane β-catenin and protein kinase C (PKC)-ζ levels were reduced in the adenoma $v.$ mucosa ($P<0·05$), and an inverse association was found between the membrane PKC-ζ in the mucosa and the adenoma number (r −0·460, $P=0·008$, n 32). Only the flaxseed diet increased lignan levels in the caecum ($P=0·002$) and in plasma ($P=0·002$) but they were not associated with tumour formation. The results suggest that the preventive effect of flaxseed on colon carcinogenesis may be due to the oil part of flaxseed, and the loss of β-catenin and PKC-ζ from the membranes of the mucosal tissue may play a permissive role in intestinal tumour development.

Colon cancer: Flaxseed: Fatty acids: Min mouse: Protein kinase C-ζ

Colorectal cancer is the third most common cancer (9·4 %) worldwide after lung and breast cancers. It ranks second in incidence and mortality in Western-type societies (Stewart & Kleihues, 2003). Lifestyle factors including diet, overweight, low physical activity and smoking may account for 70 % of colorectal cancers (Willet, 2002). The *adenomatous polyposis coli* (*APC*) gene is mutated in 80 % of sporadic colon cancers and in patients with familial adenomatous polyposis (Nishisho *et al.* 1991). The multiple intestinal neoplasia (Min) mouse, also called the *APC*$^{Min/+}$ mouse, carries one wild-type and one mutated *APC* allele in all somatic and germline cells (Su *et al.* 1992). Thus Min mice, which have multiple adenomas in the small intestine and colon, are widely used in studies on diet and colorectal cancer (Corpet & Pierre, 2003).

Dysfunction of the APC protein causes, among other things, chromosomal instability (Fodde *et al.* 2001) and improper regulation of cellular β-catenin pools (Henderson, 2000). In the cytosol, APC protein together with glycogen synthase kinase-3β and axin, post-transcriptionally regulate the level of β-catenin. Mutations in the *APC* gene lead to the accumulation of hypophosphorylated β-catenin protein in the cytosol and later in the nucleus, where β-catenin together with Tcf/Lef-transcription factor activate the expression of target genes such as *C-myc* and *cyclin-D1* (He *et al.* 1998; Tetsu & McCormick, 1999).

There are also other factors in addition to the APC–β-catenin pathway which are involved in colon tumorigenesis. The non-steroidal anti-inflammatory drugs, which inhibit cyclo-oxygenase (COX) expression and prostaglandin biosynthesis, can protect against colorectal cancer in animal models as well as in human subjects (Steinbach *et al.* 2000; Corpet & Pierre, 2003). Furthermore, the anti-tumorigenic effect of n-3 EPA on adenoma formation in Min mice was partly related to alterations in prostaglandin biosynthesis (Hansen Petrik *et al.* 2000).

Protein kinase C (PKC) isozymes are involved in diverse biological processes, including cellular proliferation, differentiation and apoptosis as well as malignant transformation (Black, 2001). Human and animal studies have shown that PKC-βII is up regulated, and PKC-α, -βI, -δ and -ζ are down regulated in colon tumorigenesis (Kahl-Reiner *et al.* 1994; Wali *et al.* 1995; Klein *et al.* 2000). Recently, atypical PKC (ζ and λ/τ) have been shown to co-localise with Par-proteins at cell–cell contacts in epithelial cells, thus establishing cell polarity, and also to phosphorylate glycogen synthase kinase-3β and subsequently induce association of APC with microtubules (Etienne-Manneville & Hall, 2003a,b).

Flaxseed contains lignans and α-linolenic acid (ALA), which have been suggested to have a preventive effect on cancer in experimental animals and human subjects (Thompson, 1998; Adlercreutz, 2002). Flaxseed contains a high amount of secoisolariciresinol diglucoside, which is metabolised into mammalian lignans enterodiol and enterolactone (ENL) by the gut microflora. Plant and mammalian lignans may protect against cancer by modulating hormone metabolism, acting as antioxidants or inhibiting angiogenesis (Adlercreutz, 1998; Kitts *et al.* 1999; Dabrosin *et al.* 2002). Studies with flaxseed have given contradicting results in animal models for colon cancer. In some studies, flaxseed has had a chemopreventive effect whereas in others no such effects have been found (Serraino & Thompson, 1992; Jenab & Thompson, 1996; van Kranen *et al.* 2003; Oikarinen *et al.* 2005). Previously, we have shown that a moderate amount of defatted flaxseed had no effect on adenoma formation in Min mice (Oikarinen *et al.* 2005). To elucidate the effect of a higher flaxseed level on adenoma formation in Min mice we used a commercial flaxseed mixture (HK-Ruokatalo, Vantaa, Finland), which contains defatted flaxseed, wheat fibre, as well as flaxseed and rapeseed oils. The diet containing flaxseed mixture was composed such that the level of defatted flaxseed was five times higher than in our previous study (Oikarinen *et al.* 2005), and the lignan (secoisolariciresinol) level was 130 mg (380 μmol)/kg diet. To elucidate the role of the oil component of the flaxseed mixture in adenoma formation, a lignan-free diet with an oil composition similar to the flaxseed-supplemented diet without the addition of flaxseed or wheat fibre was created.

Materials and methods

Diets

The control diet was a semi-synthetic AIN-93G-based (Reeves *et al.* 1993) non-fibre high-fat diet (Harlan Teklad; Madison, WI, USA; Table 1). The fat concentration (20 g/100 g) and fat composition of the control diet was designed to approximate to that in a typical Western-type diet. The flaxseed diet was supplemented with 15 % (w/w) of flaxseed mixture, which contained 26·6 g defatted flaxseed, 35·3 g wheat fibre, 44·1 g rapeseed oil and 44·1 g flaxseed oil. The flaxseed diet, prepared by diluting the non-fibre diet with the addition of a flaxseed mixture, took into account the protein, soluble carbohydrates and fat provided by components of the mixture. Wheat fibre isolate contained 78 % cellulose, 21 % hemicellulose and <1 % lignin. The flaxseed diet had 4·5 % fibre, 2·7 % defatted flaxseed, and plant lignans, mainly secoisolariciresinol (130 mg (380 μmol)/kg diet). The oil diet had a similar oil composition as the flaxseed diet without supplementation of defatted flaxseed and wheat fibre. All three diets were similar with respect to protein (20 %), carbohydrates (40 %) and fat (40 %) on an energy basis (kJ).

Animals and samples

The Laboratory Animal Ethics Committee of the University of Helsinki approved the study protocol. Min mice were bred at the Laboratory Animal Center, University of Helsinki, from the mice originally obtained from Jackson Laboratory (Bar Harbor, ME, USA). All Min mice (eleven to fourteen per group) started on the experimental diets at the age of 5 weeks. At the age of 15 weeks, mice were killed by CO_2 asphyxiation. A blood

Table 1. Nutrient composition of the experimental diets (g/kg)*

Ingredient	Control	Flaxseed	Oil
		Diet	
Casein	236·2	216·1	236·2
Dextrose	479·0	449·8	479·0
Butter	148·9	83·6	87·6
Sunflower-seed oil	13·3	7·5	7·8
Rapeseed oil	62·2	34·9	36·6
AIN-93G mineral mixture	41·6	39·7	41·6
AIN-93G vitamin mixture	11·8	11·3	11·8
L-Cystine	3·6	3·4	3·6
Choline chloride	3·6	3·4	3·6
Tertiary butylhydroxyquinone	0·014	0·014	0·014
Flaxseed mixture†	–	150·0	–
Oil mixture‡	–	–	94·2
Diet content (g/kg diet)			
Linoleic acid	24·4	30·4	31·7
α-Linolenic acid	8·0	37·1	39·0

* Casein was obtained from Kainuun Osuusmeijeri (Sotkamo, Finland), dextrose from Six Oy (Helsinki, Finland), AIN-93G mineral and vitamin mixtures from Harlan Teklad (Madison, WI, USA), and L-cystine, choline chloride and tertiary butylhydroxyquinone from Yliopiston Apteekki (Helsinki, Finland). Butter, rapeseed oil and sunflower-seed oil were from a local market. Flaxseed and oil mixtures were generously provided by HK-Ruokatalo (Vantaa, Finland).
† Flaxseed mixture (150 g) contained 26·6 g defatted flaxseed, 35·3 g wheat fibre, 44·1 g rapeseed and 44·1 g flaxseed oils, and 7·6 g carbohydrates, 45·5 g fibre, 88·2 g fat, 9·5 g protein, 1·8 g ash and 1·4 g water.
‡ Oil mixture contained 47·1 g rapeseed and 47·1 g flaxseed oils.

sample was collected from the abdominal aorta and centrifuged at 6000 *g* for 1 min, after which plasma was stored at −70°C for ENL analyses. The contents of the caecum were collected and stored at −70°C to await lignan analysis. The guts were washed with ice-cold saline, and scoring of adenomas was done as described by Mutanen *et al.* (2000). Briefly, the small intestine and colon + caecum were rinsed with ice-cold saline and spread flat on a microscope slide. The number, diameter and location of adenomas were determined with a light microscope with a magnification of 67× by two observers blind to the dietary treatment. Adenoma tissue was clipped off, and the small intestinal mucosa was scraped. Tissues were snap-frozen in liquid N_2 and stored at −70°C for further analysis.

Lignan analysis

Plasma ENL and lignan metabolites in the intestinal contents were analysed by using time-resolved fluoroimmunoassay and HPLC with coulometric electrode array detector, respectively (Adlercreutz *et al.* 1998; Stumpf *et al.* 2000; Oikarinen *et al.* 2005).

Total fatty acids of the colon tissue

About 40 mg mucosa was extracted with hexane–isopropanol (2 + 3) (Hara & Radin, 1978) and the liberated fatty acids interesterified with acidic methanol (Stoffel *et al.* 1959). The fatty acid composition was determined with a Hewlett-Packard 6890 gas chromatograph (Chemstation software A.06.03; Hewlett-Packard, Palo Alto, CA, USA) equipped with a 25 m NB-251 column (internal diameter 0·32 mm, phase layer 0·20 μm; HNU-Nordion Instruments, Helsinki, Finland) and the carrier gas was obtained from a hydrogen generator. Fatty acid peaks from 14:0 to

22 : 6 were identified in a temperature-programmed run. The identified fatty acids in each sample were normalised to 100 %.

Western blot analysis

Subcellular localisation of β-catenin and PKC is considered to be an important determinant of their function. The mucosa and adenoma tissues of the distal small intestine were therefore fractionated into nuclear, cytosolic and membranous fractions as described by Pajari *et al.* (2003). Normalised amounts of the fractionated proteins (10–20 μg), and constant amounts of rat brain or RAW cell homogenate (controls for inter-assay variation) were resolved by 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis, and each sample was run twice, the duplicates being loaded on a different gel. The proteins were blotted onto a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech, Little Chalfont, UK) at 100 V for 1 h, and blots were blocked with 5 % non-fat milk powder or 3·5 % non-fat soya flour in tris(hydroxymethyl)-aminomethane-buffered saline containing 0·1 % Tween (TBS-Tween; Sigma, St Louis, MO, USA) overnight at 4°C. Antibodies against β-catenin, sc-7199; PKC-ζ, sc-216; COX-2, sc-1745; lamin B, sc-6216, as well as all horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-β-actin monoclonal antibody (A5441) was from Sigma (St Louis, MO, USA). The blots were first probed with the primary antibodies for 2 h at room temperature, washed three times with TBS-Tween and incubated with the HRP-conjugated secondary antibody for 2 h. Signals were visualised by using the ECL reagents and film (Amersham Corp., Arlington Heights, IL, USA) according to the manufacturer's instruction. Specificities of signals were verified either with incubations without appropriate primary antibody or by using a blocking peptide provided by a supplier. The specificity of the β-catenin bands was ensured earlier by using two other commercially available antibodies (Pajari *et al.* 2003). The purity of cellular fractions was controlled by determining the nuclear lamin B levels in the cellular fractions. Both cytosol and membrane fractions were free of lamin B. COX-2 was present in the membrane (and nuclear) fraction of the adenoma tissue but was absent in the cytosol fraction. β-Actin intensity of cytosolic fraction was checked to ascertain that equal amount of protein were loaded into gel. Three animals were excluded from the statistical analysis of immunoblot results on the basis on poor signals of lamin B and β-actin. Blots were scanned and analysed using a GS-710 calibrated imaging densitometer and the Quantity One® software (Bio-Rad Laboratories, Hercules, CA, USA). The intensities of sample bands were controlled by loading on each gel a constant amount of control sample (rat brain homogenate) in duplicates, which was used to normalise the sample intensities between different blots. Results in duplicate are expressed as sample band intensity (optical density of the β-catenin or PKC-ζ or COX-2 band multiplied by band area) divided by control band intensity. Furthermore, the nuclear β-catenin signal of each sample was standardised by the signal of lamin B (nuclear marker).

Statistics

Data were analysed using the non-parametric Mann–Whitney U test to compare experimental groups with the control group and sexes within a diet group. Associations between variables were analysed with non-parametric Spearman's correlation test.

Wilcoxon signed-ranks test was used to compare the distribution of two variables. Statistical analyses were performed with SPSS software, version 10.0 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $P < 0.05$. Data are reported as medians and ranges.

Results

General observations

The Min males fed the flaxseed diet had a significantly higher body weight at the end of the 10-week feeding period than control males ($P < 0.05$), but the weight gain of the Min males fed the oil diet did not differ from the controls (Fig. 1). The weight gain and the final body weights of the Min females did not differ between dietary groups.

Adenoma results

Min mice in the flaxseed group had 31 % fewer adenomas in the small intestine than those in the control group (median 37 *v.* 54; $P = 0.023$) whereas the oil group showed a tendency to gain fewer adenomas than the control group (median 42 *v.* 54; $P = 0.095$) (Table 2). The median adenoma size in the small intestine both in the flaxseed and oil groups was significantly smaller than in the control group (Table 2). Similarly, the effect of diet was seen in the proportions (percentage from total) of small, median and large size adenomas. The males of the flaxseed group had slightly elevated adenoma size in the colon ($P = 0.034$). Otherwise, the incidence, number and size of the colon adenomas were not different between control and treatment groups.

Lignan metabolism

Flaxseed-fed mice had sixty to 100 times higher levels of caecal ENL, enterodiol, and secoisolariciresinol, and thirty times higher plasma ENL compared with the controls (Table 3). Flaxseed and
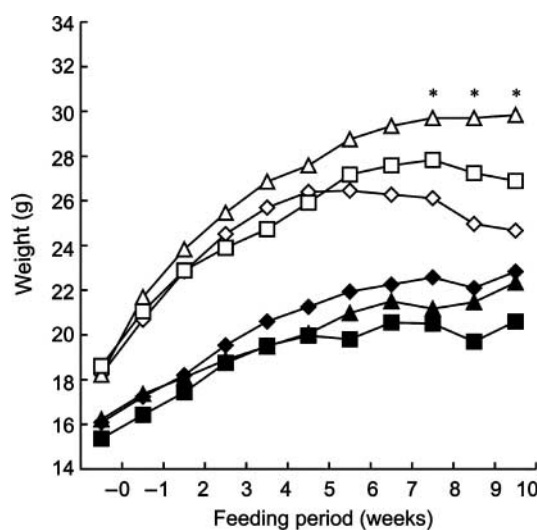

Fig. 1. Mean body weights of multiple intestinal neoplasia males and females consuming the control, flaxseed, and oil diets for 10 weeks. (−♦−), Control female; (−◇−), control male; (−▲−), flaxseed female; (−△−), flaxseed male; (−■−), oil female; (−□−), oil male. * Values were significantly different from those of the control males ($P < 0.05$).

Table 2. Tumour number and size (mm or %) in the small intestine and the colon of multiple intestinal neoplasia mice fed with a control diet or diets supplemented either with a mixture of defatted flaxseed, wheat fibre, flaxseed and rapeseed oils (Flaxseed) or a mixture of flaxseed and rapeseed oils (Oil) for 10 weeks

(Medians and ranges)

Diet …	Control (n 14)		Flaxseed (n 11)		Oil (n 11)	
	Median	Range	Median	Range	Median	Range
Small intestine						
Tumours (per mouse)	54	31−86	37*	13−81	42	15−90
Tumour size (mm)	1·2	0·9−1·3	0·9**	0·8−1·2	1·0*	0·7−1·4
Tumour size (% of total)						
<1·0 mm (%)	40	21−70	61**	25−84	56*	34−81
1·0−1·5 mm (%)	39	19−55	20*	15−58	29*	11−47
>1·5 mm (%)	22	3−28	11*	0−37	14*	5−24
Colon Incidence†						
Males	4/8		3/6		2/6	
Females	0/6		1/5		2/5	
Tumours (per mouse)						
Males	0·5	0−3	0·5	0−2	0·5	0−3
Females	−	−	0	0−2	0	0−2
Tumour size (mm)						
Males	2·9	2·6−3·1	3·5*	3·3−3·8	3·0	2·8−3·3
Females	−	−	3·3	3·3−3·3	1·7	1·1−2·3

Median values were significantly different from those of the control group: *$P < 0.05$, **$P = 0.002$.
† Number of mice with colonic adenomas.

rapeseed oils in the oil diet were not sources of plant lignans, and thus, intestinal or plasma lignan levels of the oil group did not differ from the control group. When all experimental groups were combined, no association between the intestinal lignan levels or plasma ENL and adenoma formation was found. Consumption of the flaxseed diet led to a higher level of caecal ENL level in Min males (median 91·8 nmol/g; n 6) compared with Min females (20·3 nmol/g, n 5, $P = 0.028$). Caecal secoisolariciresinol levels, by contrast, were almost significantly lower in flaxseed males (median 44·3 nmol/g) than in flaxseed females (77·3 nmol/g; $P = 0.068$). Caecal enterodiol or plasma ENL levels did not differ between flaxseed males and females.

Intestinal fatty acid composition

Both flaxseed and oil diets similarly changed the colonic fatty acid profile, a marker for fatty acid intake (Table 4). Proportions of ALA (18: 3n-3), EPA (20: 5n-3), and docosapentaenoic acid (22: 5n-3) were all increased after both experimental diets ($P < 0.001$), whereas the level of DHA (22: 6n-3) was unchanged compared with controls. Overall, the sum of n-3 PUFA increased 3-fold in the flaxseed and oil groups. The proportion of linoleic acid (18: 2n-6) in the colon mucosa was also increased in the flaxseed and oil groups, whereas the proportion of arachidonic acid (20: 4n-6) in these groups was only 50−56 % of the value in the control group. Changes in proportions of long-chain PUFA also led to changes in the n-6:n-3 PUFA ratio, which were 1·6:1 ($P < 0.001$) and 1·7:1 ($P < 0.001$) for the flaxseed and oil groups, respectively, and 4·4:1 for the control group.

Cyclo-oxygenase-2 expression

COX-2 expression was analysed in adenomas to determine whether this could explain the inhibition of adenoma growth found in the flaxseed and oil groups. A representative immunoblot on COX-2 is shown in Fig. 2 (A). Western blot analysis did not show any effect of diet on COX-2 expression in the adenoma

Table 3. Intestinal enterolactone (ENL), enterodiol (END) and secoisolariciresinol (SECO), and plasma ENL levels of multiple intestinal neoplasia mice after 10 weeks feeding of the experimental diets

(Medians and ranges)

Diet…	Control (n 5)		Flaxseed (n 11)		Oil (n 11)	
	Median	Range	Median	Range	Median	Range
Caecal level (nmol/g)						
ENL	0·5	0·1−2·5	30·5*†	14·2−129·9	0·1	0·0−0·4
END	0·3	0·1−0·5	31·9*	17·2−85·0	0·0	0·0−0·4
SECO	0·8	0·4−0·8	63·7*	28·9−96·5	0·2	0·1−0·4
Plasma ENL (nmol/l)	3·2‡	1·4−11·5	97·7*	56·2−220·5	2·0	1·2−4·1

* Median values were significantly different from those of the control group ($P = 0.002$).
† Caecal ENL level was different between the sexes (see p. 513).
‡ n 14.

Table 4. Fatty acid composition of the colonic mucosa of multiple intestinal neoplasia mice (% of total fatty acids)
(Medians and ranges)

Diet...	Control (*n* 14)		Flaxseed (*n* 11)		Oil (*n* 11)	
	Median	Range	Median	Range	Median	Range
Fatty acids (%)						
14 : 0 + 15 : 0	3·5	2·6–4·0	2·4**	1·9–2·8	2·3**	1·8–2·7
16 : 0	18·6	16·0–22·7	16·1*	12·7–19·9	15·8*	14·2–21·3
18 : 0	6·0	3·6–10·0	5·2	3·2–7·0	5·0	3·2–6·7
18 : 1*n*-9 + 18 : 1*n*-7	51·0	40·3–56·2	47·1*	44·0–51·3	49·0	44·0–53·2
18 : 2*n*-6	10·8	9·4–11·7	13·8**	11·1–14·8	13·7**	11·8–15·0
18 : 3*n*-3	1·1	0·9–1·5	7·2**	5·6–8·7	6·7**	5·5–7·5
20 : 4*n*-6	2·0	1·0–6·7	1·2*	0·6–3·4	1·2**	0·6–2·0
20 : 5*n*-3	0·2	0·1–0·5	0·6**	0·3–1·0	0·5**	0·3–0·6
22 : 5*n*-3	0·3	0·1–0·7	0·5**	0·3–1·6	0·5**	0·3–0·7
22 : 6*n*-3	1·3	0·7–3·8	1·0	0·7–2·6	1·2	0·7–1·7
Others†	4·6	3·1–6·1	3·9	2·5–5·1	4·0*	2·3–4·7
n-6 PUFA‡	13·3	10·9–17·9	14·9	12·6–17·7	14·7*	13·1–16·7
n-3 PUFA§	3·1	2·3–5·7	9·5**	8·3–11·2	8·6**	8·0–9·6
n-6:*n*-3 Ratio	4·4	3·1–5·3	1·5**	1·4–1·8	1·7**	1·5–1·9

Median values were significantly different from those of the control group: $*P=0.05$, $**P<0.001$.
† Sum of 16 : 1*n*-7, 18 : 2*trans*, 18 : 3*n*-6 and 20 : 3*n*-6.
‡ Sum of 18 : 2, 18 : 3*n*-6, 20 : 3 and 20 : 4.
§ Sum of 18 : 3, 20 : 5, 22 : 5 and 22 : 6.

tissue (Fig. 2 (B)). When the groups were combined, COX-2 expression in the adenoma tissue was, however, positively associated with the adenoma size (r 0·536, $P=0.001$, n 33) (Fig. 2 (C)).

β-Catenin expression

β-Catenin was analysed separately from the nuclear, cytosolic and membranous fractions of the adenoma and normal-appearing mucosa. As observed in our earlier study (Pajari *et al.* 2003), immunoblots of β-catenin gave one band at 98 kDa in rat brain homogenate (control), and several bands at 92 kDa (full-length β-catenin) and 72–86 kDa in the mucosa and adenoma samples of Min mice (Fig. 3 (A)). The β-catenin results are expressed as the intensity of the full-length band, which could be detected in almost all the adenoma and normal-appearing mucosa samples. The median β-catenin signal in the nucleus (standardised by the lamin B signal) was approximately four times stronger in the adenomas when compared with the mucosa. However, there was no effect of diet on the nuclear or cytosolic β-catenin signals either in the mucosa or adenoma tissues (data not shown). When compared with the normal-appearing mucosa, membrane β-catenin levels were reduced in the adenoma tissue (Table 5). There were no differences between the diet groups in the membrane β-catenin measured in the adenoma tissue. However, in the normal-appearing mucosa the membrane β-catenin levels were slightly elevated in the flaxseed group relative to the control group (median 1·8 *v.* 0·8; $P=0.09$) (Fig. 3 (D)).

Protein kinase C-ζ expression

We chose PKC-ζ of all PKC isozymes for our studies because its localisation in cell fractions can be modulated by diet (Pajari *et al.* 2000), it is down regulated during the cancer process (Klein *et al.* 2000), it mediates the effects of various anticarcinogenic substances (Roy *et al.* 1995; Wali *et al.* 1995, 1996), and it has been shown to interact with the APC pathway (Etienne-Manne-

ville & Hall, 2003*b*). PKC-ζ was analysed from the cytosolic and membranous fractions of the adenoma and normal-appearing mucosa. PKC-ζ gave three bands at 75, 70 and 50 kDa in rat brain homogenate (control), three bands at 75, 70 and 40 kDa in mucosa tissue, and four bands at 75, 70, 48 and 43 in adenoma tissue, all of which were eliminated when the blocking peptide of PKC-ζ was incubated together with PKC-ζ antibody (Fig. 3 (B)). Bands at 75 and 70 kDa represent full-length PKC-ζ with different levels of phosphorylation, whereas 43–48 kDa bands are considered as proteolytic breakdown products of full-length protein. Interestingly, in the normal-appearing mucosa, both 75 and 70 kDa bands were expressed, whereas in the adenoma tissue the 70 kDa was the main band present. We found that PKC-ζ expression (75 and 70 kDa bands) was lower in the membranes of adenoma tissue compared with the same fraction in the normal-appearing mucosa without a major reduction in PKC-ζ levels in tissues (Table 5). Diet had no effect on the cytosolic PKC-ζ (data not shown) levels either in the adenoma or mucosa tissues, nor were there any differences in low molecular weight band at 48 and 43 kDa between the diet groups (data not shown). Similarly with β-catenin localisation there was a tendency for elevated membrane PKC-ζ levels in the mucosa tissue after the flaxseed diet (median 0·6 *v.* 0·4; $P=0.09$) (Fig. 3 (E)). When the groups were combined, an inverse association was found between the membrane PKC-ζ expression in the mucosa and adenoma number in the same area in Min mice (r −0·460, $P=0.008$, n 32; Fig. 4).
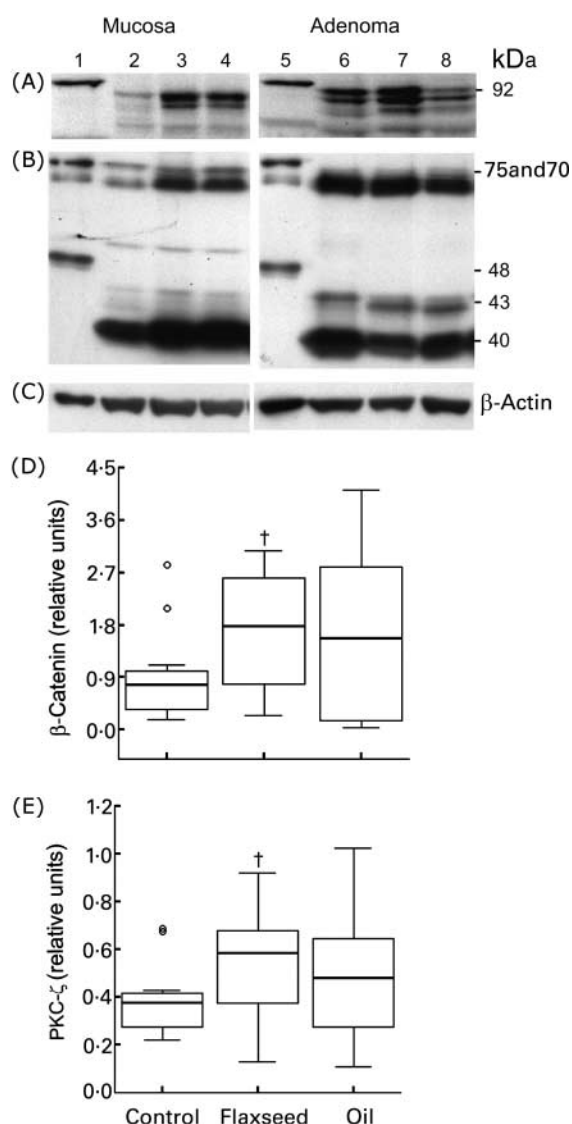
Discussion

In the present study, we have shown that the flaxseed diet containing defatted flaxseed, wheat fibre and flaxseed and rapeseed oils decreased significantly both the number and size of intestinal adenomas in Min mice. Interestingly, the oil diet having a similar oil composition as flaxseed diet, also repressed adenoma growth but the effect on adenoma number was not quite so obvious.
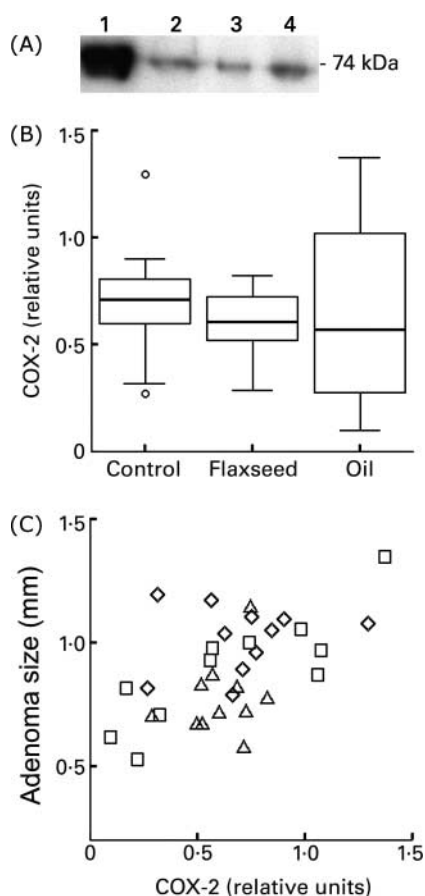
Fig. 2. (A) A representative immunoblot of cyclo-oxygenase(COX)-2 protein. Line 1 contains RAW homogenate, which was used to control inter-assay variation, and lines 2–4 contain membrane samples of the adenoma tissue of three multiple intestinal neoplasia (Min) mice. (B) Expression of COX-2 (eleven per group) in the adenoma tissue in the distal small intestine of Min mice fed the control, flaxseed, and oil diets for 10 weeks. For each group, the box represents the interquartile range, which contains 50 % of values. Medians are indicated by horizontal lines across the boxes. Outliers (○) are cases with values between 1·5 and 3 box-lengths from the upper or lower edge of the box. (C) Relationship between the adenoma size and the membrane COX-2 expression in the adenoma tissue in the small intestine of Min mice fed the control (◇), flaxseed (△), and oil (□) diets (r 0·536, $P=0·001$, n 33).

Fig. 3. Representative immunoblots of β-catenin (A), protein kinase C (PKC)-ζ (B) and β-actin (C) proteins in the normal-appearing mucosa and adenoma samples of three multiple intestinal neoplasia (Min) mice. Lanes 1 and 5 contain rat brain homogenate, which was used to control inter-assay variation. Each of the following lanes contains cytosolic samples of the normal-appearing mucosa (lines 2–4) and the corresponding adenoma tissue (lanes 6–8) from one mouse. β-Actin was used as a loading marker. Expression of membrane β-catenin (D) and membrane PKC-ζ (E) (eleven per group) in small intestinal mucosa of Min mice fed the control, flaxseed, and oil diets for 10 weeks. For each group, the box represents the interquartile range, which contains 50 % of values. Medians are indicated by horizontal lines across the boxes. Outliers (○) are cases with values between 1·5 and 3 box-lengths from the upper or lower edge of the box. † Median values of β-catenin (1·8) and PKC-ζ (0·6) in the flaxseed group tended towards being significantly different from those of the control group ($P=0·09$).

The results suggest that a protective effect on adenoma formation was partly caused by an additive effect of several chemopreventive components present in the flaxseed mixture. The results also suggest that most, but not all, of the positive effect may be due to the oil part of the mixture.

Previously the protective effect of flaxseed on tumour initiation or growth in various cancer models has been mainly linked to lignans (Serraino & Thompson, 1992; Jenab & Thompson, 1996; Thompson *et al.* 1996; Li *et al.* 1999). In the present study, substantial mammalian lignan production was found only in Min mice fed the flaxseed diet, but no association between the intestinal lignan levels or plasma ENL and adenoma formation was, however, found. The result is in line with other *in vivo* studies with Min mice (van Kranen *et al.* 2003; Oikarinen *et al.* 2005) showing no association between plasma ENL and intestinal adenoma formation in this animal model. We have found a sex difference in the intestinal ENL levels after consumption of flaxseed diets in the present and our previous study (Oikarinen *et al.*

2005). Bacterial bioconversion of the plant lignan secoisolariciresinol to the mammalian lignans or enterohepatic circulation of ENL might be different in male and female mice. However, plasma ENL levels do not reflect sex differences found in gut ENL.

The flaxseed diet also contained wheat fibre, which consists mainly of cellulose and hemicellulose isolated from wheat bran. Several studies have shown that wheat bran is protective against

Table 5. Expression of membrane β-catenin and protein kinase C (PKC)-ζ by immunoblot in the normal-appearing mucosa and adenoma tissue in the distal small intestine of multiple intestinal neoplasia mice fed the control, flaxseed, and oil diets for 10 weeks‡

(Medians and ranges)

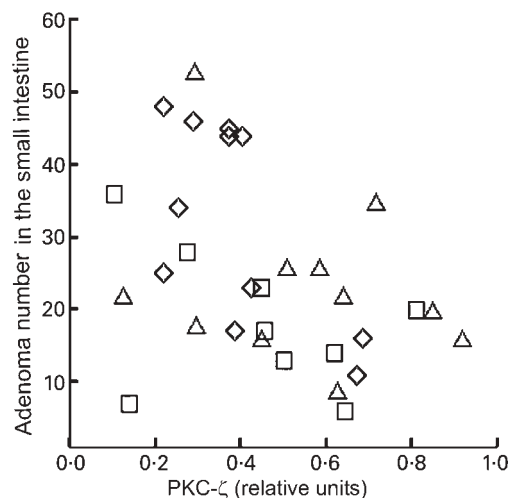| Diet...	Control		Flaxseed		Oil	
	Median	Range	Median	Range	Median	Range
Normal-appearing mucosa						
β-Catenin	0.8	0.1–2.8	1.8†	0.2–3.1	1.6	0.0–4.1
PKC-ζ	0.4	0.2–0.7	0.6†	0.1–0.9	0.5	0.1–1.0
Adenoma						
β-Catenin	0.4	0.0–1.5	0.5*	0.2–2.2	0.5*	0.0–1.3
PKC-ζ	0.2*	0.1–0.3	0.2*	0.1–0.8	0.2*	0.0–0.4

* Median values were significantly different from those of the normal-appearing mucosa ($P < 0.05$).

† Median values of β-catenin and PKC-ζ tended towards being significantly different from those of the control group ($P = 0.09$).

‡ Intensity values (relative units, eleven per dietary group) were normalised to a constant amount of internal standard.

colon cancer in carcinogen-treated rats (McIntry *et al.* 1993; Zoran *et al.* 1997; Jenab & Thompson, 1998). In Min mice, 5–10 % wheat bran or cellulose supplementation inhibited adenoma formation in the small intestine but not in the colon compared with a high-fat, non-fibre diet (Yu *et al.* 2001). $APC^{\Delta716}$ mice fed a low-risk diet (20 % wheat bran and 5 % fat) were observed to have fewer polyps in the small intestine and the colon than their peers receiving a high-risk diet (2·5 % wheat bran and 20 % fat; Hioki *et al.* 1997). However, Hioki *et al.* (1997) did not separate the effects of fat and fibre on adenoma formation. In our previous study, we found that adenoma number in the small intestine and colon of a high-fat wheat bran (10 %, w/w) group or a low-fat AIN-93G (5 % cellulose) group was not different from that of a high-fat non-fibre group (Mutanen *et al.* 2000). Therefore, while the effect of wheat fibre in the present study is somewhat unclear, wheat fibre may confer some protective effect against adenoma formation.

The oil diet inhibited the intestinal adenoma growth nearly as effectively as did the flaxseed mixture, suggesting that the oil

Fig. 4. Relationship between the adenoma number and the membrane protein kinase C(PKC)-ζ expression in the mucosal tissue in the distal small intestine of multiple intestinal neoplasia mice fed the control (◇), flaxseed (△) and oil (□) diets ($r - 0.460$, $P = 0.008$, n 32)

component is mainly responsible for the anti-tumorigenic effect of the flaxseed mixture. Indeed, the fatty acid composition of the intestinal mucosa tissue was almost identical in flaxseed and oil groups. Compared with the control group, both the flaxseed and oil groups had a 6-fold increase in the relative proportion of ALA in the mucosa. Colon cancer studies have yielded contradictory results on the effects of ALA, or ALA-rich plant oils, for example, perilla or flaxseed oils, on intestinal tumorigenesis. Perilla oil was found to decrease tumour and aberrant crypt foci formation in carcinogen-treated rats (Narisawa *et al.* 1994; Onogi *et al.* 1996), whereas lignans of flaxseed were considered more important than the oil component as an effective agent by Serraino & Thompson (1992) and Jenab & Thompson (1996). Supplementation of pure ALA-ethyl ester (31 g/kg diet) also had no significant anti-tumorigenic effect in Min mice (Hansen Petrik *et al.* 2000). We cannot fully exclude the possibility that the difference in tumour formation in the present study was due to the promotive effect of the butter in the control diet rather than the protective effect of rapeseed and flaxseed oils in the flaxseed and the oil diets. However, we previously found (Mutanen *et al.* 2000) that Min mice fed a high-fat low-fibre diet (identical to the control diet in the present study) and an AIN-93G diet (a low-fat, no-butter diet) had an equal number of adenomas in the small intestine and colon. Therefore, we think that butter did not modulate tumour formation in the present study.

COX-1 and inducible COX-2 enzymes convert arachidonic acid to prostaglandins. Compared with the normal mucosa, the levels of COX-2 mRNA and protein were shown to be highly elevated in adenomas of Min mice (Williams *et al.* 1996). Moreover, in an *APC* mutated background, mice null for COX-2 had suppressed polyp formation (Oshima *et al.* 1996). We analysed COX-2 in adenomas to determine whether this could explain the growth inhibition found in the flaxseed and oil groups. We found that the COX-2 protein level was not dependent on diets, although a positive correlation was found between COX-2 level and adenoma growth. Unfortunately, we did not measure COX-2 activity, which might also be affected by the experimental diets. In another study, the administration of 1,4-phenylene bis(methylene) selenocyanate decreased adenoma number in Min mice, and also lowered COX-2 activity in adenomas, while leaving the level of COX-2 protein unchanged (Rao *et al.* 2000).

A reduced protein expression of PKC-α, -βI, and -ζ in the adenoma tissue compared with adjacent mucosal tissue of Min mice has been demonstrated using an immunohistochemistry method (Klein *et al.* 2000). By Western blot analysis, we found that PKC-ζ expression was lower in the membranes of adenoma tissue compared with the same fraction in the normal-appearing mucosa. This is in line with a study where the majority of PKC-δ and PKC-ζ were localised in the cytosolic fraction of azoxymethane-induced tumours but not in the normal mucosa (Davies & Johnson, 2002). We also found that membrane PKC-ζ expression in the mucosa tissue was inversely associated with the adenoma number in the same area in Min mice ($r - 0.460$, $P = 0.008$, n 32). The present results suggest that membrane-associated PKC-ζ protects against cancer, although the mechanism is still to be elucidated. In normal murine intestinal epithelium, PKC-ζ was detected mainly in the post-mitotic cells of the upper crypt and surface mucosa, and in the membrane and cytoskeletal compartments (Saxon *et al.* 1994; Verstovsek *et al.* 1998). An *in vitro* experiment with Caco-2 cells has shown that epidermal growth factor-induced PKC-ζ translocation

to membranes is necessary for stabilisation of the microtubule skeleton and cell barrier function against oxidative injury (Banan *et al.* 2002). Through interaction with atypical PKC, the mammalian Par-proteins regulate the formation of epithelial cell–cell junctions, which are essential for the establishment of epithelial cell polarity (Etienne-Manneville & Hall, 2003*b*). Therefore, the presence of PKC-ζ in the membrane fraction of epithelial cells seems to be involved in the normal differentiation and maturation processes.

β-Catenin binds to E-cadherin and α-catenin in the adherent junction complexes, which form homophilic interactions between neighbouring cells and anchor to actin filaments (Cowin, 1994). Diet has been shown to affect β-catenin localisation in cellular compartments in Min mice. A decreased tumour number was associated with reduced β-catenin staining or a lower β-catenin expression in the nucleus of the small-intestinal tumours, as well as redistribution of β-catenin to a more 'normal' localisation mainly at the intercellular junctions (McEntee *et al.* 1999; Oikarinen *et al.* 2000; Schmelz *et al.* 2001). The mice fed the flaxseed diet had the lowest number of adenomas compared with the control group, and also the highest levels of mucosal membrane β-catenin as well as PKC-ζ, which might indicate a protective effect of defatted flaxseed, wheat fibre, flaxseed and rapeseed oils on mucosal integrity, and thus, on intestinal adenoma formation.

As a conclusion, both the adenoma number and size in Min mice can be reduced by the mixture of defatted flaxseed, wheat fibre, and flaxseed and rapeseed oils. This may be due to an additive effect of several chemopreventive components present in the mixture. The oil component of the mixture caused a reduction in adenoma size but not in number. This indicates that rapeseed and flaxseed oils were mainly responsible for the inhibition of tumorigenesis, particularly with regard to adenoma growth. Presumably, there are multiple cell-signalling pathways through which diet exerts its action. Loss of atypical PKC-ζ and β-catenin from the membranes of normal-appearing mucosa may play a permissive role in intestinal tumour development. The molecular mechanism responsible for plant oil-induced inhibition of adenoma formation deserves further study.

Acknowledgements

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