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Short Communication

Dietary β-carotene inhibits mammary carcinogenesis in rats depending on dietary α-linolenic acid content

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To investigate whether dietary α-linolenic acid (ALA) content alters the effect of β-carotene on mammary carcinogenesis, we conducted a chemically induced mammary tumorigenesis experiment in rats randomly assigned to four nutritional groups (15 rats per group) varying in β-carotene supplementation and ALA content. Two oil formula-enriched diets (15 %) were used: one with 6 g ALA/kg diet in an essential fatty acids (EFA) ratio of linoleic acid:ALA of 5:1 w/w (EFA 5 diet), the other with 24 g ALA/kg diet in an EFA ratio of 1:1 w/w (EFA 1 diet), both designed with a similar linoleic acid content. β-Carotene was either added (10 mg/kg diet per d) or not added to these diets. β-Carotene supplementation led to decreased tumour incidence and tumour growth when added to the EFA 5 diet, whereas it had no effect when added to the EFA 1 diet. The decreased tumour growth did not result from an involvement of lipoperoxidation (tumour malondialdehyde content being similar between the groups) or from an inhibition of tumour cell proliferation (as there was an unchanged S phase fraction in the tumours). We concluded that an adequate content of ALA in the diet is required to allow a protective effect of β-carotene in mammary carcinogenesis. Whether such an interaction between ALA and β-carotene influences the risk of breast cancer in women needs to be investigated.

β-Carotene: α-Linolenic acid: *n*-6:*n*-3 fatty acid ratio: Mammary tumours

Several epidemiological studies have consistently shown that individuals with a high intake of vegetables and/or fruits have a reduced risk of cancer, including breast cancer (Riboli & Norat, 2003). A potential explanation is that antioxidant nutrients, including carotenoids, prevent carcinogenesis by interfering with oxidative damage to DNA, lipids and proteins. The results of epidemiological studies are, however, inconclusive on the association between β-carotene and risk of breast cancer (IARC Working Group on the Evaluation of Cancer Preventive Agents, 1998*a*). Moreover, two intervention trials conducted in male smokers, the Alpha-Tocopherol Beta Carotene Cancer Prevention Study and the Beta Carotene and Retinol Efficacy Trial, which both used high-dose β-carotene supplements, found an increased incidence of lung cancer in subjects who received supplements in comparison with non-recipients (Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group, 1994; Omenn *et al.* 1996). In contrast, in a trial conducted in healthy men – the Physicians' Health Study – a high supplementation of β-carotene on alternate days had no effect on incidence of cancer (Hennekens *et al.* 1996). No clear mechanistic explanation has yet been provided to explain these conflicting findings. Nevertheless, some hypotheses have been advanced, involving the form of β-carotene (synthetic or natural, *trans* or *cis*), the amount of β-carotene (physiological or pharmacological), individuals exposed or not exposed to high risk factors for cancer, genetic factors interfering with nutrition, and the possible interaction between β-carotene and other nutrients.

The supplementation of antioxidant vitamins to mammary tumour-bearing rodents has generated contradictory results (IARC Working Group on the Evaluation of Cancer Preventive Agents, 1998*b*). It is still unclear whether contrasting results are due to differences in animal models, differences in supplement doses, interference of the antioxidants used with other dietary compounds or the combined effects of these confounding factors. In a model of chemically induced mammary tumours in rats, adding the antioxidant vitamin E to a diet rich in α-linolenic acid (ALA, 18:3*n*-3, the essential fatty acid of the *n*-3 family) led to enhanced mammary tumour growth, whereas it had no effect when added to a diet devoid of ALA (Cognault *et al.* 2000). These data suggest that an

Abbreviations: ALA, α-linolenic acid; EFA, essential fatty acid; MDA, malondialdehyde.

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interaction between antioxidant compounds and dietary *n*-3 fatty acids is a determinant of mammary tumour growth.

To determine whether the ALA content of diet alters the effect of β-carotene on mammary carcinogenesis, we examined the effects of two oil formula-enriched diets differing in terms of their ALA content (6 and 24 g/kg diet), with ratios of linoleic acid (18:2*n*-6) to ALA of 4·66 and 1·05 respectively, in absence or presence of β-carotene (10 mg/kg diet per d) on the characteristics of mammary carcinogenesis, and found that the *n*-3 lipid environment of diet modified the effect of β-carotene.

Material and methods

Animals and experimental carcinogenesis

Sixty 40-d-old female Sprague-Dawley rats were purchased from Harlan France (Gannat, France). The animals were cared for in accordance with institution guidelines. Rats were housed three per cage and maintained at constant temperature (22°C) and humidity with a 12-h light/dark cycle. Mammary tumours were induced by a single dose of N-nitroso-N-methylurea as previously described (Colas *et al.* 2004). Rats were randomly separated into four dietary groups (15 rats per group). Three weeks after initiating carcinogenesis and for 15 weeks, animals were palpated once weekly to detect mammary tumours. The length, width and depth of each tumour were measured with a calliper, and the tumour area was calculated as the product of the two largest parameters. The tumour incidence (the percentage of rats bearing at least one malignant mammary tumour) and the tumour growth (the mean of tumour areas per tumour-bearing rat each week) were determined. After 17 weeks of monitoring the rats, the animals were killed.

Diets

Until administration of the N-nitroso-N-methylurea, rats were fed a diet recommended for the breeding and rearing of rodents (Harlan Teklad TRM Rat/Mouse Diet; Harlan Teklad, Gannat, France). They were then fed the experimental diets, composed of a common basal diet (APAE, Jouy-en-Josas, France) as already described (Cognault *et al.* 2000) and 15 % (w/w) of an oil mixture. The diets, designed with a similar linoleic acid content, were as follows: the essential fatty acid (EFA) 5 diet, containing a mixture of 60·2 % African peanut oil and 39·8 % European rapeseed oil (Bailly, Aulnay sous Bois, France), resulting in a 4 % ALA content in the recommended EFA ratio (linoleic acid:ALA) for rats of nearly 5 (4·66; Potier de Courcy *et al.* 1989); the EFA 1 diet, containing a mixture of 69 % African peanut oil and 31 % linseed oil (ALA-enriched oil; Daudruy, Dunkerque, France) leading to a 16 % ALA content in an EFA ratio of 1·05 (Table 1). β-Carotene (type I; Sigma, St Quentin Fallavier, France) was added (10 mg/kg diet per d) or not added (controls) to these diets. Animals received commercial and experimental diets and water *ad libitum*.

The weight of the rats was monitored weekly until the end of experiment.

Biochemical analyses

The fatty acid composition of adipose tissue was determined as previously described (Colas *et al.* 2004). After total lipid extraction, triacylglycerols were purified by preparative TLC, and fatty acids were methylated with BF_3 and analysed by GC (Trace GC; Thermofinnigan, Courtaboeuf, France) with a 60-m polar capillary column (BPX 70; SGE, Courtaboeuf, France).

Table 1. Fatty acid composition of commercial and experimental diets and triacylglycerols in rats' adipose tissue (Mean values and standard deviations)

		EFA 5 diet			EFA 1 diet		
			Adipose tissue§			Adipose tissue§	
Fatty acids (mol % total fatty acids)	TRM diet* Diet†	Diet‡	Mean	SD	Diet‡	Mean	SD
Saturates							
16 : 0	16·3	8·5	15·4	0·4	9·3	17·5	0·5
18 : 0	2·7	2·4	2·6	0·07	3·4	2·9	0·09
Total‖	20·5	13·7	19·5	0·4	15·8	21·9	0·6
Monounsaturates							
18 : 1*n*-9$_{cis}$	20·6	58·6	56·7	0·4	47·1	48·2	1·5
Total¶	24·7	63·0	62·4	0·3	49·8	54·6	1·4
n-6 PUFA							
18 : 2*n*-6$_{cis}$	48·4	18·3	13·8	0·2	16·7	13·2	1·1
Total**	48·4	18·4	14·3	0·2	17·3	13·5	1·1
n-3 PUFA							
18 : 3*n*-3	4·7	3·9	1·3	0·05	16·3	7·0	0·4
Total††	4·8	3·9	1·4	0·05	16·4	7·4	0·4
Ratio							
18 : 2*n*-6$_{cis}$/18 : 3*n*-3	10·3	4·66	10·63	0·4	1·05	1·9	0·1

* Harlan Teklad TRM Rat/Mouse Diet (Harlan, Gannat France).
† Fatty acid composition given by the supplier.
‡ Fatty acid composition of one sample of each diet.
§ Ten rats were randomly selected in each dietary group to provide tissues for fatty acid analysis.
‖ Including 14 : 0, 15 : 0, 17 : 0, 20 : 0, 21 : 0, 22 : 0, 23 : 0 and 24 : 0.
¶ Including 14 : 1, 16 : 1, 17 : 1, 18 : 1*n*-7$_{cis}$, 20 : 1, 22 : 1 and 24 : 1.
** Including 18 : 3*n*-6, 20 : 2*n*-6, 20 : 3*n*-6, 20 : 4*n*-6, 22 : 2*n*-6 and 22 : 4*n*-6.
†† Including 20 : 3*n*-3, 20 : 5*n*-3, 22 : 5*n*-3 and 22 : 6*n*-3.
For details of diets and procedures, see this page.

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The β-carotene absorption was monitored for each nutritional group by measuring the hepatic β-carotene content. After extraction (Lyan *et al.* 2001), β-carotene was analysed by an HPLC system (Spectra System; Thermofinnigan) with two Adsorbosphere HS C18 3 μm cartridges (100 mm × 4·6 mm and 150 mm × 4·6 mm; Alltech, Templemars, France) at 37°C and a photodiode array detector (UV6000LP; Thermofinnigan) as already described (Steghens *et al.* 1997).

Lipoperoxidation was evaluated by measuring total malondialdehyde (MDA) content in the tumours. At the time of autopsy, necrotic tissues were carefully removed from tumours before freezing. Fifteen tumours (similarly distributed according to their age and size) were used for the analysis. A fragment of tumour was cut and thawed at 4°C on Tris-HCl 100 mM, pH 7·4 (KCL 100 mM, EDTA 1 mM, butylhydroxytoluene 0·1 mM, Triton X-100 and 0·1 % phenylmethanesulfonylfluoride 0·1 mM). The total lysat was centrifuged at 10 000 ***g*** for 5 min and the supernatant extracted for analysis. The protein content of the tumours was measured and standardised at 15–20 mg/ml for each sample. As previously described (Steghens *et al.* 2001), MDA was derivatisated with diaminonaphtalene in an acid medium at 37°C to form an MDA diazepinium. Analyses were carried out with a HPLC diode array system and an online mass spectrophotometer for confirmation (Thermo Electron, Courtaboeuf, France). Results were expressed as nmol/g protein, instead of nmol/g tumour, to avoid variations in the MDA content owing to the weight of the tumours.

Cell cycle

The distribution of cells within the cell cycle was assessed by flow cytometry after staining for DNA content with propidium iodide, as previously described (Cognault *et al.* 2000).

Statistical analyses

The effects of dietary conditions on carcinogenesis and biochemical parameters were evaluated, using Statistica 6.0 software (StatSoft, Inc., Maisons-Alfort, France), by the following tests ($P<0·10$): Pearson χ^2 test for tumour incidence; repeated-measures ANOVA with grouping factor (time) for tumour growth; Mann–Whitney test for biochemical analyses and cell cycle. Data are expressed as means with their standard errors.

Results

During the experiment, no significant difference in weight gain between the dietary groups was observed (data not shown).

The fatty acid composition of the rat adipose tissue (an indicator of dietary fatty acid intake) is presented in Table 1. Because β-carotene supplementation did not change this composition whatever the ALA content, Table 1 presents the results for the groups without β-carotene. We showed that the EFA ratio was 5·6-fold greater in rats fed the EFA 5 diet than in rats fed the EFA 1 diet and that the linoleic acid content was similar between groups.

β-Carotene was detected only in the livers of rats receiving dietary β-carotene supplementation. This content was not significantly different between rats fed the EFA 5 diet (0·21 (SE 0·04) μg/g tissue) and the EFA 1 diet (0·27 (SE 0·04) μg/g tissue; $P=0·35$).

No significant difference in tumour incidence at the end of the experiment was found between groups not receiving β-carotene: EFA 5 diet, 96 % incidence, fourteen of fifteen rats with a tumour; EFA 1 diet, 73·3 %, eleven of fifteen rats ($P=0·14$). β-Carotene supplementation led to a reduced tumour incidence in rats fed the EFA 5 diet (60·0 %, nine of fifteen; $P=0·03$) but not in rats fed the EFA 1 diet: (86·7 %, thirteen of fifteen; $P=0·36$) compared with their respective controls (96 % and 73·3 %). No difference in tumour growth was observed between rats fed the EFA 5 and EFA 1 diets without β-carotene (Fig. 1). β-Carotene supplementation led to a decreased (approximately 50 % decrease) in tumour growth in rats fed the EFA 5 diet but not in rats fed the EFA 1 diet (Fig. 1).

The measure of S phase fraction in the tumours was not significantly different between groups: 3·8 (SE 0·8) % and 4·7 (SE 0·9) % without and with β-carotene respectively in groups fed the EFA 5 diet; 4·4 (SE 0·7) % and 3·7 (SE 0·9) % without and with β-carotene respectively in groups fed the EFA 1 diet (all $P>0·2$).

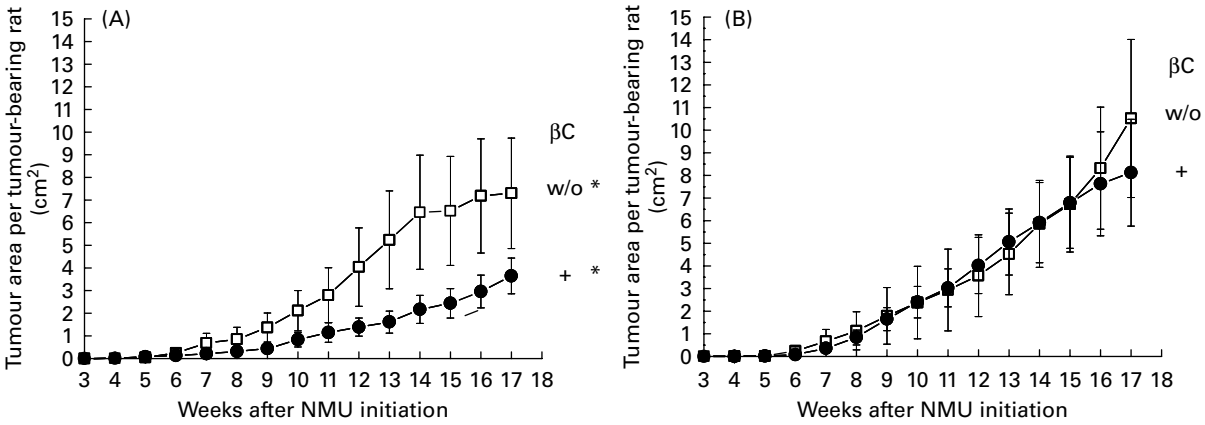

Fig. 1. Effects of β-carotene (βC) on mammary tumour growth in response to the essential fatty acid (EFA) 5 (A) and EFA 1 (B) diets. After chemical induction of mammary tumours, rats were randomly assigned to four nutritional groups (15 rats per group). Rats were fed either the EFA 5 diet (with an EFA ratio of linoleic:α-linolenic acid of 5) or the EFA 1 diet (with an EFA ratio of 1), supplemented (10 mg/kg diet per d, +) or not supplemented (w/o) with β-carotene. Tumour growth was not significantly different between rats fed the EFA 5 and EFA 1 diets without β-carotene or between rats fed the EFA 1 diet with and without β-carotene ($P=0·99$, repeated-measures ANOVA with time as a grouping factor). NMU, N-nitroso-N-methylurea. *In rats fed the EFA 5 diet, tumour growth (mean and standard error of the mean) is significantly different between animals receiving and not receiving β-carotene ($P=0·09$, repeated-measures ANOVA with time as a grouping factor). For details of diets and procedures, see p.19.

The average MDA content of the tumours was 220·6 pmol/mg protein and was not significantly different between dietary conditions (data not shown).

Discussion

The objective of the present study was to determine whether dietary ALA content altered the effect of β-carotene on mammary carcinogenesis. We provide evidence that β-carotene had an inhibitory effect on tumour incidence and growth in rats fed the recommended EFA ratio of 5 (6 g ALA/kg diet), but failed to act as a protective agent in rats fed an EFA ratio of 1 (24 g ALA/kg diet), which is above physiological levels in human diets. These data suggest that such a protective effect of β-carotene on tumour growth may be dependent on the ALA content of the diet, although other nutritional factors associated with ALA in oils might interfere.

Because β-carotene has been shown to possess antioxidant (Sies & Stahl, 1995) as well as prooxidant (Palozza, 1998) properties, we assessed the involvement of lipoperoxidation in the decrease of tumour growth. We found that the MDA content of the tumours was not modified by β-carotene supplementation whatever the dietary EFA ratio. In agreement with our data, Chew *et al.* (1999) did not find any significant difference in lipoperoxide products in the transplanted mammary tumours of mice fed β-carotene compared with controls without supplementation. In contrast, β-carotene was found to decrease the MDA content of colon adenocarcinoma cells supplemented with EPA (Palozza *et al.* 2000). These data suggest that β-carotene could act as an antioxidant in the presence of long-chain *n*3 fatty acids, which are more susceptible to peroxidation than ALA.

The S phase fraction was determined in tumours as an index of cell proliferation. We did not find any significant difference in S phase fraction between the groups, suggesting that decreased tumour growth in rats fed the EFA 5 diet with β-carotene was a consequence of cell loss rather than an inhibition of cell proliferation.

Pathways implicated in the effects of β-carotene in the present study are not known. Several mechanisms have, however, been proposed, notably including the modulation of apoptotic signalling (Palozza *et al.* 2004), of the immune response (Chew & Park, 2004) and of the gap junction communications, or regulation of the detoxifying enzymes (Stahl *et al.* 2002).

We conclude that dietary ALA content alters the effect of β-carotene on mammary carcinogenesis. Whether the ALA content of the diet modifies the protective effect of β-carotene on breast cancer prevention in women needs to be determined. It also implies that more research needs to be carried out in order to understand the effect of dietary β-carotene supplementation along with *n*-3 fatty acids in breast cancer.

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