

## Review article

# Antimutagenic and some other effects of conjugated linoleic acid

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Conjugated linoleic acid (CLA) is a collective term for positional and geometric isomers of octadecadienoic acid in which the double bonds are conjugated, i.e. contiguous. CLA was identified as a component of milk and dairy products over 20 years ago. It is formed as an intermediate in the course of the conversion of linoleic acid to oleic acid in the rumen. The predominant naturally occurring isomer is the *cis*-9, *trans*-11 modification. Treatment of linoleic acid-rich oils such as safflower oil, soyabean oil, or maize oil with base and heat will result in the formation of CLA. Two isomers predominate in the synthetic preparation, c9,t11 and t10,c12. CLA has been shown to inhibit chemically-induced skin, stomach, mammary or colon tumours in mice and rats. The inhibition of mammary tumours in rats is effective regardless of type of carcinogen or type or amount of dietary fat. CLA has also been shown to inhibit cholesterol-induced atherosclerosis in rabbits. When young animals (mice, pigs) are placed on CLA-containing diets after weaning they accumulate more body protein and less fat. Since CLA is derived from the milk of ruminant animals and is found primarily in their meat and in products derived from their milk there is a concerted world-wide effort to increase CLA content of milk by dietary means. Its effect on growth (less fat, more protein) is also a subject of active research. The mechanisms underlying the effects of CLA are still moot.

### Conjugated linoleic acid: Antimutagens: Cancer: Atherosclerosis

Conjugated linoleic acid (CLA) is a collective term for a mixture of positional and geometric isomers of octadecadienoic acid (18:2) in which the double bonds are conjugated, i.e. contiguous, rather than the double bonds of linoleic acid which are separated by a methylene group. CLA is not a recent discovery. Bartlett & Chapman (1961) reported that CLA was an intermediate in the microbial hydrogenation of linoleic acid. A few years later Kepler *et al.* (1966) showed that a rumen bacterium, *Butyrivibrio fibrisolvens*, converted linoleic acid to oleic acid with CLA as an intermediate. The predominant CLA isomer found in foods is the c9 to t11 modification. CLA can be found in the meat of ruminant animals, cheese and dairy products. It is also present in turkey meat, which appears to be an anomaly (Chin *et al.* 1992) (Table 1).

#### Antimutagenic effects

The discovery of one biological (antimutagenic) effect of CLA was made by Pariza and his co-workers in the course

of a study of heterocyclic amine formation during grilling of beef. Cooking of high-protein foods is known to produce heterocyclic amines or polynuclear hydrocarbons which are mutagens and carcinogens (Wakabayashi *et al.* 1992; Dipple, 1983). Pariza *et al.* (1979) were studying formation of carcinogenic compounds in fried ground beef and discovered one fraction of the extract of heated ground beef that inhibited mutagenesis. The antimutagenic activity was assayed using the Ames test. They found that the antimutagenic activity was also present in raw ground beef. It is to their credit that they pursued this observation before they knew the identity of the antimutagenic material as it would have been easy to dismiss it as an anomalous result.

In a subsequent study, the partially purified mutagenesis inhibitor was assayed in the Ames test against two known mutagens, 2-amino-3-methylimidazo(4,5-f)quinoline (IQ) and 2-aminofluorene. Mutagenicity of IQ was inhibited in tests with uninduced rat liver S9 fraction or with S9 induced by phenobarbital or Aroclor 1254. Mutagenicity of 2-aminofluorene

**Abbreviations:** CLA, conjugated linoleic acid; DMBA, 7,12-dimethylbenz(a)anthracene; IQ, 2-amino-3-methylimidazo(4,5-f)quinoline; SCID, severe combined immunodeficient.

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**Table 1.** Concentration of conjugated linoleic acid (CLA) and linoleic acid (LA) in representative foods

Food	CLA (g/kg fat)*	c9,t11 isomer (%)*	LA (g/kg fat)†
<b>Meat</b>			
Beef, fresh ground	4.3	85	24
Chicken	0.9	84	179
Lamb	5.6	92	58
Pork	0.6	82	79
Turkey	2.5	76	218
Veal	2.7	84	53
<b>Seafood</b>			
Salmon	0.3	nd	102
Shrimp	0.6	nd	16
Trout	0.5	nd	26
<b>Cheese</b>			
Cheddar	3.6	92	17
Cottage	4.5	83	22
Parmesan	3.0	90	11
Ricotta	5.6	84	24
Romano	2.9	92	11
<b>Dairy products</b>			
Butter	4.7	88	23
Milk, homogenized	5.5	92	22
Yogurt	4.8	84	20
<b>Vegetable oils</b>			
Maize	0.2	39	58
Olive	0.2	47	79
Peanut	0.2	46	32
Safflower	0.7	44	74

nd, not detectable.

\* After Chin *et al.* (1992).

† Data from United States Department of Agriculture (1999) nutrient database.

was inhibited when uninduced S9 was used. The fraction was ineffective when phenobarbital was used and enhanced mutagenicity when Aroclor 1254-induced S9 was used. As a consequence of these results, Pariza *et al.* (1983) began to refer to the partially purified beef extract as 'mutagenesis modulator'. The first test of the 'mutagenesis modulator' as an anticarcinogen in animals was carried out using the (7,12-dimethylbenz(a)anthracene) DMBA mouse epidermal papilloma model. It was shown that treatment of the mouse skin with 'mutagenesis modulator' 5 min before DMBA treatment reduced the number of mice with papillomas as well as the number of papilloma-bearing mice (Pariza & Hargraves, 1985). Two years later the anticarcinogen from fried ground beef was identified as a mixture of four isomeric CLA, these were the c9,t11, t9,t11, t10,c12, and t10,t12 modifications (Ha *et al.* 1987).

Once the inhibitor was identified it was tested in the benzo(a)pyrene-induced mouse forestomach neoplasia model. The CLA used was prepared from linoleic acid by alkali isomerization and the four principal products were the c9,t11, t10,c12, t9,t11, and t10,t12 isomers. Mice were given orally either olive oil (0.1 ml) or CLA (0.1 ml) plus olive oil (0.1 ml) or linoleic acid (0.1 ml) plus olive oil (0.1 ml). In addition, all mice were given orally 2 mg benzo(a)pyrene in 0.2 ml olive oil once weekly for 4 weeks. The results are presented in Table 2. It is evident that CLA significantly reduced tumour incidence (percentage of mice bearing tumours) as well as tumour multiplicity (no. of tumours per tumour-bearing mouse) (Ha *et al.* 1990).

**Table 2.** Effect of dietary conjugated linoleic acid (CLA) on benzo(a)pyrene-induced forestomach tumours in mice\* (Mean values with standard errors from three experiments)

Treatment	Mice (n)	Tumour incidence (%)	
		Mean	SE
Olive oil	23	95.6	2.6
CLA	22	79.2	8.3
Linoleic acid	19	89.6	6.1

\* After Ha *et al.* (1990).

Up to this time, CLA had been shown to inhibit tumourigenesis when applied directly to the tumour site but evidence of a direct dietary effect was still lacking. In an elegant series of experiments Ip and his colleagues showed that dietary CLA could inhibit chemically-induced mammary tumours in rats independently of other dietary fat or type of carcinogen. Ip *et al.* (1991) fed DMBA-treated rats on diets containing 5–15 g CLA/kg. Even at the lowest concentration of CLA, tumour incidence was suppressed significantly (Table 3). Ip *et al.* (1994) then showed that CLA was equally effective against the action of a direct-acting carcinogen (methylnitrosourea) as it was against DMBA, a carcinogen which requires metabolic activation. At 10 g CLA/kg diet, tumour incidence was reduced in DMBA-treated rats by 35 % and that in methylnitrosourea-treated rats by 32 % ( $P < 0.01$  in both cases). The data suggested a direct effect of CLA on the target organ, and immunohistochemical staining showed an effect of CLA on the development of mammary lobulo-alveolar structures which are derived from the terminal end buds. An effect on development of the target organ was also suggested by the finding that short-term feeding of CLA (5 weeks from weaning) was enough to offer significant protection in a 4-month experiment. Further investigation into the morphological effects of CLA (Thompson *et al.* 1997) showed that it caused a 20 % reduction in the density of the ductal-lobular tree. Recovery of desaturation and elongation products of CLA, eighteen C atoms (18:3) or twenty C atoms with three conjugated double bonds (20:3) suggested that CLA metabolism might play a defining role in reducing risk.

CLA-feeding leads to its incorporation into mammary neutral lipid and phospholipid. Uptake of CLA into neutral

**Table 3.** Influence of dietary conjugated linoleic acid (CLA) on 7,12-dimethylbenz(a)anthracene(DMBA)-induced mammary tumours in rats†

Group	Dietary CLA (g/kg)	(Mean values for thirty rats per group)	
		Tumour incidence (%)	Adenocarcinomas (total no.)
1	0	80.0	81
2	5	66.7	55*
3	10	46.7*	36*
4	15	40.0*	31*

Mean values were significantly different from those of control group: \*  $P < 0.05$ .† After Ip *et al.* (1991).

lipid plateaus after 4 weeks and declines rapidly after CLA withdrawal. Less CLA is found in the phospholipids, its uptake is slower and declines after removal of CLA somewhat more slowly than seen in the case of triacylglycerol (Ip *et al.* 1997).

Feeding studies in mice (Belury & Kempa-Steczko, 1997) show that CLA may be incorporated into neutral and hepatic liver phospholipids at the expense of linoleate. Oleate was increased and arachidonate decreased in the neutral lipids of livers of CLA-fed mice. A possible effect on eicosanoid production could be adduced. When rats were fed 10 g CLA/kg for 14 d, levels of arachidonate in liver phosphatidyl choline, phosphatidylethanolamine or phosphatidylserine were essentially unchanged but the level of arachidonate in liver phosphatidyl inositol was 10% lower than in rats fed on linoleic acid and in liver cardiolipin it was 83% higher. Prostaglandin E<sub>2</sub> levels in serum and spleen were reduced by 47 and 38% respectively (Sugano *et al.* 1997).

Virtually all of the studies with CLA were carried out using it as a component of triacylglycerol, the form in which it appears naturally. Comparison of 10 g CLA/kg diet fed as triacylglycerol or as the free fatty acid in methylnitrosourea-treated rats gave almost the same result (Ip *et al.* 1995). Tumour incidence in rats fed 10 g triacylglycerol-CLA/kg was reduced by 33% (compared with control rats) and that in rats fed on 10 g free CLA/kg was reduced by 38%. That study also showed that CLA-feeding for only 3 weeks post-weaning (21–42 d old) was sufficient to reduce significantly tumour incidence and total tumour yield.

The efficacy of CLA inhibition of mammary tumorigenesis is independent of the amount or type of dietary fat; the studies cited earlier were carried out using a semi-purified diet containing 50 g maize oil/kg. Since linoleic acid enhances experimental mammary carcinogenesis (Welsch, 1992) the possibility that higher levels of this acid might swamp the CLA effect was addressed by Ip *et al.* (1996): using a fat-blend designed to reflect the fatty acid composition of the American diet, the ratio of saturated:monounsaturated:polyunsaturated fatty acids in the dietary fat was 1:1:1.

Inhibition of DMBA-induced mammary tumours was virtually the same in rats fed on 10 g CLA/kg and 100, 133, 167 or 200 g fat/kg. The extent of tumour inhibition by CLA was also the same in rats fed on 200 g maize oil/kg or 80 g maize oil/kg + 120 g lard/kg. Similarly, the amount of linoleate present in the diet (20 or 120 g/kg) did not affect the inhibitory effect of CLA. Testing levels of CLA of 5, 1, 15 and 20 g CLA/kg showed significant protection against DMBA-induced carcinogenesis at the lowest level (5 g CLA/kg) and no added protection beyond 15 g CLA/kg (Ip & Scimeca, 1997).

The inhibition of mammary tumorigenesis in rats by CLA has been studied thoroughly but there have also been studies of other tumour types or other experimental models. Liew *et al.* (1995) investigated the effect of CLA on IQ-induced colon carcinogenesis in male F344 rats. The carcinogen was administered at weeks 3 and 4 orally (100 mg/kg body weight). CLA and the safflower oil from which it was formed by alkali isomerization were also given on alternate

days orally at levels designed to reflect 5 g CLA/kg in the diet. CLA and IQ treatments were discontinued at 4 weeks and the rats killed 12 weeks later. The number of aberrant crypt foci per rat were 4.3 (SEM 2.4) in the IQ-fed controls; 3.2 (SEM 1.7) in rats given IQ plus safflower oil; and 1.1 (SEM 1.3) in rats given IQ + CLA. The number of aberrant crypts per rat in the three groups were 14.2 (SEM 11.6), 12.0 (SEM 7.0), and 4.0 (SEM 4.6) respectively.

Belury *et al.* (1996) studied effects of increasing levels of dietary CLA on phorbol ester (12-*o*-tetradecanoyl phorbol-13-acetate)-promotion of skin tumours in mice. Tumour yield fell significantly with increasing levels of dietary CLA, from 6.71 in controls to 5.92, 4.83, and 4.67 in mice fed on 50, 10 or 15 g CLA/kg respectively.

The severe combined immunodeficient (SCID) mouse provides a model for growing human tumour cells (Cesano *et al.* 1992). Subcutaneous inoculation of tumour cells into the SCID mouse leads to tumour growth at the site of injection and metastatic spread as well. SCID mice were fed on a diet containing 10 g CLA/kg for 2 weeks before inoculation with 10<sup>7</sup> human breast adenocarcinoma cells (MDA-MB468). CLA feeding was continued for the duration of the study (14 weeks). At the end of the study tumour weight (g) and volume (mm<sup>3</sup>) were significantly reduced in the treated mice. Systemic spread of the tumour into the lungs, peripheral blood and bone marrow was abrogated completely (Visonneau *et al.* 1997). In a subsequent study effects of CLA and linoleic acid were compared in SCID mice inoculated subcutaneously with 5 × 10<sup>6</sup> human prostatic cancer cells (DU145). As in the earlier study, CLA and linoleic acid were fed as 10 g/kg diet beginning at 2 weeks before inoculation of the DU145 cells and the mice were observed for another 12 weeks. Tumour volume (mm<sup>3</sup>) was increased significantly by linoleic acid and decreased dramatically (*P* < 0.01) by CLA. Tumour mass followed the same pattern. Metastatic spread of the tumour to the lungs was observed in 80–100% of the control or linoleic acid-fed mice and in only 10% of the mice fed on CLA (Cesano *et al.* 1998). CLA has also been shown to inhibit growth of a number of human tumour cell lines *in vitro* (Shultz *et al.* 1992a; Schonberg & Krokan, 1995; Visonneau *et al.* 1996; Cunningham *et al.* 1997).

#### *Mechanisms through which conjugated linoleic acid inhibits tumorigenesis*

The mechanism(s) through which CLA inhibits tumorigenesis are moot. Ha *et al.* (1990) suggested an antioxidant mechanism. Ip's data show an effect on growth and development of certain types of mammary cell (Ip *et al.* 1994). Reduced formation of carcinogen-DNA adducts has been implicated (Liew *et al.* 1995; Schut *et al.* 1997; Josyula *et al.* 1998; Josyula & Schut, 1998). Durgam & Fernandes (1997) suggest influences on the oestrogen response system. Other suggested mechanisms of action include effects on eicosanoid metabolism (Cunningham *et al.* 1997; Liu & Belury, 1997) and apoptosis (Schultz *et al.* 1992a,b).

Farquharson *et al.* (1999) have reported that when human prostate cancer cells (PC3) are treated with CLA (100 mM)

for 24 h the mRNA for glutathione peroxidase is increased by about 50% and diacylglycerol content is reduced by about 50% as well. Diacylglycerol production by malignant prostate tissue is regarded as a tumour-promoting factor. The levels of the adhesion molecules ICAM-1 and E selection were reduced by 50% in human umbilical vein endothelial cells when incubated with 10 mM CLA. When Cesano *et al.* (1998) fed 10 g CLA/kg to SCID mice engrafted subcutaneously with human prostatic cancer cells (DU145), levels of SICAM-1 antigen were reduced significantly ( $P < 0.01$ ) compared with the controls. In mice fed on linoleic acid the antigen levels were significantly elevated ( $P < 0.02$ ).

It is not impossible that CLA effects are influenced by tissue type and mode of carcinogenesis. At the moment we can agree that CLA is definitely anticarcinogenic but we do not yet know precisely how it works.

### Miscellaneous effects

Reports that CLA exhibited antioxidant properties *in vivo* (Ip *et al.* 1990) and *in vitro* (Ha *et al.* 1990) and knowledge that oxidized derivatives of cholesterol (Cook & MacDougal, 1968; Imai *et al.* 1976) and oxidized LDL (Steinberg *et al.* 1989) were atherogenic prompted an inquiry into CLA effects on atherosclerosis. Rabbits fed on a semipurified diet containing 10 g cholesterol/kg were fed on 0.5 g CLA/d. After 22 weeks lipid deposition and connective tissue development were less severe in the aortas and CLA-fed rabbits than in controls. Plasma lipid levels were lower in the CLA-fed group but plasma peroxide levels were the same in CLA-fed rabbits and controls (Lee *et al.* 1994). In a subsequent study the effects of CLA on progression and regression of atherosclerotic lesions in rabbits fed on 2 g cholesterol/kg were examined. Addition of 10 g CLA/kg to a semipurified atherogenic diet (2 g cholesterol/kg) did not influence lipaemia but reduced severity of atherosclerosis by 36%. Rabbits with pre-established lesions were placed on a cholesterol-free diet containing 60 g maize oil/kg or the same diet containing 50 g maize oil and 10 g CLA/kg. After 90 d atherosclerosis in the control group was virtually unchanged from that seen at cessation of cholesterol feeding but that in the CLA-fed group was reduced by 29%

(compared with cholesterol-free control) and 30% compared with the cholesterol-fed group. To test the lowest level at which dietary CLA might influence atherosclerosis, rabbits were then fed on a semipurified diet containing 2 g cholesterol/kg or the same diet with 1, 5 or 10 g CLA/kg added. After 90 d CLA at levels of 1, 5 or 10 g/kg had reduced atherosclerosis by 34, 64, and 58% respectively. Addition of CLA had no effect on serum lipid levels (Table 4). The effect of graded levels of CLA on pre-established lesions was also studied. Feeding 10 g CLA/kg in a cholesterol-free diet reduced lesion severity by 35% but 1 or 5 g CLA/kg had no effect. CLA has also been shown to reduce aortic sudanophilia in hamsters fed on 1.2 g cholesterol/kg (Nicolosi *et al.* 1997).

Recently Munday *et al.* (1999) have reported that CLA enhanced aortic sudanophilia in C57BL/6 mice. Paigen *et al.* (1987) found that a diet containing 10 g cholesterol/kg and 5 g cholic acid/kg fed for 14 weeks caused aortic sudanophilia in C57BL/6 mice. The level of sudanophilia observed by Paigen *et al.* (1987) was 0.66 (SD 0.14) mm<sup>2</sup>. Munday *et al.* (1999) found that control mice fed on the sudanophilic diet for 15 weeks exhibited a level of sudanophilia of 0.13 (SD 0.13) mm<sup>2</sup>. At levels of 2.5 and 5.0 g CLA/kg, sudanophilia observed was 0.33 (SD 0.27) and 0.25 (SD 0.22) mm<sup>2</sup> respectively. The sudanophilia observed on the diet containing 2.5 g CLA/kg was significantly different from the control value, but that on the diet containing 5 g CLA/kg was not. It is possible that in this model, a level of 10 g CLA/kg, or higher is required to reduce sudanophilia.

The mechanism whereby CLA reduces atherosclerosis is also unclear. In rabbits CLA does not affect lipaemia and may function at the level of cellular metabolism.

Many other aspects of CLA effects are under investigation but to date the published data are scant. Based on studies of oxidation of 1-palmitoyl-2-linoleoyl phosphatidyl choline, the role of CLA as an antioxidant has been challenged (van den Berg *et al.* 1995). CLA feeding partially overcomes the catabolic responses to endotoxin injection in mice (Miller *et al.* 1994). Dietary CLA (10 g/kg) reduces levels of monounsaturated fatty acids and *n*-6 fatty acids in a number of rat tissues and increases levels of *n*-3 fatty acids and saturated fatty acids, possibly by inhibition  $\Delta^9$  desaturase activity in the liver (Li & Watkins, 1998).

**Table 4.** Influence of graded levels of conjugated linoleic acid (CLA) on severity of atherosclerosis in rabbits fed on 2 g cholesterol/kg for 90 days\*

(Mean values with standard errors for eight rats per group)

Variable	CLA (g/kg diet)							
	0 (Control)		1		5		10	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Serum cholesterol (mmol/l)	25.4	3.1	33.2	3.0	32.7	2.7	28.5	3.5
Serum triacylglycerol (mmol/l)	2.1	0.4	2.8	0.5	2.3	0.5	2.4	0.4
Aortic lesions†								
Aortic arch	2.36 <sup>a</sup>	0.39	1.69 <sup>a</sup>	0.23	0.88 <sup>b</sup>	0.20	1.00 <sup>b</sup>	0.28
Thoracic aorta	2.21 <sup>a</sup>	0.42	1.31	0.28	0.75 <sup>b</sup>	0.21	0.94 <sup>c</sup>	0.27

<sup>a,b,c</sup> Mean values within a row with unlike superscript letters were significantly different ( $P < 0.05$ ).

\* After D. Kritchevsky (unpublished results).

† Graded visually on a scale of 0–4 (Duff & McMillan, 1949).



**Table 5.** Influence of 5 g conjugated linoleic acid (CLA)/kg diet fed to mice for 32 days on their body composition\*

	Group				Statistical significance of difference between means: <i>P</i>
	Control		CLA		
	Mean	SE	Mean	SE	
Empty carcass weight (g)	32.4	1.1	32.2	0.8	
Fat (g/kg)	101.3	11.7	43.4	4.0	0.001
Protein (g/kg)	177.6	3.0	185.8	1.4	0.01
Water (g/kg)	663	8.0	709	4.0	0.001
Ash (g/kg)	30.8	1.4	32.4	0.5	0.05

\* After Park *et al.* (1997).

Dietary CLA (15 g/kg) normalizes impaired glucose tolerance and lowers plasma insulin levels in the Zucker diabetic fatty fa/fa rat (Houseknecht *et al.* 1998). That effect may be due in part to the activation of peroxisome proliferator-activated receptor  $\gamma$ ). This appears to exert potent anti-proliferative effects (Sarraf *et al.* 1998). This observation may explain some of the anti-tumour properties of CLA.

CLA has been shown to be growth factor for rats (Chin *et al.* 1994) and also has a remarkable effect on the body composition of mice. Weanling mice fed on 5 g CLA/kg had about 60 % less body fat and 14 % more lean body mass after a month of feeding (Park *et al.* 1997) (Table 5). These data suggest again an effect of CLA on growing tissue.

### Future developments

The availability of purified isomers of CLA will make it possible to determine if all isomers of this fatty acid possess the same physiological properties or if specific observations may be due to specific isomeric forms. Park *et al.* (1999), for instance, have reported that the t10,c12 isomer affects body composition of mice whereas the c9,t11 isomer has no effect. There is also interest in linoleic acids containing three conjugated double bonds. The next few years should provide insights into the mechanisms whereby CLA exerts its many effects. The findings that CLA affects tumorigenicity and atherosclerosis at low concentrations holds promise that it may be found effective at normal dietary levels and not in the pharmacological quantities used now. Animal fat, which has been maligned for so long, may actually contribute a potent therapeutic component to our diet.

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