Inhibition of Carcinogenesis by Conjugated Linoleic Acid: Potential Mechanisms of Action[1]

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ABSTRACT Conjugated linoleic acid (CLA) is composed of positional and stereoisomers of octadecadienoate (18:2); it is found in foods derived from ruminants (beef and lamb as well as dairy products from these sources). When a mixture of isomers is fed to experimental animals, chemically induced tumorigenesis of mammary, skin and colon is reduced. Importantly, many isomers of CLA are readily metabolized to desaturated/elongated products as well as β-oxidized products, suggesting that these metabolites may be important anticancer compounds. Mechanisms of inhibition of carcinogenesis may include reduction of cell proliferation, alterations in the components of the cell cycle and induction of apoptosis. In addition, CLA modulates markers of immunity and eicosanoid formation in numerous species as well as lipid metabolism and gene expression. It is likely that CLA exerts inhibitory properties in carcinogenesis via one or more of these pathways with some tissue specificity. This review will explore recent advances in putative mechanisms of reduction of carcinogenesis by CLA. J. Nutr. 132: 2995–2998, 2002.

KEY WORDS: • conjugated linoleic acid • carcinogenesis • CLA isomers • anticarcinogenic

Conjugated linoleic acid (CLA)[3] refers to a group of polyunsaturated fatty acids that exist as positional and stereoisomers of octadecadienoate (18:2). CLA is found in foods such as beef and lamb as well as dairy foods derived from these ruminant sources (1,2). The double bonds of CLA may be in the positions of 7,9; 8,10; 9,11; 10,12; or 11,13 and the 3-dimensional geometric combinations of *cis* and/or *trans* configurations. The major isomers in foods are in the following rank order: *c*9*t*11-CLA (also called rumenic acid) > *t*7*c*9-CLA > 11,13-CLA (*c*/*t*) > 8,10-CLA (*c*/*t*) > *t*10*c*12-CLA isomer > other isomers (2–4). Importantly, the majority of experiments performed in experimental animals have used a synthetic mixture of CLA isomers containing primarily *c*9*t*11-CLA and *t*10*c*12-CLA (**Fig. 1**) [reviewed in 5)].

Numerous physiologic properties have been attributed to CLA including action as an anticarcinogenic, antiatherosclerotic, antiadipogenic and antidiabetogenic agent [reviewed in 5–7)]. In addition, CLA modulates immunity and thrombosis as well as fatty acid biochemistry, lipid metabolism and gene expression in the liver, muscle and adipose tissues (6). There are several recent reviews on the effects and mechanisms of CLA in biological systems, including cancer (7). Therefore, this review will focus on recently identified mechanisms by which CLA inhibits carcinogenesis.

Dietary CLA inhibits carcinogenesis in experimental animals

As a component of semipurified diets, CLA inhibits cancer in several animal models. In particular, CLA inhibits dimethylbenz(a)anthracene-induced tumorigenesis of skin, mammary and forestomach neoplasia (8–10). In addition, when a synthetic mixture of CLA isomers (~45% *c*9*t*11-CLA, ~42% *t*10*c*12-CLA with several other remaining isomers comprising minor amounts) is provided in diets (0.5–1.5 g/100 g) either during or after initiation, chemically induced skin tumor promotion or mammary and colon tumorigenesis are inhibited (10–13). Furthermore, CLA inhibits the growth of transplanted cell lines derived from mammary (14) and prostate (15) cancers. Although the role of CLA in inhibiting carcinogenesis is convincing, not all studies have shown inhibition. In fact, CLA did not alter the growth of transplanted prostate (7) and breast (16) cancer cells and did not reduce tumorigenesis in an intestinal model of colon carcinogenesis using the Apc^{Min} mouse (17). No studies have yet reported that CLA enhances tumorigenesis.

In conjunction with identifying the inhibitory properties of CLA in various tumor models, efforts have been made to elucidate the role of CLA in modulating the stages of carcinogenesis known as initiation, promotion and progression. In particular, the anticarcinogenic property of CLA was first identified during the initiation stage of skin carcinogenesis, a stage associated with a genetic alteration in a subset of cells in the target tissue (8). During initiation, CLA modulates events such as free radical–induced oxidation, carcinogen metabolism and carcinogen-DNA adduct formation in some tissues (7). Findings have been ambiguous. In fact, a recent study in male rats demonstrated tissue-specific effects of CLA on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine–induced mutation frequencies (18): Dietary CLA (0.5 g/100 g) reduced mutation frequency in the distal colon, but had no effect or enhanced mutation frequency in the proximal colon and cecum of rats.

In addition to tissue- and/or tumor model–specific effects of CLA on tumor initiation, several studies demonstrated that CLA inhibits carcinogenesis postinitiation (10,11,13,19,20). In chemically induced mammary carcinogenesis, there may be an optimal time for exposure to CLA, i.e., the inhibitory properties of CLA on chemically induced mammary carcinogenesis were most profound when CLA was fed during mammary gland maturation [between 21 and 42 d of age (19)]. During the promotion stage of skin carcinogenesis, CLA re-

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E-mail: Belury@u.washington.edu.
[3] Abbreviations used: BrdU, bromodeoxyuridine; CLA, conjugated linoleic acid; COX, cyclooxygenase; PG, prostaglandin; PPAR, peroxisome proliferator-activated receptors.

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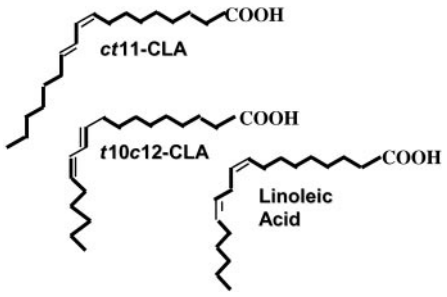

FIGURE 1 Structures of *c*9*t*11-conjugated linoleic acid (CLA), *t*10*c*12-CLA and linoleic acid [18:2(n-6)].

duces the yield of mouse skin tumors by a mechanism distinct from its anti-initiator activity (10).

Although a great deal of evidence demonstrates that dietary CLA inhibits the initiation and postinitiation and/or promotion stages of carcinogenesis, its role in the progression stage of carcinogenesis has not been established definitively. Using transplantable tumor models, dietary CLA reduced the growth rates of mammary and prostate cancer cells when implanted in vivo in mice (14,15). In addition, at least one study demonstrated that CLA (0.5–1.0 g/100 g) inhibited the ability of transplanted mammary cancer cells to form secondary tumors in mice (21). Furthermore, the CLA-responsive chemically induced mammary carcinogenesis model (10) is a model for human breast cancer ductal carcinomas in situ. Therefore, data showing that CLA inhibits tumorigenesis in this model are consistent with the possibility that CLA will reduce breast cancer metastasis. However, no studies have addressed the role of CLA in the prevention of metastatic cancer. It is critical to understand how CLA modulates malignant tumor formation and metastasis because the growth of secondary tumors is the major cause of morbidity and mortality in people with cancer.

CLA modulates cell proliferation and apoptosis

In an attempt to identify mechanisms of action, recent efforts have focused on elucidating how CLA modulates events that occur postinitiation and/or during promotion. The promotion stage involves the clonal expansion of initiated cells to form a benign tumor. This stage of carcinogenesis represents a premalignant state in which tumors form as a result of imbalances between dysregulated differentiation, enhanced cell proliferation and/or reduced apoptosis (or programmed cell death). CLA reduces the proliferation of numerous cell types grown in culture [reviewed in 7)]. In vivo, dietary CLA (1.0 g/100 g) reduces proliferation of terminal end bud and lobuloalveolar bud structures, the sites at which tumors form in both rat and human mammary cancers (22). Furthermore, mammary adenocarcinomas induced by PhIP contained significantly fewer proliferating cell nuclear antigen positive cells in rats fed dietary CLA (0.1 g/100 g) compared with rats fed a control diet without CLA (13). Recent work by Ip and colleagues (23) demonstrated that CLA or *c*9*t*11-CLA–rich butter fat reduces the rate of incorporation of bromodeoxyuridine (BrdU) and the expression of cyclins A and D. These two cyclins regulate the conversion of G1 → S phase of the cell cycle (**Fig. 2**). In addition, diets with CLA moderately increased levels of p16 and p27 proteins. These data suggest that CLA reduces cell proliferation by blocking DNA synthesis and cell cycle proteins that regulate this process (13,23). In contrast to findings in mammary carcinogenesis, dietary CLA does not reduce cell proliferation in phorbol ester–induced tumor promotion of mouse skin as measured by hyperplasia or ornithine decarboxylase activity, although c-myc mRNA was modestly reduced (24). Furthermore, dietary CLA enhances cell proliferation and/or ornithine decarboxylase in livers of rats and mice (25,26).

As a counterbalance to cell proliferation, apoptosis offers protection against carcinogenesis via programmed cell death (Fig. 2). Dietary CLA induces apoptosis in numerous tissues including mammary gland (27), liver (25), colon (28) and adipose (29) tissues. In terminal end buds of rat mammary tissue initiated with methylnitrosurea, dietary CLA induces apoptosis in a site-specific manner. The ability of CLA to induce apoptosis in terminal end buds and the premalignant lesions known as intraductal proliferation lesions (27) may have implications for development of this epithelial tissue. Induction of apoptosis by CLA was associated with reduction of Bcl-2 protein within the lesions. The Bcl-2 gene family has differential effects on apoptosis; for example, Bcl-2 and Bcl-x_L suppress apoptosis, whereas others, such as Bax and Bak, promote apoptosis. The ability of Bax to induce apoptosis appears to involve a countereffect on Bcl-2. Although CLA reduced Bcl-2, there was only a moderate effect of CLA on induction of Bax protein. Therefore, it appears that CLA may support elevated apoptosis primarily by reducing the suppressor of apoptosis, bcl-2. Because the inhibitory effects of CLA or *c*9*t*11-CLA on reductions of BrdU incorporation in mammary epithelium were dependent on the proliferative (vs. quiescent) status of mammary epithelial cells (23), effects of CLA on pivotal signaling events regulating both cell proliferation and apoptosis (e.g., cyclin dependent kinases or check point proteins such as p53) warrant further study.

Effects of CLA on phospholipid metabolism and regulation of gene expression

The cellular mechanisms of modulation of carcinogenesis by CLA are numerous and complex. Several studies have shown that diets with CLA are associated with altered phospholipid-associated fatty acid metabolism and eicosanoid for-

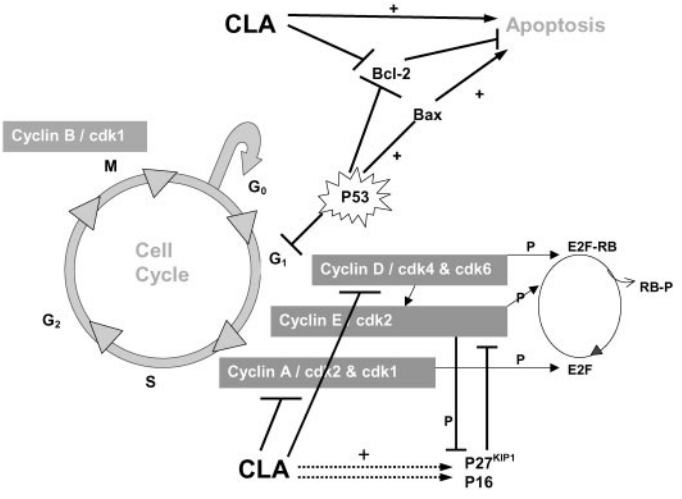

FIGURE 2 Schematic diagram of how conjugated linoleic acid (CLA) may modulate the cell cycle and apoptosis. CLA significantly reduces levels of cyclin A and cyclin D and induces apoptosis in the mammary epithelium (23,27). The tumor suppressor, p53, induces apoptosis and modulates the cell cycle in some cell types under various conditions. Solid lines (—): significant ($P < 0.05$); dotted lines (·····): modest ($P < 0.05$). Abbreviation: cdk, cyclin-dependent kinase.

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mation. Eicosanoids modulate tumorigenesis in many tissues including mammary gland, skin, prostate and colon [reviewed in (30)]. Events in carcinogenesis that appear to be particularly sensitive to eicosanoids include cell proliferation, inflammation, local and systemic immunity, platelet aggregation and tissue differentiation. Diets with CLA result in an accumulation of CLA, especially the 9,11-CLA (*c*/*t*; *t*/*c*) isomer in phospholipids of tissues [e.g., liver (31), mammary (32), skin (24) and others] and lipid fractions of human sera (M.A. Belury, unpublished data). In addition, when fed as the free fatty acid, dietary CLA alters the relative amounts of numerous other fatty acids in phospholipid fractions (24,28). These findings raise the possibility that CLA, when fed as the free fatty acid, competes with other fatty acids for incorporation into phospholipids and modifies subsequent eicosanoid production (especially from arachidonate, 20:4). In fact, dietary CLA reduces prostaglandin (PG)-E_2 and/or other eicosanoids derived from enzymatic oxidation of arachidonic acid in some tissues (24,28). However, only one study has shown that dietary CLA reduces phospholipid-associated arachidonate (28). In addition, when fed as triglyceride-esterified fatty acid (in CLA-rich butter), CLA does not alter phospholipid associated nonconjugated fatty acids (33). Furthermore, some studies demonstrated that when dietary CLA altered the levels of nonconjugated fatty acids, these changes occurred in neutral lipid fractions of tissues [e.g., adipose (M.A. Belury, unpublished data), skin (24), liver (31) and mammary (32). The relevance of reduced neutral lipid–associated arachidonate levels to altered arachidonate–derived eicosanoids is unclear at present.

Interestingly, when CLA lowers arachidonate-derived eicosanoids such as PGE_2 and $PGF_{2\alpha}$ in colon and skin (24,28), it also reduces tumorigenesis in these tissues. In contrast, at least one study has shown a relationship between an inability of dietary CLA to alter arachidonate-derived eicosanoids with a lack of its inhibition of intestinal tumorigenesis in Min mice (17). Together, these studies indirectly suggest that the mechanism by which CLA inhibits carcinogenesis in some tissues may involve the modulation of arachidonate-derived eicosanoids.

CLA may reduce arachidonate-derived eicosanoids such as prostaglandin-E_2, $PGF_{2\alpha}$, leukotriene-B_4 and leukotriene-C_4 by one of two mechanisms. First, CLA may displace arachidonate incorporation into phospholipids as shown in cultured keratinocytes (34). In addition, dietary CLA displaces the arachidonate precursor, linoleate, in a dose responsive manner in livers of mice fed various doses of CLA (0.5–1.5 g/100 g) in one study (31) but not others (33,35 Belury, M. A. unpublished data). A recent study demonstrated that dietary CLA reduces phospholipid-associated arachidonate in the colonic mucosa of rats (28).

A second explanation for the reduction of arachidonate-derived eicosanoids by CLA may be through inhibition of the constitutive enzyme, cyclooxygenase (COX)-1, and/or the inducible form, COX-2, at the level of mRNA, protein, or activity. CLA or elongated and desaturated products from CLA (e.g., conjugated "arachidonate" or conjugated eicosatetraenoate) may act as antagonists for COX thereby reducing available enzyme (at the level of expression or activity) for arachidonate. Using an in vitro activity assay, CLA or individual isomers inhibited the rate of oxygenation of arachidonate in the presence of COX-1 (36). Furthermore, *c*9*t*11-CLA and *t*10*c*12-CLA reduced COX-2 at the levels of mRNA and protein in a cultured macrophage cell line (37).

Although CLA is readily metabolized by Δ^6 desaturase to form numerous downstream products (31–33,38,39), little is known about how CLA modulates metabolism of nonconjugated fatty acids via enzymatic systems such as Δ^6 desaturase-elongase-Δ^5 desaturase. CLA reduces levels of linoleate (18:2) and its desaturated and elongated product, arachidonate (20:4) in mammary tissue (32). In contrast, one study has shown that CLA may modestly enhance levels of neutral lipid–associated arachidonate in the epidermis of mice (24). Furthermore, other studies showed no effect of CLA on arachidonate levels in fat pads (40), liver (33) or small intestine (17). The ability of CLA to alter arachidonate levels may depend on the form of CLA (free fatty acid vs. esterified) as well as tissue- and species-specific effects. The relevance of altered arachidonate levels in neutral lipids vs. phospholipid as a modulator of lipid metabolism and eicosanoid formation is not clear at the present time.

CLA may modulate lipid metabolism in part by a mechanism dependent on the activation of the nuclear hormone receptors, peroxisome proliferator-activated receptors (PPAR) [reviewed in (5)]. In particular, the PPARγ isoform is found in extrahepatic tissues such as adipose, prostate, colon, mammary gland and others. PPARγ2 is a required transcription factor in adipose tissue differentiation [reviewed in (41)]. In addition, thiazolidinediones, high affinity ligands for PPARγ, modulate carcinogenesis in mammary gland, colon and prostate tissues [reviewed in (42)]. Isomers of CLA have moderate affinity for binding to and activating PPARγ (43). Dietary CLA appears to modulate transcription of genes responsive to PPARγ in adipose tissue in vivo [reviewed in (6)] and in vitro (37). Our current attempts to study the ability of CLA to activate PPARγ have focused on downstream metabolites of Δ^6 desaturase metabolism of *c*9*t*11-CLA or *t*10*c*12-CLA. In these studies, we have used approaches to block desaturase activity to determine whether reducing metabolites alters activation of PPARγ (43). CV-1 cells were transiently transfected with murine PPARγ, luciferase-peroxisome proliferator responsive element reporter and β-galactosidase, and treated with *c*9*t*11-CLA or *t*10*c*12-CLA. The activation of PPARγ was determined by measuring luciferase activity. By blocking Δ^6 desaturase using the synthetic inhibitor, SC-26196 (44), the ability of CLA isomers to activate PPARγ was reduced ($P < 0.05$). These data indirectly suggest that activation of PPARγ by CLA is increased by the formation of the Δ^6-desaturated products from CLA, *c*6*c*9*t*11-CLA or *c*6*t*10*c*12-CLA. However, the activation of PPARγ by these products is yet to be measured.

In addition to evidence showing that CLA may induce PPARγ-responsive genes in vivo, CLA may induce the level of PPARγ itself (45). Because PPARγ2 is thought to be one of several transcription factors required for adipose tissue differentiation (41), and because new evidence suggests that activators of PPARγ are protective against cancers arising in the mammary gland, colon and prostate (42), it is possible that some of the molecular mechanisms of action of CLA on carcinogenesis are mediated by PPARγ. Perhaps the ability of PPARγ to mediate effects of CLA is through increased levels of PPARγ protein (45) and/or through activation of PPARγ by downstream metabolites of CLA [e.g., desaturase and elongase products (43)].

In summary, an inverse relationship has been observed between CLA accumulation and outcomes of breast cancer in postmenopausal women (46). However, a preventive role for CLA in human cancer (breast and possibly others) is still unproven. To date, all intervention studies have been conducted in experimental animal models of carcinogenesis. It has been estimated that dietary factors contribute to approximately one third of deaths due to cancer in the United States (47). Because CLA inhibits carcinogenesis in numerous animal models and at multiple stages, this group of fatty acids offers the possibility that several types of cancers in humans

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may be prevented with a diet rich in a diversity of chemopreventive compounds, including CLA. More work is required to understand fully the implications of dietary CLA and the possibility of lowering the risk for human cancer development.

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