

Conjugated Linoleic Acid (CLA) Inhibits Akt Phosphorylation in HT-29 Human Colon Cancer Cells: A Mechanism for the Growth Inhibitory Effect of CLA

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INTRODUCTION

Colorectal cancer is one of the most common malignancies in the western world, and the incidence of colorectal cancer is increasing. Carcinogenesis is a complex, multi-step process that progresses over many years. Since it is exceptionally difficult to cure malignant tumors (Saha et al., 2002), cancer prevention may be a more effective strategy to control and, ultimately, overcome cancer. A promising and important group of potential cancer preventive agents are those derived from natural products, particularly dietary substances because of their low toxicity and apparent benefit in other chronic diseases.

Conjugated linoleic acid (CLA) is a collective term used to denote a mixture of positional and geometric isomers of octadecadienoic acid that is found naturally in dairy products and meat from ruminant animals. CLA possesses potent anti-tumor properties in number of experimental cancer models (Belury, 2002). We previously have shown that a diet containing 1% CLA significantly decreased colon tumor incidence in rats treated with 1,2-dimethylhydrazine (Park et al., 2001). The decrease in colon tumor incidence was accompanied by decreased cellular proliferation and increased apoptosis of the colonic mucosa (Park et al., 2004).

The insulin-like growth factor (IGF) system consists of the peptide growth factors IGF-I and IGF-II, the type I and II IGF receptors, the IGF-binding proteins (IGFBPs), and their corresponding proteases (Jones and Clemmons, 1995). Initially identified as potent physiological mitogens, IGFs are now known as polypeptides to have effects on cell proliferation, differentiation, apoptosis, and transformation (Baserga et al., 1997). The actions of IGFs are mediated through the IGF-I receptor (IGF-IR). Similar to the insulin receptor in structure, the IGF-IR is a heterotetrameric glycoprotein with two extracellular α -subunits and two transmembrane β -subunits. Ligand binding to the receptor induces receptor autophosphorylation in the intracellular domain of the β -subunit and results in activation of the intrinsic tyrosine kinase of the IGF-IR. Signaling pathways known to be activated by IGF-IR activation include pathways involved in activation of the ERK subfamily of mitogen-activated protein kinases (MAPKs) and a pathway involved in the activation of phosphatidylinositol 3-kinase (PI3K) (Dupont and LeRoith, 2001).

Insulin-receptor substrates (IRS)-1, IRS-2, and Shc are immediate substrates of the IGF-IR and phosphorylated after binding to the activated receptor through its phosphotyrosine-binding domain. IRS-1 is phosphorylated on multiple tyrosine residues that serve as docking sites for a variety of signaling molecules including the p85 regulatory subunit of PI3K (Dupont and LeRoith, 2001). Tyrosine-phosphorylated IRS-1 binds p85 and thereby activates the associated catalytic subunit (p110) of the enzyme (Reviewed in Ref. (Le Roith et al., 2001)). The serine/threonine kinase Akt or protein kinase B (PKB) appears to lie at the crossroads of a variety of cellular signaling pathways and serves as a transducer of multiple functions initiated by growth factor receptors that activate PI3K. Tyrosine phosphorylated Shc activates the Ras-ERK signaling pathway through a Grb2-SOS (Son

of Sevenless) complex (Boguski and McCormick, 1993). PI3K, through its downstream target Akt/PKB, as well as the MAPK pathway promotes growth factor-mediated mitogenesis and blocks programmed cell death or apoptosis (Kandel and Hay, 1999; Parrizas et al., 1997).

The aberrant activation of the IGF-IR induces growth, neoplastic transformation, and tumorigenesis (Baserga, 1999). The critical role played by the IGF-IR in the development of tumors suggests that this receptor might be an attractive target for dietary intervention for cancer prevention. Colon cancer is one of the most frequent malignant diseases in the developed world and experimental and clinical data implicate the IGF-IR in colon cancer etiology. Compared with equivalent normal tissues, the IGF-IR is over-expressed by tumors in colorectal cancer (Guo et al., 1992; Weber et al., 2002). In addition, IGF-I protects colon cancer cells from death factor induced apoptosis (Remacle-Bonnet et al., 2000). Furthermore, IGF-II mRNA is over-expressed in human colon carcinoma compared to normal adjacent tissues (Zhang et al., 1997). Therefore, the discovery of agents that inhibit the IGF-I signaling pathway could lead to the development of highly successful prevention strategies for colon cancer.

During the last several years, we performed several in vitro studies to examine if CLA inhibits cell proliferation and induces apoptosis of HT-29 cells, the human colon adenocarcinoma cell line. We also examined if CLA alters the cell cycle progression of HT-29 cells, since unrestrained cell proliferation is the characteristic of cancer, and tumor cells have usually acquired damage to genes that directly regulate their cell cycles. HT-29 cells synthesize and secrete IGF-II, IGFBP-2, -4, and -6 (Oh et al., 2001), and IGF-II acts as an autocrine growth regulator of these cells (Pommier et al., 1992). Therefore, we examined whether the growth inhibitory effect of CLA is related to changes in the IGF system in HT-29 cells.

DISCUSSION

To examine whether CLA inhibits HT-29 cell proliferation and/or induces apoptosis, we cultured HT-29 cells in serum-free medium in the presence of various concentrations (0~20 μ M) of CLA. CLA inhibited growth of the HT-29 cells in a dose-dependent manner. CLA inhibited DNA synthesis and induced apoptosis of HT-29 cells. CLA utilized in these studies consists of several possible geometric isomers of 9,11- and 10,12-octadecadienoic acid; with *cis*-9,*trans*-11 (*c9t11*) and *trans*-10,*cis*-12 CLA (*t10c12*) being the principal isomers (Pariza et al., 1999). Of the two main isomers *t10c12* decreased viable cell number, whereas *c9t11* had no effect. *T10c12* inhibited DNA synthesis and induced apoptosis of HT-29 cells. However, *c9t11* had no effect.

To study the mechanisms by which CLA induces apoptosis, we treated HT-29 cells with various concentrations (0~20 μ M) of CLA and performed Western blot analysis. Treatments of HT-29 cells with increasing concentrations of CLA led to a dose-dependent increase in the levels of phospho-p38 MAPK, phospho-stress-activated protein kinase/Jun-terminal kinase (SAPK/JNK), and phosphor-c-Jun. CLA did not alter the protein levels of Bax or Bcl-2. Western immunoblot analysis of subcellular fractions revealed that CLA increased translocation of Bax to mitochondria and induced release of Smac/Diablo and cytochrome c from mitochondria. CLA also increased Bad levels in mitochondria in a dose-dependent manner. The redistribution of these proteins occurred in concert with proteolytic cleavage of caspase-3, -7, and -9 and poly (ADP-ribose) polymerase. These results indicate that CLA increases the apoptosis of HT-29 colon cancer cells by caspase-3 dependent mechanisms, which may, at least in part, be due to the translocation of Bax and Bad to the mitochondria.

To examine the mechanism by which CLA regulates cell proliferation, we stained CLA-treated cells with propidium iodide, and analyzed using flow cytometry. A dose-dependent increase in the percentage of cells

arrested in G1 was detected following treatment with CLA. This was accompanied by corresponding reductions in the percentage of cells in S and G2 phases of the cell cycle. CLA increased protein and mRNA expression of p21^{CIP1/WAF1}, the cyclin dependent kinase (CDK) inhibitor, in a concentration dependent manner. CLA decreased cyclin A, D1, and E protein levels in a concentration-dependent manner, but did not alter CDK2 or CDK4 protein levels. Results from *in vitro* kinase assays revealed that CLA decreased the activity of both CDK2 and CDK4 in a dose-dependent manner. Hyperphosphorylated retinoblastoma (Rb) protein levels were decreased and hypophosphorylated Rb protein levels were increased in CLA-treated cells. CLA inhibited expression of proliferating cell nuclear antigen (PCNA) in a concentration-dependent manner. Immunoprecipitation/Western blot studies revealed that CLA increased binding of p21^{CIP1/WAF1} to PCNA in a concentration-dependent manner. These results indicate that CLA blocks cell cycle progression of HT-29 colon cancer cells in G1 by inducing p21^{CIP1/WAF1} and decreasing cyclin protein levels. Increased p21^{CIP1/WAF1} expression may inhibit cell cycle progression through its interaction with cyclin-CDK complexes and PCNA in HT-29 colon cancer cells.

Since the insulin-like growth factor (IGF) system regulates the growth of HT-29 cells by an autocrine mechanism, we next examined whether the growth inhibitory effect of CLA was related to changes in the IGF system in HT-29 cells. To examine if CLA inhibits IGF-II production, HT-29 cells were incubated in serum-free medium in the presence of various concentrations of CLA. CLA decreased protein levels of both mature and pro IGF-II and IGF-II transcripts. While exogenous IGF-I and IGF-II led to an increase in cell number, neither IGF-I nor IGF-II was able to counteract the negative growth regulatory effect of CLA. RT-PCR and Western blot analyses of total cell lysates revealed that CLA decreased IGF-I receptor (IGF-IR) transcript and protein levels in a dose-dependent manner. Immunoprecipitation/Western blot studies revealed that CLA inhibited IGF-I-induced phosphorylation of IGF-IR and insulin-receptor substrate (IRS)-1, recruitment of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) to IGF-IR, IGF-IR-associated PI3K activity, and phosphorylated Akt and extracellular signal-regulated kinase (ERK)-1/2 levels.

CONCLUSION

In conclusion, we have demonstrated that CLA decrease the growth of HT-29 cell growth, which was due to the induction of apoptosis and cell cycle arrest at G0/G1 phase. CLA-induced cell cycle arrest is mediated by the up-regulation of p21^{CIP1/WAF1}, and down-regulation of cyclins, which results in the decreased activity of CDKs. In addition, p21 may inhibit activities of PCNA. The activation of the caspase-3 pathway may be one of the mechanisms by which CLA induces apoptosis. CLA negatively regulates levels of IGF-II and mature IGF-IR and subsequent activation of Akt and MAPK pathways in HT-29 cells. The cell-survival oncoprotein Akt is abnormally activated in several human malignancies and suppresses apoptosis (Kennedy et al., 1997). Akt is involved in cell survival and cell cycle regulation by preventing GSK3-mediated phosphorylation and degradation of cyclin D1 (Diehl et al., 1998) and by phosphorylating and regulating the cellular localization of p21^{CIP1/WAF1}. The results reported herein indicate that inhibition of Akt phosphorylation may be a major mechanism by which CLA inhibits cell proliferation and induces apoptosis. Since Akt takes center stage in cell cycle-deregulation in cancer these results raise the possibility that CLA can be used as an agent to prevent and treat colon cancer.

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