Published in final edited form as:

Biochem Biophys Res Commun. 2007 May 18; 356(4): 1044–1049.

Conjugated linoleic acid induces apoptosis of murine mammary tumor cells via Bcl-2 loss

Lihui Ou¹, Clement Ip², Barbara Lisafeld¹, and Margot M. Ip^{1,*}

¹Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA

²Department of Chemoprevention, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263, USA

Abstract

Conjugated linoleic acid (CLA) is a powerful anticancer agent in a number of tumor model systems; however, its precise mechanism of action remains elusive. Here, we report that t10,c12 CLA, a component of synthetic CLA supplements, induced apoptosis and G₁ arrest of p53 mutant TM4t murine mammary tumor cells. Furthermore, t10,c12-CLA induced a time- and concentration-dependent cleavage of caspases-3 and -9, and release of cytochrome c from mitochondria to cytosol. Levels of Bcl-2 protein were decreased both in total cellular lysates and in mitochondria after t10,c12-CLA treatment; however, there was no significant change in Bax or Bak. Overexpression of Bcl-2 attenuated apoptosis in response to t10,c12-CLA treatment. These results demonstrate that t10,c12-CLA triggers apoptosis of p53 mutant murine mammary tumor cells through the mitochondrial pathway by targeting Bcl-2.

Keywords

CLA; apoptosis; caspase; mitochondria; Bcl-2; mammary tumor

Introduction

CLA is a collective term used to describe a mixture of positional and geometric isomers of C18 unsaturated fatty acids with two conjugated double bonds. The c9,t11 isomer of CLA is the major form of naturally occurring CLA, and is found in dairy products and ruminant meats. Additionally, a mixture of the c9,t11- and t10,c12-CLA isomers is available in health food stores as a dietary supplement, and is being evaluated clinically for its potential benefit in controlling obesity, improving immune function and insulin sensitivity, and altering lipid metabolism [1–3]. CLA has also been shown to be an anticancer agent in a number of rodent and human tumor model systems, including carcinogen-induced and transplantable mammary tumor models [4]. A number of biochemical and molecular mechanisms to explain its anticarcinogenic effect have been suggested [4], but at the cellular level, elimination of preneoplastic and neoplastic cells by apoptosis is a key event [5,6].

Apoptosis is a form of programmed cell death that is usually dependent on the activity of caspases, which are synthesized as proproteins, and then cleaved to active proteases after the

* Corresponding author. Fax: +1-716-845-5865. E-mail: Margot.Ip@RoswellPark.org

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

cell receives an apoptotic signal. In the mitochondrial pathway, release of cytochrome c from the mitochondria is a key initial step, triggering the formation of the apoptosome, a complex of cytochrome c, Apaf-1, dATP and procaspase-9. As a result, caspase-9 is activated, initiating the downstream apoptotic cascade. Release of cytochrome c, which is essential [7], although not sufficient for this apoptotic pathway [8], is controlled by members of the Bcl-2 family. The proapoptotic members Bax and Bak are believed to form oligometric channels in the mitochondrial outer membrane which facilitate the exit of cytochrome c to the cytosol [9]. In contrast, antiapoptotic family members such as Bcl-2 and Bcl-X_L are thought to sequester proapoptotic proteins, prevent the formation of protein-conducting channels, and hence inhibit apoptosis [10].

Previously, we demonstrated that Bcl-2 expression was decreased in mammary gland preneoplastic lesions of rats fed CLA [5]. In the current study, we used the p53 mutant TM4t mouse mammary tumor cell line [11] as an *in vitro* model to investigate the role played by Bcl-2 in CLA-induced apoptosis. This cell line forms a well differentiated adenocarcinoma when injected into the mammary gland of syngeneic BALB/c mice (Ou, L. and Ip, M.M., unpublished), and thus represents an excellent model for studying the mechanisms by which CLA inhibits the *de novo* development of mammary tumors. Importantly, this cell line also allowed us to determine if CLA could induce apoptosis in the presence of a mutant p53, and to determine the role of the mitochondrial pathway in the apoptotic response.

Materials and methods

Cell Culture

TM4t mouse mammary tumor cells, derived from tumorigenic outgrowths of the TM4 preneoplasia cell line [12], were cultured in DMEM-F12 supplemented with 2% adult bovine serum, 10 µg/ml insulin, 5 ng/ml EGF and 5 µg/ml gentamicin. t10,c12-CLA, ~98% pure, was obtained from Larodan Fine Chemicals (Malmö, Sweden) and prepared as the sodium salt [13] for use in culture. For each experiment, 5×10⁵ cells were plated in 100 mm dishes (5×10⁴ cells/ml of medium) and cultured overnight to allow attachment, prior to addition of CLA. Viable cell number was measured by trypan blue dye exclusion. TM4t/Neo and TM4t/Bcl-2 cells were obtained by transfection of parental TM4t cells with a vector containing a neomycin resistance gene or the same vector containing the mouse Bcl-2 (wild type) cDNA under the control of the CMV promoter (Bcl-2 cDNA expression kit, Upstate Biotechnology, Lake Placid, NY). The LipofectamineTM 2000 kit was used for the transfection, and 400 µg/ml G418 was used for selection.

Annexin V-Biotin and propidium iodide (PI) Double Staining

Apoptosis was measured in asynchronously growing cells using the RAPID protocol in the Annexin V-Biotin Apoptosis Detection kit (Oncogene Research Products, Boston, MA). The samples were analyzed by flow cytometry using the FACScan flow cytometer (Becton, Franklin Lakes, NJ), and apoptosis quantitated using Winlist Version 5.0 software (Verity Software House, Topsham, ME). The cell cycle was determined by DNA content as measured by PI staining and analyzed using ModFit 3.0 software (Verity).

Antibodies

Primary antibodies against cleaved poly (ADP-ribose) polymerase (PARP) (#9544), caspase-3 (#9662), cleaved caspase-9 (#9504) and Bax (#2772) were purchased from Cell Signaling Technology (Danvers, MA). Bak (#D16601-6), actin (#CP01) and voltage-dependent anion channel (VDAC; #529532) antibodies were purchased from Calbiochem (La Jolla, CA). The Bcl-2 antibody (#D038-3) was purchased from Medical & Biological Laboratories CO. (Woburn, MA). Cytochrome c antibody (SC-13156) was purchased from Santa Cruz

Biotechnology (Santa Cruz, CA). The antibodies were used at the dilutions recommended by each of the companies.

Immunoblotting

After CLA treatment, attached and floating cells were collected and lysed with 300 μ l lysis buffer [1% (v/v) Triton X-100, 50 mM Tris pH 8.0, 150 mM NaCl, 2 mM EDTA, 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 5 mM sodium vanadate, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 20 μ g/ml leupeptin, 100 μ g/ml soybean trypsin inhibitor, 120 μ g/ml pepabloc, 1 μ M DTT, 1 μ g/ml pepstatin A, 10 μ g/ml aprotinin, 1 μ M NaF]. Protein concentration was measured using the Dc Protein Assay (Bio-Rad Laboratories). Lysates were separated by electrophoresis on a SDS/12% polyacrylamide gel, and then transferred to PVDF Western Blotting Membranes (Roche, Indianapolis, IN). Primary antibodies were diluted in blocking buffer or 5% BSA (bovine serum albumin), according to the data sheet provided with the antibodies.

Separation of mitochondria and cytosol

Cells were cultured without or with various concentrations of t10,c12-CLA for 3 days. Separation of mitochondrial and cytosolic fractions was according to the method reported by Yang et al [14].

Statistical analysis

ANOVA followed by Bonferroni's procedure was used to determine statistical differences between groups. A value of $P < 0.05$ was considered statistically significant. Data are presented as mean $\pm$ SEM.

Results

CLA induces apoptosis and cell cycle arrest of TM4t mammary tumor cells

Treatment of cells with t10,c12-CLA for 3 days resulted in a decrease in viable cell number from $7.3 \pm 1.4 \times 10^6$ in the control to $3.5 \pm 0.2 \times 10^6$, $1.3 \pm 0.2 \times 10^6$, and $1.0 \pm 0.1 \times 10^6$ in the 10, 20 and 40 μ M CLA groups, respectively. Microscopic observation of CLA-treated cells suggested the occurrence of apoptosis (Fig. 1A). Notably, cells rounded up and lifted from the plate, and cell shrinkage and blebbing were evident (Fig. 1A). To determine if CLA were indeed inducing apoptosis, the annexin V-PI double staining flow cytometric technique was utilized. As seen in Fig. 1B, CLA induced apoptosis in a concentration-dependent fashion, with the percentage of apoptotic cells increasing from 7.6% at 10 μ M to 25.4% at 40 μ M. Fig. 1C shows that CLA also increased the percentage of cells in G₁, accompanied by a corresponding reduction in the S and G₂/M phases. However, this cell cycle arrest effect of CLA was modest and observed only at the highest CLA concentration tested (40 μ M).

CLA induces cleavage of caspases and the release of cytochrome c into the cytosol

Consistent with the annexin V-PI double staining data, CLA induced cleavage of caspase-3 (Fig. 2A,B), a signature executioner caspase. Caspase-9, an initiator caspase, was also cleaved in CLA-treated cells (Fig. 2A,B). This suggested that CLA may induce apoptosis through the mitochondrial apoptotic pathway, a notion supported by the observation that CLA treatment induced a concentration and time-dependent release of cytochrome c into the cytosol (Fig. 2C,D).

CLA decreases cellular and mitochondrial levels of Bcl-2

To determine their potential involvement in the CLA effect, we examined the expression of Bak, Bax and Bcl-2 in whole cell lysates. Since Bcl-2 is an integral membrane protein located

mainly on the outer membrane of mitochondria, we also determined whether CLA induced changes in mitochondrial Bcl-2. When TM4t cells were treated with CLA for 3 days, Bak and Bax protein levels were not substantially altered by CLA treatment (Fig. 3A,B). However, Bcl-2 protein levels were markedly decreased both in total cell lysates (Fig. 3C), and in mitochondrial fractions (Fig. 3D). Together, these results suggested that CLA might induce apoptosis by downregulating Bcl-2.

Overexpression of Bcl-2 attenuates CLA-induced apoptosis

To further determine the role of Bcl-2 in CLA-induced apoptosis, we asked whether apoptosis could be attenuated by Bcl-2 overexpression. TM4t cells stably overexpressing Bcl-2 (TM4t/Bcl-2) and TM4t/Neo cells were treated with 40 μ M CLA for 3 days. Using PI-Annexin V double staining flow cytometry, we found a significantly reduced apoptosis in CLA-treated TM4t/Bcl-2 cells, when compared with the TM4t/neo cells (Fig. 4A). Furthermore, cleavage of PARP and caspase-3 was markedly reduced in TM4t/Bcl-2 cells treated with CLA (Fig. 4B). A similar observation was made in two different stable clones. These data suggest that the overexpression of Bcl-2 in TM4t cells is sufficient to attenuate CLA-induced apoptosis.

Discussion

Previous studies demonstrated that dietary CLA could induce apoptosis of p53 wild type preneoplastic lesions in the rat mammary gland [5], but not of the normal mammary epithelium nor in p53 wild type DMBA-induced mammary tumors [15]. *In vitro*, t10,c12-CLA was shown to induce apoptosis of p53 wild type TM12 mouse mammary hyperplasia cells (Lisafeld, B., Ou, L. and Ip, M.M., unpublished) and HCT-116 colorectal adenocarcinoma cells [6]. The current studies demonstrate that the t10,c12 isomer of CLA induces apoptosis of TM4t mouse mammary tumor cells, in which the p53 gene has a deletion from 524 to 544 on one allele and a mutation at 571 (C→G) on the second [11]. This mutation inhibits the normal tumor suppressor function of p53, at least in part, since tumorigenesis from implanted TM4 mammary hyperplasia cells is accelerated compared to that from the corresponding p53 wild type cells; moreover, pre-tumorigenic outgrowths of the TM4 cell line are insensitive to radiation-induced apoptosis [16]. Our data therefore suggest that wild type p53 is not required for CLA-induced apoptosis. A possible explanation for this is our recent observation that CLA induces CEBP-homologous protein (CHOP) in TM4t cells (Ou, L. and Ip, M.M., unpublished). This protein, which can be induced in a p53-independent manner [17], has been shown to down-regulate Bcl-2 [18], and may explain the Bcl-2 loss in our study. Elevated CHOP levels may also contribute to the CLA-induced apoptosis, a hypothesis we are currently evaluating.

In contrast to the marked induction of apoptosis in TM4t cells, CLA had only a modest effect on cell cycle progression, and indeed an inhibition of G1-S progression was only seen at the highest concentration of CLA examined (40 μ M). This observation is consistent with that reported in other p53 mutant cell lines, namely HT-29 and SW-480 human colon cancer cells [19,20], strengthening the argument that the effects of CLA do not require wild type p53. Together these data support the notion that CLA may be a promising candidate for prevention or therapy of breast cancer, which arises, in a significant proportion of cases, from cells with a mutated p53 [21,22].

Since we found that a stimulation of apoptosis was a greater determinant of CLA efficacy in decreasing TM4t cell number than an inhibition of cell proliferation, we focused our efforts on elucidating the mechanism by which CLA induced apoptosis. t10,c12-CLA stimulated release of cytochrome c from the mitochondria to the cytosol, decreased mitochondrial Bcl-2 levels, and activated the caspase-9/caspase-3 proteolytic cascade. This demonstrates activation of the intrinsic mitochondrial apoptotic pathway, an effect that was time- and concentration-dependent. Our data confirm and extend previous studies in colon cancer and hepatoma cells

which reported that this isomer stimulated the activities of caspases-3 and -9 [23,24] and release of cytochrome c into the cytosol [23,25]. Importantly, our studies point to a critical role for Bcl-2 in mediating the apoptotic effects of CLA in TM4t cells. Previous Western blot studies indicated that t10,c12-CLA induced either a modest 20–25% loss, or no loss, of total cellular Bcl-2 protein in cultured colon, prostate or breast cancer cells [23,26,27]. However, using mitochondrial fractions, we found that t10,c12-CLA induced a marked concentration-dependent decrease of mitochondrial Bcl-2 after 3 days of treatment (10% of control levels relative to VDAC at 40 μ M). This suggested that loss of Bcl-2 might be an important mechanism by which CLA induced apoptosis, a notion further supported by our observation that overexpression of Bcl-2 in TM4t cells almost completely blocked CLA-induced apoptosis. The decreased Bcl-2 is physiologically relevant based on our previous *in vivo* observation that dietary CLA reduced Bcl-2 levels and increased apoptosis in premalignant lesions in the rat mammary gland [5].

No effects of t10,c12-CLA were observed on levels of Bax or Bak, nor on translocation of Bax to the mitochondria. However, loss of Bcl-2 would relieve a critical restraint, allowing activation of Bax and Bak and thus formation of the protein channel through which proapoptotic factors such as cytochrome c are released into the cytosol [28,29]. Truncated Bid (tBid), a known amplifier of the mitochondrial apoptotic pathway [30], may collaborate in CLA-induced apoptosis, since we found that caspase-8, the enzyme responsible for the proteolytic cleavage of Bid to tBid, was activated by t10,c12-CLA in TM4t cells in a time- and concentration-dependent manner (data not shown). In support of this theory, Yamasaki *et al* [25] reported that full length Bid was decreased in t10,c12-CLA-treated hepatoma cells, although this group did not show whether tBid was increased concurrently.

The t10,c12 isomer of CLA is only one of several CLA isomers. It and the predominant natural isomer c9,t11-CLA, have been studied most extensively since they are each available commercially at relatively high purity. Although both CLA isomers were found to be equally efficacious in inhibiting rat mammary carcinogenesis [31] and angiogenesis [32], t10,c12-CLA is the more potent isomer in most *in vitro* studies. In TM4t mammary tumor cells, for example, we found that t10,c12-CLA is approximately five times more potent than c9,t11-CLA in decreasing viable cell number. Moreover, concentrations of c9,t11-CLA up to 120 μ M only minimally induce apoptosis of TM4t cells, as evidenced by the induction of PARP cleavage (Ou, L. and Ip, M.M., unpublished), whereas the current study demonstrates that 20 μ M t10,c12-CLA induces apoptosis in the same cells. Since dietary CLA is incorporated into mammary adipocyte triglycerides, it may be possible to achieve an effective local concentration of c9,t11-CLA in the *in vivo* setting. This is an important consideration, given the potentially serious side effects of t10,c12-CLA seen in some, although not all studies, including our recent observation that t10,c12-CLA stimulates mammary tumorigenesis and metastasis in mice which overexpress erbB2 in the mammary epithelium ([33] and references within). Work is continuing in our laboratory on the mechanism by which the individual CLA isomers exert their effects, with the goal of defining an optimal CLA strategy for chemoprevention.

Acknowledgements

This study was supported by NIH CA61763, by pre-doctoral DOD Grant W81XWH-06-1-0288 (LO), and by the shared resources of the NCI Roswell Park Cancer Center Support Grant CA 16056. We are grateful to Dr. Daniel Medina (Baylor College of Medicine, Houston, TX) for providing us with the TM4t mammary tumor cell line.

References

1. Gaullier JM, Halse J, Høy K, Kristiansen K, Fagertun H, Vik H, Gudmundsen O. Conjugated linoleic acid supplementation for 1 y reduces body fat mass in healthy overweight humans. *American Journal of Clinical Nutrition* 2004;79:1118–1125. [PubMed: 15159244]

2. Tricon S, Burdge GC, Kew S, Banerjee T, Russell JJ, Grimble RF, Williams CM, Calder PC, Yaqoob P. Effects of cis-9,trans-11 and trans-10,cis-12 conjugated linoleic acid on immune cell function in healthy humans. *Am J Clin Nutr* 2004;80:1626–1633. [PubMed: 15585778]
3. Moloney F, Yeow TP, Mullen A, Nolan JJ, Roche HM. Conjugated linoleic acid supplementation, insulin sensitivity, and lipoprotein metabolism in patients with type 2 diabetes mellitus. *Am J Clin Nutr* 2004;80:887–895. [PubMed: 15447895]
4. Ip MM, Masso-Welch PA, Ip C. Prevention of mammary cancer with conjugated linoleic acid: Role of the stroma and the epithelium. *J Mamm Gland Biol Neopl* 2003;8:103–118.
5. Ip C, Ip MM, Loftus T, Shoemaker SF, Shea-Eaton W. Induction of apoptosis by conjugated linoleic acid in cultured mammary tumor cells and premalignant lesions of the rat mammary gland. *Cancer Epidemiol Biomarkers Prev* 2003;9:689–696. [PubMed: 10919739]
6. Lee SH, Yamaguchi K, Kim JS, Eling TE, Safe S, Park Y, Baek SJ. Conjugated linoleic acid stimulates an anti-tumorigenic protein NAG-1 in an isomer specific manner. *Carcinogenesis* 2006;27:972–981. [PubMed: 16286461]
7. Matapurkar A, Lazebnik Y. Requirement of cytochrome c for apoptosis in human cells. *Cell Death Differ* 2006;13:2062–2067. [PubMed: 16729029]
8. Di Sano F, Ferraro E, Tufi R, Achsel T, Piacentini M, Cecconi F. Endoplasmic reticulum stress induces apoptosis by an apoptosome-dependent but caspase 12-independent mechanism. *J Biol Chem* 2006;281:2693–2700. [PubMed: 16317003]
9. Burlacu A. Regulation of apoptosis by Bcl-2 family proteins. *J Cell Mol Med* 2003;7:249–257. [PubMed: 14594549]
10. Borner C. The Bcl-2 protein family: sensors and checkpoints for life-or-death decisions. *Mol Immunol* 2003;39:615–647. [PubMed: 12493639]
11. Jerry DJ, Ozbun MA, Kittrell FS, Lane DP, Medina D, Butel JS. Mutation in p53 are frequent in the preneoplastic stage of mouse mammary tumor development. *Cancer Res* 1993;53:3374–3381. [PubMed: 8324748]
12. Kittrell FS, Oborn CJ, Medina D. Development of mammary preneoplasias in vivo from mouse mammary epithelial cell lines in vitro. *Cancer Res* 1992;52:1924–1932. [PubMed: 1372532]
13. Ip MM, Masso-Welch PA, Shoemaker SF, Shea-Eaton WK, Ip C. Conjugated linoleic acid inhibits proliferation and induces apoptosis of normal rat mammary epithelial cells in primary culture. *Exp Cell Res* 1999;250:22–34. [PubMed: 10388518]
14. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, Peng TI, Jones DP, Wang X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 1997;275:1129–1132. [PubMed: 9027314]
15. Thompson H, Zhu ZJ, Banni S, Darcy K, Loftus T, Ip C. Morphological and biochemical status of the mammary gland as influenced by conjugated linoleic acid: Implication for a reduction in mammary cancer risk. *Cancer Res* 1997;57:5067–5072. [PubMed: 9371504]
16. Meyn RE, Stephens LC, Mason KA, Medina D. Radiation-induced apoptosis in normal and pre-neoplastic mammary glands *in vivo*: Significance of gland differentiation and p53 status. *Int J Cancer* 1996;65:466–472. [PubMed: 8621229]
17. Li J, Lee B, Lee AS. Endoplasmic reticulum stress-induced apoptosis: multiple pathways and activation of p53-up-regulated modulator of apoptosis (PUMA) and NOXA by p53. *J Biol Chem* 2006;281:7260–7270. [PubMed: 16407291]
18. McCullough KD, Martindale JL, Klotz LO, Aw TY, Holbrook NJ. Gadd153 sensitizes cells to endoplasmic reticulum stress by down-regulating Bcl2 and perturbing the cellular redox state. *Mol Cell Biol* 2001;21:1249–1259. [PubMed: 11158311]
19. Cho HJ, Kim EJ, Lim SS, Kim MK, Sung MK, Kim JS, Park JH. Trans-10,cis-12, not cis-9,trans-11, conjugated linoleic acid inhibits G1-S progression in HT-29 human colon cancer cells. *J Nutr* 2006;136:893–898. [PubMed: 16549447]
20. Lim DY, Tyner AL, Park JB, Lee JY, Choi YH, Park JH. Inhibition of colon cancer cell proliferation by the dietary compound conjugated linoleic acid is mediated by the CDK inhibitor p21CIP1/WAF1. *J Cell Physiol* 2005;205:107–113. [PubMed: 15880444]
21. Soussi T, Beroud C. Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer* 2001;1:233–240. [PubMed: 11902578]

22. Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, Pawitan Y, Hall P, Klaar S, Liu ET, Bergh J. An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. *Proc Natl Acad Sci U S A* 2005;102:13550–13555. [PubMed: 16141321]
23. Miller A, Stanton C, Devery R. *Cis* 9, *trans* 11- and *trans* 10, *cis* 12-conjugated linoleic acid isomers induce apoptosis in cultured SW480 cells. *Anticancer Research* 2002;22:3879–3887. [PubMed: 12553008]
24. Yamasaki M, Chujo H, Koga Y, Oishi A, Rikimaru T, Shimada M, Sugimachi K, Tachibana H, Yamada K. Potent cytotoxic effect of the *trans*10, *cis*12 isomer of conjugated linoleic acid on rat hepatoma dRLh-84 cells. *Cancer Letters* 2002;188:171–180. [PubMed: 12406562]
25. Yamasaki M, Miyamoto Y, Chujo H, Nishiyama K, Tachibana H, Yamada K. *Trans*10, *cis*12-conjugated linoleic acid induces mitochondria-related apoptosis and lysosomal destabilization in rat hepatoma cells. *Biochim Biophys Acta* 2005;1735:176–184. [PubMed: 16005259]
26. Ochoa JJ, Farquharson AJ, Grant I, Moffat LE, Heys SD, Wahle KWJ. Conjugated linoleic acids (CLAs) decrease prostate cancer cell proliferation: different molecular mechanisms for *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers. *Carcinogenesis* 2004;25:1185–1191. [PubMed: 14976130]
27. Majumder B, Wahle KW, Moir S, Schofield A, Choe SN, Farquharson A, Grant I, Heys SD. Conjugated linoleic acids (CLAs) regulate the expression of key apoptotic genes in human breast cancer cells. *FASEB J* 2002;16:1447–1449. [PubMed: 12205043]
28. Danial NN, Korsmeyer SJ. Cell death: critical control points. *Cell* 2004;116:205–219. [PubMed: 14744432]
29. Adams JM, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 2007;26:1324–1337. [PubMed: 17322918]
30. Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA, Green DR, Newmeyer DD. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 2005;17:525–535. [PubMed: 15721256]
31. Ip C, Dong Y, Ip MM, Banni S, Carta G, Angioni E, Murro E, Spada S, Melis MP, Saebo A. Conjugated linoleic acid isomers and mammary cancer prevention. *Nutr Cancer* 2002;43:52–58. [PubMed: 12467135]
32. Masso-Welch PA, Zangani D, Ip C, Vaughan MM, Shoemaker SF, McGee SO, Ip MM. Isomers of conjugated linoleic acid differ in their effects on angiogenesis and survival of mouse mammary adipose vasculature. *J Nutr* 2004;134:299–307. [PubMed: 14747664]
33. Ip MM, McGee SO, Masso-Welch PA, Ip C, Meng X, Ou L, Shoemaker S. The t10,c12 isomer of conjugated linoleic acid stimulates mammary tumorigenesis in transgenic mice overexpressing erbB2 in the mammary epithelium. *Carcinogenesis* 2007;28:1269–1276.10.1093/carcin/bgm018 [PubMed: 17259656]

Figure 1.

CLA induces apoptosis and G₁ arrest of p53 mutant TM4t mammary tumor cells. (A) Light microscopy of TM4t cells treated with 0, 10, 20 or 40 μ M CLA for 3 days. Photographs were taken under the 10x objective. An arrow points to cells undergoing apoptosis. (B) Cells were treated with 0, 10, 20 or 40 μ M CLA for 3 days, and apoptosis evaluated using Annexin V-Biotin and PI double staining flow cytometry. Both early (annexin V positive, PI negative) and late (annexin V positive, PI positive) apoptotic cells were counted. Values are means $\pm$ SEM (n=6 separate experiments). Means without a common letter differ ($P<0.05$). (C) Cell cycle distribution was examined by flow cytometry. Values are means $\pm$ SEM (n=4 separate experiments). * Significantly ($P<0.05$) different from others within its group.

Figure 2.

CLA induces cleavage of caspases and the release of cytochrome c into the cytosol. (A and B) TM4t mammary tumor cells were treated with different concentrations of t10,c12-CLA for 3 days or 40 μ M CLA for the indicated times, and then evaluated by Western blot analysis. (C and D) TM4t mammary tumor cells were treated with different concentrations of CLA for 3 days or 40 μ M CLA for the indicated times, and the cytosol isolated. The release of cytochrome c into the cytosol was examined by Western blot analysis. Actin was used as a control for protein loading. Each Western blot is representative of 2-3 separate experiments.

Figure 3.

CLA does not alter Bax or Bak, but decreases Bcl-2. (A, B and C) Effect of CLA on the levels of Bak, Bax and Bcl-2 proteins in total cell lysates. Cells were treated with different concentrations of CLA for 3 days, then harvested and analyzed by Western blot. Actin was used as a control for protein loading. (D) Effect of CLA on the levels of Bcl-2 and Bax in cytosolic and mitochondrial fractions. Cells were treated with different concentrations of CLA for 3 days. VDAC, a mitochondrial membrane protein, was used as a mitochondrial loading control. Each Western blot is representative of 2-3 separate experiments.

Figure 4.

Bcl-2 overexpression attenuates CLA-induced apoptosis. (A) Effect of overexpression of Bcl-2 on induction of apoptosis by CLA. Cells were cultured without or with 40 μ M CLA for 3 days. Apoptosis was determined using an annexin V-PI double staining flow cytometric technique. Values are means $\pm$ SEM (n=3 separate experiments). Means without a common letter differ ($P<0.05$). (B) Effect of overexpression of Bcl-2 on induction of cleavage of caspase-3 and PARP. Cells were cultured without or with 40 μ M CLA for 3 days. PARP and caspase-3 proteins were detected in whole cell lysates by Western blot analysis. Actin was used as a control for protein loading.