Conjugated linoleic acids (CLAs) decrease prostate cancer cell proliferation: different molecular mechanisms for *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers

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The aims of this study were to examine the anti-proliferative effects of different concentrations of a commercial preparation of conjugated linoleic acids (CLA) mixture of isomers [*cis*-9, *trans*-11 CLA (*cis*9:*cis*11 CLA); *trans*-10, *cis*-12 CLA (50:50)] and their constituent isomers on PC-3, a human prostatic carcinoma cell line, and to study their effects on gene expression (mRNA and protein levels) of different enzymes and oncoproteins involved in oncogenesis and progression of prostate cancer. This includes pathways for arachidonic acid metabolism [cyclooxygenase 1 (COX-1), 2 (COX-2) and 5-lipoxygenase (5-LOX)], apoptosis (bcl-2) and cell cycle control (p21WAF1/Cip1). Our results indicate a significant decrease in *PC-3* proliferation elicited by CLA, although with high variability between isomers. The *trans*-10, *cis*-12 CLA was the most effective isomer (55% inhibition). This isomer was also able to decrease bcl-2 gene expression and to increase p21WAF1/Cip1 mRNA levels (60% increase at highest concentration). In contrast, *cis*-9, *trans*-11 had no effect on these proteins but had a clear effect on 5-LOX expression and to a lesser degree on COX-2 protein level isomers. In conclusion, the anti-proliferative effects on PC-3 of CLA mixture and their constituent isomers are not equivalent, due to the different pathways involved for individual isomers. *Trans*-10, *cis*-12 seems to work preferentially through modulation of apoptosis and cell cycle control, while *cis*9:*cis*11 CLA isomer affects arachidonic acid metabolism.

Introduction

Prostate cancer is the most commonly diagnosed and second leading cause of cancer mortality in men living in the western world (1) and it is increasing rapidly due, in part, to the increase in the number of older people (2,3). Despite the high incidence of this cancer, little is known about its aetiology. Multiple genetic and epigenetic factors have been implicated in the oncogenesis and progression of prostate cancer, much as for other cancers, and include alterations in arachidonic acid metabolism (4–6), decreased apoptosis and increased cell cycle progression (7–9). Surgery, chemo- and radiation therapy save many lives, but many men still develop metastatic prostate cancer, which progresses, in many cases, to hormone-refractory disease, which displays resistance to hormone and chemotherapy.

Conjugated linoleic acid (CLA) is a dietary fatty acid predominant in ruminant food products that has received considerable attention because of its anti-mutagenic and anticarcinogenic properties (14–17). CLA is the generic term of a group of positional and geometric isomers of the omega-6 essential fatty acid linoleic acid (LA). Experimental studies in animal models and cells have shown that unlike the parent LA, which stimulates tumour growth, CLA is an effective inhibitor of many human cancers, including prostate cancer (17,18). However, the cellular mechanisms by which CLAs elicit these anticancer effects are not clear at present. Proposed mechanisms include modulation of eicosanoids synthesis and signal transduction, up-regulation of genes dependent on the transcription factors peroxisome proliferator-activated receptor (PPAR), inhibition of DNA adduct formation induced by exposure to carcinogens and induction of apoptosis (14–17). In addition, recent findings suggest that not only does CLA affect many different pathways, but that individual isomers of CLA act differently and that some effects are induced and/or enhanced by these isomers apparently acting synergistically (15).

As a result of its inhibitory effects on tumorigenesis, CLA could be a novel dietary supplement for individuals with increased risk of prostate cancer or patients undergoing prostate cancer treatment. However, compared with other major cancers, such as breast or colon, the literature on CLA and prostate cancer is not extensive (18–20). A better understanding of the anticarcinogenic properties of CLA preparations and their constituent isomers in prostate cancer could prove to be a novel nutritional management and an adjunct therapy in this disease.

Our aims are: (i) to evaluate the effects of a commercial preparation of a CLA mixture of isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers in approximately a 50:50 ratio) and the individual isomers on the proliferation of prostate cancer cells in culture. (ii) To assess the effect of these fatty acids on gene expression (mRNA and protein levels) of different enzymes and oncoproteins involved in prostate cancer cell proliferation/progression. This includes enzymes of arachidonic
acid metabolism/eicosanoid synthesis like cyclooxygenase 1 (COX-1), cyclooxygenase 2 (COX-2) and 5-lipoxygenase (5-LOX), and the oncoproteins involved in apoptosis (bcl-2) and cell cycle control (p21[\textsuperscript{WAF1}]/Cip1), using the androgen-independent human prostate cancer cell line, PC-3. (iii) To determine whether individual isomers have the same regulatory effects on these enzymes and proteins as the mixture.

Materials and methods

Cell culture and treatment

Androgen-independent human prostate cancer cell line, PC-3, was obtained from the American Type Culture Collection (Rockville, MD). The cells were grown in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (Lonza Ltd, Watford, UK) and antibiotics. PC-3 cells were sub-confluent and were used as a control, CLA-free medium. When the cells were sub-confluent, the respective media were replaced with new fresh medium containing CLA in various concentrations. The modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to quantify cell viability/proliferation. It is a widely used ‘in vitro’ assay for the measurement of cell proliferation that determines a reduction in cell viability (which equates with a reduction in cell numbers and therefore proliferation), based on the reduction of the tetrazolium salt by actively growing cells to produce a blue formazan product.

The MTT assay was purchased from Sigma (Sigma-Aldrich, Poole, Dorset, UK) and standard manufacturer’s protocol was followed for cell incubations and spectrophotometric determination of purple formazan dye. Briefly, cells were incubated in 96 well multiplates in the appropriate complete medium with standardised densities/well for 24 h as a pre-incubation process. The medium was then replaced and replaced by medium containing different CLA concentrations. After 24 h of treatment the medium was removed and cells were allowed to grow for an additional 48 h in new, fresh medium without CLA. After this period of time, MTT was added to each well (20 μl of 5 mg/ml in PBS solution) and plates were incubated for 4 h at 37°C. Absorbance was measured at 570 nm. Data were analysed by plotting cell numbers versus absorbance, allowing quantification of changes in cell viability/proliferation.

Cell proliferation/viability assessment

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Statistical analysis

For all groups, data are presented as the mean of the different experiments ± standard error of the mean (SEM). Comparison of mean values was assessed by one-way ANOVA followed by a post hoc Duncan’s test. P values <0.05 were considered significant. Data were analysed using SPSS statistical software package (SPSS for Windows, 11.0.1, 2001; SPSS, Chicago, IL).

Results

Effect of CLA on COX-1, COX-2 and 5-LOX expression

Figure 1 shows the effects of different concentrations (25, 50, 100 and 150 μM) of a commercial preparation of CLA mixture of isomers (cis-9, trans-11 and trans-10, cis-12 isomers in approximately a 50:50 ratio) (CLA-mix) on gene expression (Figure 1A and B, mRNA and protein levels, respectively) of the different enzymes involved in arachidonic acid metabolism (COX-1, COX-2 and 5-LOX) in PC-3 cells. The CLA-mix did not elicit any effects on gene expression (neither mRNA nor protein levels) of the COX-1 and COX-2 enzymes at any concentrations. Only at the relatively high concentration of 150 μM was the CLA-mix able to decrease the levels of both mRNA (83.5 ± 2.1% of control) and protein (78.3 ± 7.6% of control) of 5-LOX significantly (P <0.05) with respect to the control values.

The effect of each individual isomer cis-9,11 CLA and trans-10, cis-12 CLA (trans-10, cis-12 CLA (t10,c12 CLA) on the mRNA levels of these enzymes are shown in Table I. Individual isomers, the same as the CLA-mix, did not show any significant effect on the levels of COX-1 and COX-2 mRNA at any of the concentrations

\[5\text{-CAAAGGCCCGCTCTACATCTTCTGCCTTAGTCTCA-3} \text{ and } 5\text{-CACGCCCCCATCGCATCCGGACCAGCGTGC-3}\] and \[5\text{-CAGCGCCCATACGATCCGGACCAGCGTGC-3}\] and \[5\text{-CAGCGCCCATACGATCCGGACCAGCGTGC-3}\] with \[\text{[12]}\text{PC}	ext{d}	ext{oxyctydine triphosphate by 5-end labelling using T4 polynucleotide kinase from New England Biolabs (Hitchin, UK). Membranes were then washed to remove non-specifically bound probe and specific hybridization was then detected by electronic autoradiography using a Canberra Packard InstantImager (Packard, Pangbourne, Berks, UK) and densitometric analysis. After analysis, individual membranes were stripped by washing in 0.1% (w/v) SDS for 5–10 min at 95°C before re-hybridization to other specific probes.

The housekeeping 18 S probe was used last in order to correct any variation between loading of RNA on the gel or transfer to the nylon membrane.

Western blot analysis (protein)

After 24 h of treatment, cells were lysed in buffer (20 mM Tris pH 7.5, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM sodium orthovanadate, 50 mM sodium β-glycerophosphate, 50 mM sodium fluoride, 1% (v/v) protease inhibitor cocktail (Sigma-Aldrich, Poole, Dorset, UK) and 1% (v/v) Triton X-100). The lysate was centrifuged at 14 000 r.p.m. and 4°C for 30 min, and the cleared supernatants were stored at −80°C until use. Protein concentrations were determined using the Bio-Rad (Hemstead, UK) assay.

Samples containing equal amounts of proteins (20 μg) were then subjected to electrophoretic fractionation on a 10% polyacrylamide gel under reducing conditions. Separated fractions were transferred onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia Biotech, Buckinghamshire, UK) by using a semi-dry transfer method (Transblot SD, Bio-Rad). Membranes were blocked with TBS-T (Tris-buffered saline-0% Tween 20) containing 5% non-fat milk and incubated for 1 h with the human-specific antibodies (COX-2 and 5-LOX from Transduction Laboratories, Becton Dickinson, Oxford, UK) and COX-1, bcl-2 and p21WAF1/Cip1 from Santa Cruz Biotechnology (Santa Cruz, CA); they were then diluted according to the manufacturer’s instructions. Following incubation with the antibodies, the membranes were washed three times for 5 min in TBS-T and incubated with the specific Horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotechnology) following the manufacturer’s instructions. After washing two times for 5 min with TBS-T and one time with TBS, specific proteins were detected using an enhanced chemiluminescence system (Super Signal, Pierce Chemical, Rockford, IL).

Ponceau S and Coomassie brilliant blue (Sigma-Aldrich, Poole, Dorset, UK) staining were used for markers of the protein loading and protein transfer control, respectively.
Only c9,t11 CLA at high concentrations (100 and 150 μM) showed a significant (P < 0.05) decrease of 5-LOX mRNA levels, about a 20 and 28% inhibition, respectively, with respect to the control values.

The effects of individual CLA isomers on COX-2 protein levels are given in Figure 2, only c9,t11 CLA at the highest concentration showed a decrease of COX-2 protein of ~20% of the control. The t10,c12 isomer elicited
an increase although it was not significant with respect to the control.

**Effect of CLA on bcl-2 and p21WAF1/Cip1 expression**

The CLA-mix did not elicit any effect on the levels of bcl-2 mRNA (Figure 3A) in PC-3. However, this fatty acid decreased the levels of bcl-2 protein (Figure 3B), although this decrease was only statistically significant ($P < 0.05$) at the highest concentration (81.1 ± 4.4% of control).

c9,11 CLA isomer, was the same as the CLA-mix and did not have any effect on bcl-2 mRNA levels at any of the concentrations used (Table II). In contrast to the CLA-mix, this isomer did not show any effect on bcl-2 protein level (Figure 4). The effect of the t10,c12 isomer was opposite to that of the CLA-mix and the c9,11 isomer and did elicit a significant decrease of bcl-2 mRNA levels (Table II) (18% inhibition) with respect to the control at 150 μM. This isomer also produced a decrease in the bcl-2 protein levels at 100 and 150 μM (77.6 ± 4.9% of control and 69.2 ± 8.8% of control, respectively), which were significantly different to the control values ($P < 0.05$).

p21WAF1/Cip1 mRNA levels were increased after 24 h of treatment with CLA-mix (Figure 3A), although only significant ($P < 0.05$) with respect to the control values at 100 and 150 μM (121.4 ± 6.1% of control and 130.7 ± 5.4% of control, respectively). The protein levels (Figure 3B) followed the same trend as the mRNA levels, with significant differences with respect to control values at 100 and 150 μM (132.6 ± 10.6% of control and 152.1 ± 14.7% of control, respectively).

In contrast to the CLA-mix, c9,11 CLA did not produce any effect on mRNA levels of p21WAF1/Cip1 at any concentration (Table II).
Effect of CLA on PC-3 proliferation (MTT assay)

The literature relating to CLA and its effects on prostate cancer is important if we want to develop suitable dietary supplements for cancer prevention or for patients undergoing cancer treatment.

Treatment with trans-10, cis-12 CLA elicited a significant increase in mRNA levels of p21\textsuperscript{WAF1/Cip1} (Table II) \((P < 0.05)\), with respect to the control values at 50, 100 and 150 \(\mu\)M (about a 23, 45 and 60% increase, respectively).

**Table II.** Effect of c9,t11 CLA and t10,c12 CLA isomers on mRNA levels of bcl-2 and p21\textsuperscript{WAF1/Cip1} in prostate cancer cell line PC-3

<table>
<thead>
<tr>
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<th>c9,t11 CLA</th>
<th>t10,c12 CLA</th>
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<tr>
<td></td>
<td>Control</td>
<td>25 (\mu)M</td>
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<tr>
<td>bcl-2 mRNA</td>
<td>100.0 ± 2.2</td>
<td>102.3 ± 3.5</td>
</tr>
<tr>
<td>p21\textsuperscript{WAF1/Cip1} mRNA</td>
<td>100.0 ± 6.6</td>
<td>112.5 ± 8.7</td>
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Values are expressed as mean \((n = 8)\) ± SEM (% of control). Asterisk indicates significant difference versus the appropriate control \((P < 0.05)\).

**Discussion**

Prostate cancer remains an enormous healthcare problem (1). As with other cancers, diet, particularly dietary fat, has been shown to be a major risk factor for prostate cancer (10–13). Identification of micro-and macro-nutrients, which could suppress the initiation and development of this cancer would provide important information for the development of preventive nutritional strategies or adjuvant treatments. In this sense, CLA has received a great deal of attention due to its reported anti-mutagenic and anticarcinogenic properties in animal models and cells in culture (14–17). The underlying molecular and cellular mechanisms by which CLA can elicit these anticancer effects are not clearly understood at present (14–17). In addition, CLA is the common term for a group of positional and geometric isomers of LA, which are thought to act independently or synergistically with one another (15).

The literature relating to CLA and its effects on prostate cancer is not extensive (18–20) and although it has been shown that CLA has anti-proliferative effects \textit{in vitro} in human prostate cancer cells and \textit{in vivo} in animal models of human prostate cancer (18–20), the underlying cell mechanisms are not well understood. Therefore, a better understanding of the molecular anticarcinogenic cell mechanisms affected by CLA mixtures and their constituent isomers in this type of cancer is important if we want to develop suitable dietary supplements for cancer prevention or for patients undergoing cancer treatment.

The colorimetric MTT assay was used to quantify cell viability and reduced proliferation, using a long-term incubation (1 day plus 2 days), which allows the determination of cells that remain viable and are capable of proliferating and those that remain viable but cannot proliferate and/or the detection of delayed programmed cell death (21). Our results show a decrease in PC-3 proliferation elicited by CLA, although significant differences were observed between the isomers studied.

However, the MTT assay, although widely used to measure cell proliferation, will only give general information about the viability of cells and little about the possible mechanisms that could change the cell capacity for proliferation and viability. Therefore, in an effort to try to clarify the possible basic mechanisms of these different anti-proliferative properties of CLA, we have studied for the first time, their effects on gene expression of different enzymes and proteins involved in arachidonic acid metabolism (COX-1, COX-2 and 5-LOX), apoptosis (bcl-2) and cell cycle (p21\textsuperscript{WAF1/Cip1}), all regarded...
as important pathways involved in the oncogenesis and progression of prostate cancer (4–9).

Increased eicosanoid biosynthesis in relation to enhanced prostate cancer development has been documented (4,6) making the enzymes involved in this metabolic pathway an attractive new target area for prostate cancer therapy. The pathways for eicosanoid synthesis have received a great deal of attention as possible targets for cancer inhibition, especially COX-2 (5,23,24), and lipoxygenase products, especially 5-LOX metabolites (6,25,26). Previous reports showed that inhibition or attenuation of COX-2 or 5-LOX expression or activities by various agents decreased prostate cancer proliferation by different pathways, such as apoptosis and cell cycle control (4–6,23–26). Modulation of eicosanoids synthesis has been proposed as one of the major mechanisms by which CLA can elicit its anticancer effects (14–17). However, information about CLA effects on gene expression of major enzymes involved in arachidonic acid metabolism to eicosanoids in cancer cells/tissues is extremely sparse.

In the experimental conditions reported here the mixture of CLA isomers used was only able to decrease the gene expression of 5-LOX at the highest concentration used and did not show any effect on cyclooxygenase gene expression (COX-1 and COX-2) at this concentration. This effect appeared to be largely due to the cis-9, trans-12 CLA isomer in the mix which, when used alone, decreased 5-LOX mRNA at 100 and 150 μM and COX-2 protein levels but was without effect on COX-2 mRNA at the highest concentration. This could be due to instability of the COX-2 mRNA (27). In contrast, trans-10, cis-12 did not have any inhibitory effect on gene expression of these enzymes. Even at the highest concentrations used there was actually a non-significant increase in COX-2 protein. These results, in part, agree with those obtained by other authors who showed differences between these isomers in their effects on eicosanoid synthesis. They indicate that the main reason for reduced product formation is likely to be reduced gene expression of the important COX and LOX enzymes (28,29).

Another proposed mechanism by which CLA exerts its anticancer effects is through the induction of apoptosis (14–17,30). Increased expression of Bcl-2, an important anti-apoptotic oncogene product, has been associated with the development of androgen-independent prostate cancer, and its down-regulation was associated with a decrease in prostate cancer proliferation (31). In our study, CLA was able to significantly decrease bcl-2 expression, which was regarded as an anticancer effect. This was apparently due largely to the trans-10, cis-12 isomer, which, when used individually, decreased bcl-2 mRNA and protein levels at 100 and 150 μM. Palombo et al. (19) showed recently that the trans-10, cis-12 isomer increased caspase 3 activity and induced apoptosis in PC-3 cells. However, our findings show an effect at a different level, specific oncogene expression, in the apoptotic cascade.

Finally, cell cycle checkpoints play a crucial role in maintaining tissue homeostasis. Loss of this control mechanism may contribute to the development of the malignant phenotype. Induction of p21WAF1/Cip1, a cyclin-dependent kinase inhibitor, which is capable of contributing to regulation of cell division (32), in prostate cancer cells, has been shown to reduce cell proliferation (33,34) and to induce apoptosis (33). This protein was shown by our group to be induced by CLA in breast cancer cells (30); however, there is no similar information for prostate cancer cells. In the present study we clearly show that CLA can also induce p21WAF1/Cip1 in prostate cancer cells and that the trans-10, cis-12 CLA isomer is apparently responsible for this effect, even at 50 μM.

In conclusion, our results show an anti-proliferative, anti-angiogenic effect of CLA on the androgen-independent human prostate cancer cell line PC-3. However, the anti-proliferative/anti-angiogenic effects of CLA mixtures and their constituent isomers on this cell line are not equivalent. These differences are apparently due to the different pathways modulated by the individual isomers. The trans-10, cis-12 CLA isomer appears to elicit the greatest effect and apparently works preferentially through modulation of genes involved in apoptosis and cell cycle control. The c9,t11 CLA isomer, in contrast, elicits its effects through regulation of genes involved in arachidonic acid metabolism and the subsequent attenuation of eicosanoid synthesis. These results are important because they highlight the fact that cancer is not a disease with a single aetiology for which there will be a single cure (7). CLA has clearly been implicated as a possible dietary factor for reducing both breast and prostate cancer through studies with animal models of the human disease and human cell studies in vitro (see above). If CLA is to be regarded as a suitable dietary adjuvant cancer therapy, it is important to understand the type of cancer cells

Fig. 5. Effect of CLA mixture (CLA-mix) and individual isomers (c9,t11 CLA and t10,c12 CLA) on PC-3 proliferation. Values are expressed as mean (n = 9) ± SEM (% of control). Asterisk indicates significant difference versus the appropriate control (P < 0.05).
targeted, the specific cell mechanisms affected and the type and concentration of CLA isomer to be used. In the case of androgen-independent prostate cancer cell it seems that the best CLA isomer for a possible adjuvant cancer therapy is the trans-10, cis-12 isomer, at least in our conditions.

Acknowledgements

J.J.O. was supported by a grant from the Ministry of Science and Technology of Spain and by the Scottish Executive Environmental Rural and Agriculture Department (SEERRAD).

References


Received October 9, 2003; revised February 4, 2004; accepted February 6, 2004