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Conjugated linoleic acid (CLA)-enriched milk fat inhibits growth and modulates CLA-responsive biomarkers in MCF-7 and SW480 human cancer cell lines

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Milk enriched in conjugated linoleic acid (CLA) was obtained from cows on pasture supplemented with full-fat rapeseeds (FFR; 2·26 g *cis* 9, *trans* 11 (*c*9, *t*11)-CLA/100 g fatty acid methyl esters) and full-fat soyabeans (1·83 g *c*9, *t*11-CLA/100 g fatty acid methyl esters). A control milk fat (1·69 g *c*9, *t*11-CLA/100 g fatty acid methyl esters) was obtained from cows fed on pasture only. The present study assessed the potency of the CLA-enriched milk fats to modulate biomarkers that had previously been observed to respond to *c*9, *t*11-CLA in the MCF-7 and SW480 cell lines. Cell numbers decreased ($P<0·05$) by up to 61 and 58 % following the incubation of MCF-7 and SW480 cells, respectively, for 4 d with milk fats (yielding CLA concentrations between 60·2 and 80·6 μM). The FFR milk fat, containing the highest CLA content, increased ($P<0·05$) [^{14}C]arachidonic acid (AA) uptake into the monoacylglycerol fraction of MCF-7 and SW480 cells while it decreased ($P<0·05$) uptake into the phospholipid fraction of the latter. This milk fat also decreased ($P<0·05$) [^{14}C]AA conversion to prostaglandin (PG) E_2 while increasing conversion to $PGF_2\alpha$ in both cell lines. All milk-fat samples increased ($P<0·05$) lipid peroxidation as measured by 8-epi-$PGF_2\alpha$ in both cell lines. In SW480 cells the milk-fat samples decreased ($P<0·05$) bcl-2 and cytosolic glutathione levels while increasing ($P<0·05$) membrane-associated annexin V levels. All milk-fat samples decreased ($P<0·05$) the expression of ras in SW480 cells. These data suggest that milk-fat CLA was effective at modulating synthetic CLA-responsive biomarkers.

Milk fat: Conjugated linoleic acid: Eicosanoids: Apoptosis

A growing number of dietary components with putative health-promoting properties are being identified and a new class of foods, so-called functional foods, has emerged (Milner, 1999). Research is unveiling milk fat as a source of bioactive components including minerals, peptides derived from milk proteins and lipid components (Boland *et al.* 2001; Calder, 2002). Of particular interest is the fatty acid conjugated linoleic acid (CLA), which has consistently been shown to inhibit chemically induced tumour development in animal models at a number of sites including skin (Belury *et al.* 1996), mammary gland (Ip *et al.* 1994), forestomach (Ha *et al.* 1990) and colon (Liew *et al.* 1995). In addition, cell culture studies have shown CLA to inhibit the growth of human cancer cell lines including those of the colon (Miller *et al.* 2001; Palombo *et al.* 2002), breast (Park *et al.* 2000; Miller *et al.* 2001) and prostate (Palombo *et al.* 2002) at micromolar concentrations. CLA is produced in ruminant animals and as a result milk fat is among the richest natural sources of CLA, with the *cis* 9, *trans* 11 (*c*9, *t*11)-CLA isomer being the predominant form, accounting for up to 90 % of total milk-fat CLA (Chin *et al.* 1992). While CLA is formed in ruminant animals as a first intermediate in the microbial biohydrogenation of linoleic acid (LA) by the action of a bacterial LA isomerase (Kepler & Tove, 1967), endogenous synthesis from *trans*-vaccenic acid (TVA) represents a more significant source of *c*9, *t*11-CLA in milk fat (Griinari *et al.* 2000).

Because of the health-promoting properties associated with CLA, attempts have been made to enrich its content in milk fat. Animal diet has a major impact on the CLA content of milk fat and several studies have shown that it can be elevated by modifying the dietary regimen of the dairy cow (Lawless *et al.* 1998; Chilliard *et al.* 2000), thus offering the possibility of producing CLA-enriched

Abbreviations: AA, arachidonic acid; *c*9, *t*11, *cis* 9, *trans* 11; CLA, conjugated linoleic acid; FFR, full-fat rapeseed; FFS, full-fat soyabean; GSH, reduced glutathione; LA, linoleic acid; MG, monoacylglycerol; PBST, PBS containing 0·1 % (v/v) Tween 20; PG, prostaglandin; TVA, *trans*-vaccenic acid.

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dairy products. Ip *et al.* (1999) have reported that feeding CLA-enriched butterfat (41 mg/g fat) alters mammary gland morphogenesis and reduces mammary cancer risk in rats by the same magnitude as a synthetic mixture of CLA isomers and a synthetic *c*9, *t*11-CLA preparation. The group consuming the butterfat consistently accumulated more total CLA in their tissues compared with the group consuming the synthetic CLA preparations and the authors suggested that TVA in the butterfat may have been a precursor for the endogenous synthesis of CLA. In a more recent study, this CLA-enriched butterfat effectively suppressed the proliferative activity and the expression of cell cycle regulating proteins (cyclin D_1 and A) in the developing rat mammary epithelium (Ip *et al.* 2001).

It has previously been reported that the mammary MCF-7 and colon SW480 cancer cell lines were sensitive to the cytotoxic effect of a CLA mixture of isomers and the *c*9, *t*11-CLA isomer (Miller *et al.* 2001). The CLA-induced cytotoxicity was related to an increase in lipid peroxidation (O'Shea *et al.* 1999; Miller *et al.* 2001). The cytotoxicity was also related to alterations in the mobilisation and metabolism of arachidonic acid (AA) (Miller *et al.* 2001), a reduction in the level of the anti-apoptotic bcl-2 protein, which triggered a cascade of events leading to apoptosis (Miller *et al.* 2002) and the modulation of ras expression (McGrath *et al.* 2001). CLA-enriched milk fat was previously reported to be taken up by MCF-7 cells and to be more effective than synthetic CLA at decreasing MCF-7 cell numbers and increasing lipid peroxidation after an 8 d incubation period (O'Shea *et al.* 2000). These effects were independent of the variable composition of the milk-fat samples, suggesting that CLA may be the active ingredient responsible for the cytotoxic effect in MCF-7 cells. The present study investigated whether fatty acids (LA, TVA and oleic acid) present in CLA-enriched milk fats could influence the growth of MCF-7 and SW480 cells. The potency of CLA-enriched milk fats to modulate CLA-responsive biochemical and molecular biomarkers previously observed in the MCF-7 and SW480 cell lines (Miller *et al.* 2001, 2002) was assessed. Specifically, the effects of CLA-enriched milk fats on: (1) AA uptake, distribution and conversion to eicosanoid classes in MCF-7 and SW480 cell lines; (2) lipid peroxidation; (3) apoptotic markers in the SW480 cells line (reduced glutathione (GSH) levels, membrane annexin V levels and bcl-2 expression); (4) levels of ras expression in SW480 cells were assessed.

Materials and methods

Cell culture conditions

Human breast (MCF-7) and colon (SW480) cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Culture media and supplements were purchased from Sigma-Aldrich Ireland Ltd. (Dublin, Republic of Ireland). The SW480 cells were maintained in Dulbecco's minimum essential medium supplemented with fetal bovine serum (5 %, v/v), 0·2 mM-L-glutamine, 1 mM-N′-(2-hydroxyethyl)piperazine-N-2-ethanesulfonic acid, and 1 unit each of penicillin and streptomycin/ml. The MCF-7 cells required an additional supplement of 10 mM-sodium pyruvate. Cells were grown in Falcon T-75 cm^2 flasks and maintained as previously described (O'Shea *et al.* 1999).

Milk-fat samples

The milk fats used in the present study were obtained following the supplementation of lactating dairy cows on pasture (control) or on pasture supplemented with full-fat rapeseeds (FFR) or full-fat soyabeans (FFS) for 33 and 34 d, respectively (Lawless *et al.* 1998). Table 1 summarises the concentrations of CLA, oleic acid, TVA and LA in the milk-fat samples. The full fatty acid composition was as described by O'Shea *et al.* (2000).

Quantification of cell numbers

Cells were seeded in six-well plates and the MCF-7 and SW480 cells were seeded at densities of 1×10^5/well and 5×10^4/well respectively. Cells were cultured for 24 h to allow the cells to attach to the substratum. The medium was then replaced with media containing either FFR milk fat, FFS milk fat or control milk fat at a concentration of 1 mg/ml to yield CLA concentrations of 60·2, 65·2 and 80·6 μM, respectively. Control wells were supplemented with equivalent volumes of ethanol (0·1 % final concentration, v/v). In a separate experiment, the milk-fat content of the medium was varied between 0·8 and 1·2 mg/ml to yield a CLA concentration of 71·3 μM in all milk-fat samples. Cells were also incubated in the presence of *c*9, *t*11-CLA (95 % pure; Natural ASA, Hovdebygda, Norway), LA, TVA or oleic acid (all 99 %; Sigma-Aldrich Ireland Ltd.) at concentrations similar to those found in the milk-fat samples. For all viability experiments, the cells were harvested in the presence of PBS containing 0·25 % (v/v) trypsin. Viable cell numbers were quantified using the trypan blue exclusion (0·4 %, w/v) assay.

Uptake of [^{14}C]arachidonic acid and conversion to eicosanoids

Cells were seeded in T-25 cm^2 flasks at a density of 2×10^5/flask and grown to 90 % confluency. The medium was then replaced with a medium containing [^{14}C]AA at 0·2 μCi along with the milk-fat samples control, FFS or FFR (all added at a milk-fat concentration of 1 mg/ml yielding CLA concentrations of 60·2, 65·2 and 80·6 μM, respectively) or an equivalent volume of ethanol. After 24 h incubation, the cells were harvested to determine the uptake of

Table 1. Fatty acid composition of milk-fat samples (g/100 g fatty acid methyl esters)

Fatty acid	Control milk fat	FFS milk fat	FFR milk fat
CLA	1·69	1·83	2·26
Trans-vaccenic acid	3·14	4·41	4·64
Oleic acid	20·28	23·37	26·92
Linoleic acid	1·25	4·28	1·69

FFS, full-fat soyabean; FFR, full-fat rapeseed; CLA, conjugated linoleic acid.

[^{14}C]AA and the media were collected. Total cellular lipids were extracted from the cell pellet and then separated into triacylglycerol, monoacylglycerol (MG) and phospholipid fractions as described by Miller *et al.* (2001). A sample of each fraction was counted in a Beckman LS6500 scintillation counter (Beckman Instruments, Galway, Republic of Ireland). Eicosanoids were extracted as described by Miller *et al.* (2001). Eicosanoid extracts were dried under N_2, re-dissolved in ethyl acetate separated using normal-phase TLC as described previously (Miller *et al.* 2001). Bands of prostaglandin (PG) E_2, $PGF_2\alpha$, and PGD_2 were removed from the TLC plates and placed in vials for counting by liquid scintillation. The isoprostane 8-epi-$PGF_2\alpha$ was extracted from the media as described by Watkins *et al.* (1999) and a competitive horseradish peroxidase enzyme-linked immunoassay kit (BIOXYTECH 8-Isoprostane assay system; Bio-Stat Ltd, Stockport, Cheshire, UK) was used to quantify 8-epi-$PGF_2\alpha$ levels according to the manufacturer's instructions. 5-Hydroperoxyeicosatetraenoate was measured using a colorimetric method developed by Waslidge & Hayes (1995).

Measurement of reduced glutathione and annexin V levels

Cells were seeded in T-75 cm^2 flasks at a density of 1×10^6 cells/flask and incubated for 24 h. The medium was then replaced with fresh media containing the milk-fat samples; control, FFS or FFR (1 mg/ml) as described earlier and incubated for 4 d, after which both floating and adherent cells were collected and pooled. Cells were then re-suspended in a PBS–phenylmethylsulfonylfluoride buffer (10 mM-sodium phosphate buffer (pH 7·2), 100 mM-NaCl, 0·2 mM-phenylmethylsulfonylfluoride) containing 0·1 mM-leupeptin and 0·2 μg aprotinin/ml (both from Sigma-Aldrich Ireland Ltd.) as protease inhibitors, sonicated on ice and centrifuged at 100 000 ***g*** for 1 h at 4°C. The supernatant fraction (cytosolic fraction) was analysed for GSH levels according to the method of Hissin & Hilf (1976). The pellet was re-suspended in a lysis buffer (10 mM-sodium phosphate buffer (pH 7·2), 0·2 mM-phenylmethylsulfonylfluoride and 100 mM-NaCl) containing 0·1 mM-leupeptin and 0·2 μg aprotinin/ml and incubated on ice for 20 min followed by centrifugation at 15 000 ***g*** for 15 min at 4°C. The resulting supernatant fraction (membrane fraction) was analysed for annexin V content using an Annexin V ELISA kit (Alexis Biochemicals, San Diego, CA, USA) according to the manufacturer's instructions. The GSH and annexin V concentrations of the samples were determined from standard curves and expressed relative to the protein content, as determined using the Bio-Rad protein assay (Biorad, Hemel Hempstead, Herts, UK).

Measurement of bcl-2 and ras expression

SW480 cells were seeded at 5×10^6 cells/150 cm^2 flask and were cultured for 24 h to allow the cells to attach to the substratum. The medium was then replaced with fresh media containing the milk-fat samples; control, FFS or FFR (1 mg/ml). After 4 d of incubation, both floating cells and adherent cells were collected and pooled. Cells were washed twice in ice-cold PBS and re-suspended in the lysis buffer (described earlier). Lysates were sonicated using a Vibra Cell VC502 (Sonics, Newtown, CT, USA) on ice and concentrated using Microcons® (Millipore, Cork, Republic of Ireland). Protein concentrations were determined using the Bio-Rad protein assay (Biorad). SDS-PAGE and Western blots were performed essentially by the methods of Laemmli (1970) and Towbin *et al.* (1979), respectively. Lysates containing approximately 70 μg protein were solubilised in a sample buffer (10 % (w/v) SDS, 600 mM-tri(hydroxymethyl)-aminomethane-HCl (pH 6·7), and 50 % (w/v) glycerol) containing β-mercaptoethanol and 50 μg bromophenol blue/ml. Samples were boiled for 2 min and proteins resolved by electrophoresis and blotted onto Hybond enhanced chemiluminescence membrane (Amersham, Little Chalfont, Buckinghamshire, UK) in a Trans-blot Electrophoretic transfer cell (Biorad). Blots were stained with Ponceau S solution (0·2 %, w/v) to ensure the transfer of proteins was complete and to determine if equivalent amounts of protein were loaded in each lane. The blots were destained with PBS containing 0·1 % (v/v) Tween 20 (PBST) and blocked with 5 % (w/v) non-fat dry milk dissolved in PBST. Blots were then incubated with monoclonal antibodies: anti-bcl-2 diluted to 1:1000 (Sigma-Aldrich Ireland Ltd.) or anti-ras diluted to 1:40 (Oncogene Science, Manhasset, NY, USA) in PBST containing 0·5 % (w/v) non-fat dry milk. Blots were washed extensively in PBST and re-incubated with a horseradish peroxidase-linked secondary antibody (Amersham) diluted 1:2000 in PBST containing 0·5 % (w/v) non-fat dry milk. The blots were then thoroughly washed in excess PBST and probed with the Super Signal detection system (Pierce, Rockford, IL, USA) and exposed to autoradiography films (Amersham) according to the manufacturer's instructions. Densitometry (using NIH Image software; Research Services Branch, Bethesda, MD, USA) was performed on Ponceau S scans and autoradiographs.

Statistical analysis

Three independent experiments were performed in triplicate. One-way ANOVA, followed by *post hoc* testing by Tukey's honestly significant difference test, was used to analyse the data. For treatments with more than one factor, factorial ANOVA followed by *post hoc* testing by Tukey's honestly significant difference test was carried out.

Results

The effects of conjugated linoleic acid-enriched milk fat on cell viability

MCF-7 and SW480 cells were incubated for 4 d in the presence of milk fat (1 mg/ml) to yield CLA concentrations in the range from 60·2 to 80·6 μM. This allowed the examination of the effect of increasing milk-fat CLA concentration while the milk fat content was kept constant. A previous study had shown that there was a proportional increase in CLA uptake from these milk fats by MCF-7 cells (O'Shea *et al.* 2000), suggesting a cellular lipolytic competence for milk-fat triacylglycerols. Cell numbers following 4 d

of incubation with all three milk fats were significantly ($P<0{\cdot}05$) lower than the number of untreated control cells (Figs. 1 (A) and 1 (B)). A dose-dependent decrease in cell number was observed with increasing CLA content in the milk fats, with both FFS and FFR being significantly ($P<0{\cdot}05$) more potent than the control milk fat. Maximal growth inhibition of 61 and 58 % occurred in the MCF-7 and SW480 cells, respectively, following treatment with the highest milk-fat CLA concentration. A significant ($P<0{\cdot}05$) inhibitory effect on cell number was obtained following the incubation of both cell lines with *c*9, *t*11-CLA at 60·2, 65·2 and 80·6 μM, representing the concentrations present in the control, FFS and FFR milk fats, respectively (Figs. 1 (A) and 1 (B)). The respective percentages by which cell viability was reduced following the addition of *c*9, *t*11-CLA to the medium were 43, 46 and 49 in the MCF-7 cell line and 41, 44 and 50 in the SW480 cell line. In the MCF-7 cell line, milk fat CLA (60·2 and 65·2 μM) was significantly ($P<0{\cdot}05$) more effective at decreasing cell numbers when compared with the synthetic *c*9, *t*11-CLA at the same concentrations. In the SW480 cell line no significant difference was observed between the milk-fat CLA and synthetic *c*9, *t*11-CLA treatments suggesting that lipase activities may differ between cell lines and may possibly be rate limiting in the MCF-7 cell line.

The milk-fat content of the medium was then varied between 0·8 and 1·2 mg/ml to yield a final CLA milk-fat concentration of 71·3 μM. All milk-fat samples significantly ($P<0{\cdot}05$) lowered cell numbers compared with the untreated control cells after 4 d incubation by approximately 58 and 53 % in MCF-7 and SW480 cells respectively (Figs. 2 (A) and 2 (B)). No significant differences in final cell numbers were obtained for all milk-fat treatments in either cell line.

To elucidate the effects of individual fatty acids present in the milk fat on cell viability, cells were incubated in the presence of pure LA, TVA or oleic acid at concentrations similar to those found in the milk-fat samples and viability was assessed after 4 d incubation (Figs. 3 (A) and 3 (B)). TVA significantly decreased ($P<0{\cdot}05$) cell viability by approximately 22–37 % in both cell lines when added at the concentrations present in the three milk-fat samples (111·2, 156·1 and 164·2 μM in the control, FFS and FFR, respectively). The MCF-7 cells were more sensitive to the growth inhibitory effects of TVA. When cells were incubated in the presence of LA at the concentrations present in the three milk-fat samples (44·6, 152·5 and 60·2 μM in the control, FFS and FFR, respectively) differential effects on growth were observed. In the MCF-7 cell line, LA at a concentration of 44·6 μM significantly ($P<0{\cdot}05$) stimulated cell growth by 26 % but at a concentration of 152·5 μM LA was cytotoxic to the cells inhibiting cell growth by 43 %. LA at 60·2 μM had no significant effect on final cell numbers. In the SW480 cell line, LA at 44·6 and 60·2 μM stimulated cell growth but the increase was only significant at the higher LA concentration. When LA was added at a concentration of 152·5 μM, SW480 cell growth was significantly ($P<0{\cdot}05$) inhibited by 40 %. The latter cell line was more sensitive to the growth-modulatory effects of oleic acid than the MCF-7 cell line. Incubation with oleic acid significantly decreased cell numbers in the MCF-7 only at the highest concentration of 952·9 μM, which was representative of the concentration found in the FFR milk-fat sample. In contrast,

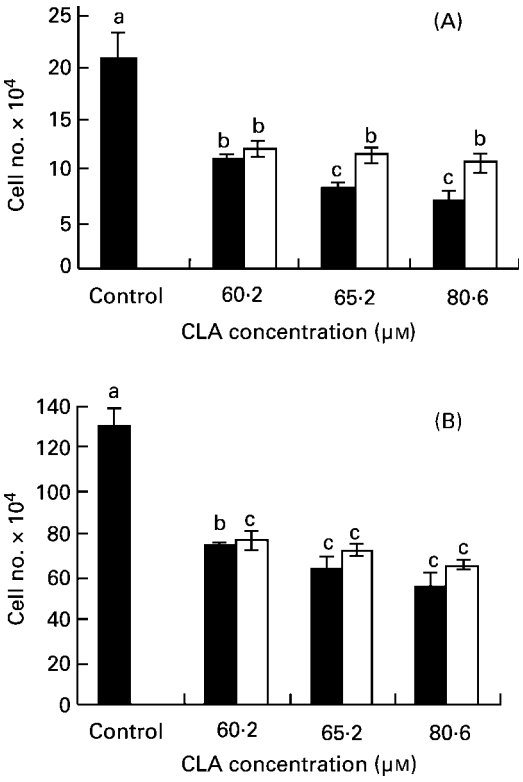

Fig. 1. Growth of (A) MCF-7 and (B) SW480 cells incubated with milk-fat samples (■) or synthetic *cis* 9, *trans* 11-conjugated linoleic acid (CLA) (□) containing varying levels of CLA for 4 d. [a,b,c] Mean values within a concentration with unlike superscript letters were significantly different ($P<0{\cdot}05$) or significantly different from the control ($P<0{\cdot}05$).

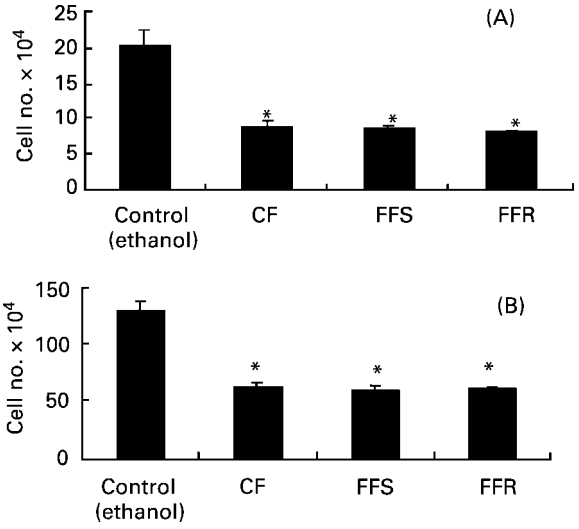

Fig. 2. Growth of (A) MCF-7 and (B) SW480 cells incubated with milk-fat samples containing 20 μg conjugated linoleic acid (CLA)/ml (71·3 μM-CLA) for 4 d. CF, control fat; FFS, full-fat soyabean; FFR, full-fat rapeseed. * Mean values were significantly different to those for the control cells ($P<0{\cdot}05$).

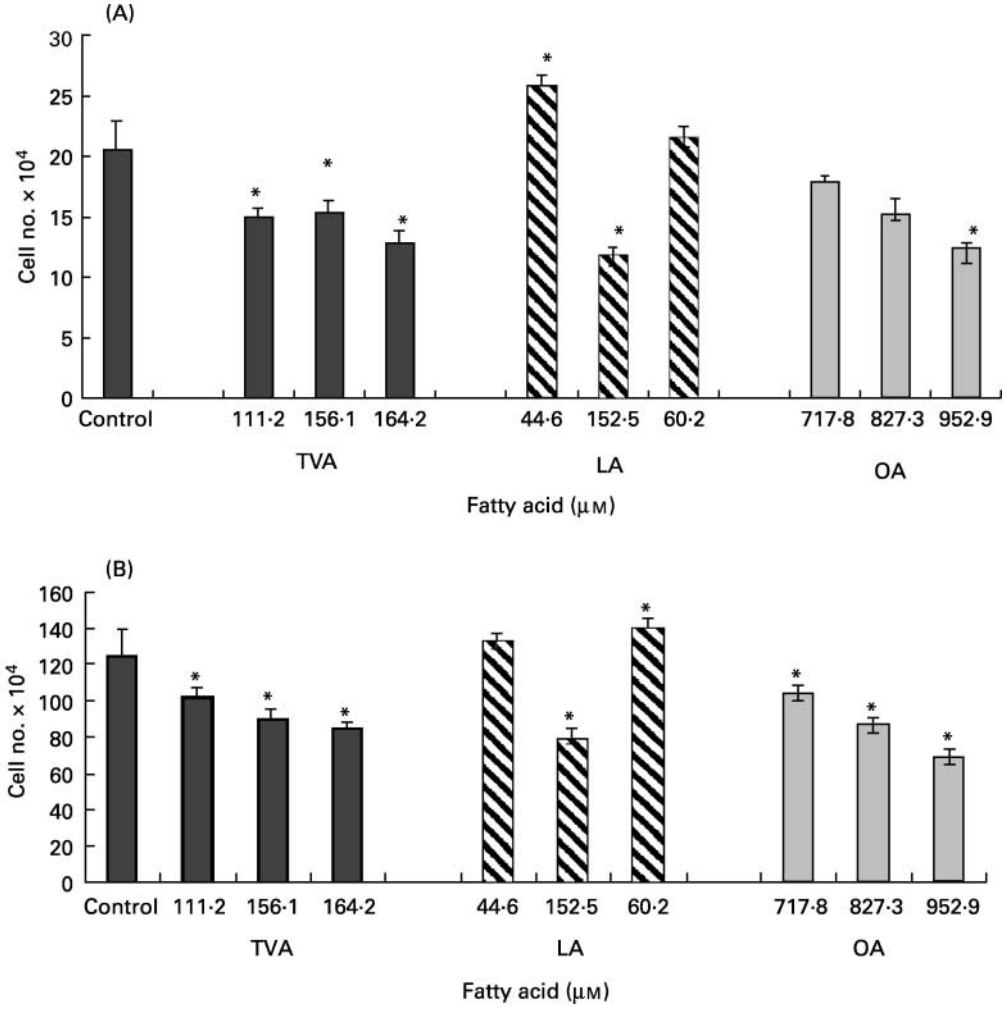

Fig. 3. Growth of (A) MCF-7 and (B) SW480 cells incubated with *trans*-vaccenic acid (TVA), linoleic acid (LA) and oleic acid (OA) at concentrations similar to those present in the milk-fat samples. * Mean values were significantly different to those for the control cells ($P<0{\cdot}05$).

incubation of SW480 cells with oleic acid at 717·8, 827·3 and 952·9 μM, representing the concentrations present in control, FFS and FFR milk fats respectively, significantly ($P<0{\cdot}05$) decreased cell viability by 13, 26 and 39 %, respectively.

Effect of conjugated linoleic acid-enriched milk fat on incorporation of [^{14}C]arachidonic acid into cellular lipid fractions

In order to examine if growth inhibition by milk fat could be attributed to an altered pattern of AA distribution and eicosanoid formation, the effects of the three milk-fat samples on the incorporation of [^{14}C]AA into the cellular lipid fractions of MCF-7 and SW480 cells were investigated. The control fat, FFS and FFR milk fats were added at 1 mg/ml milk fat to yield CLA milk-fat concentrations of 60·2, 65·2 and 80·6 μM, respectively. Levels of [^{14}C]AA uptake into phospholipid, triacylglycerol and MG were 64, 27 and 9 %, respectively, in control MCF-7 cells and 74, 22 and 4 %, respectively, in control SW480 cells (Figs. 4 (A) and 4 (B)). These patterns of AA incorporation are similar to those previously reported in these cell lines (Miller *et al.* 2001). In both cell lines, only incubation with the FFR milk fat, containing the highest CLA levels (80·6 μM), caused perturbations in [^{14}C]AA uptake (Figs. 4 (A) and 4 (B)). In the MCF-7 cell line, treatment with FFR milk fat significantly ($P<0{\cdot}05$) increased [^{14}C]AA uptake into the MG fraction by 10 %. In SW480 cells, FFR milk-fat treatment also resulted in a significant ($P<0{\cdot}05$) increase in [^{14}C]AA uptake into the MG fraction (by 8 %) and this was accompanied by a significant ($P<0{\cdot}05$) decrease in uptake into the phospholipid fraction (by 12 %).

Effect of conjugated linoleic acid-enriched milk fat on eicosanoid and 8-epi-prostaglandin $F_{2\alpha}$ synthesis

The effects of the control, FFS and FFR milk-fat treatments (all added at 1 mg milk fat/ml) on the enzymic conversion of AA to selected eicosanoids (PGD_2, PGE_2, and $PGF_{2\alpha}$ and 5-hydroperoxyeicosatetraenoate) and on the oxidation to 8-epi-$PGF_2\alpha$ were examined. In both cells lines, only the FFR milk-fat treatment altered the eicosanoid profile (Figs. 5 (A) and 5 (B)). Following the incubation of both cell lines with FFR milk fat, [^{14}C]AA conversion to

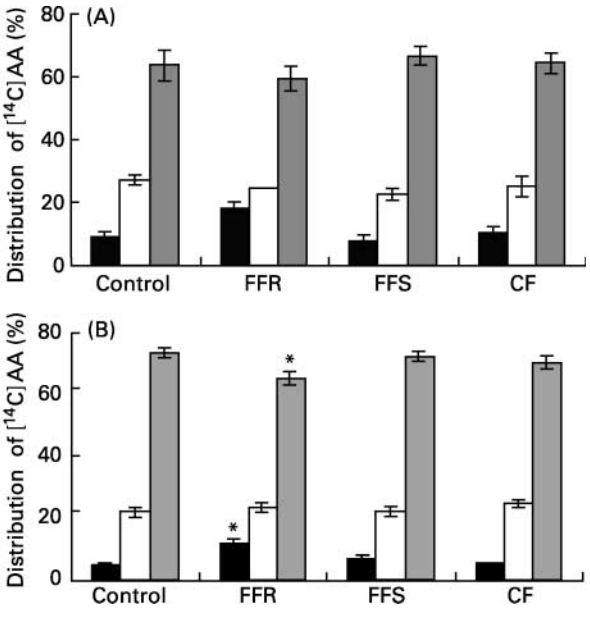

Fig. 4. Percentage distribution of [^{14}C]arachidonic acid (AA) radioactivity into phospholipids (▨), triacylglycerol (□) and monoacylglycerol (■) following 24 h treatment of MCF-7 (A) and SW480 (B) cells with milk-fat samples (1 mg/ml). FFR, full-fat rapeseed; FFS, full-fat soyabean; CF, control fat. *Mean values were significantly different to those for the control cells ($P<0{\cdot}05$).

PGE_2 was significantly ($P<0{\cdot}05$) decreased (by approximately 21–25 %) while the conversion to $PGF_{2\alpha}$ was significantly ($P<0{\cdot}05$) increased (by 23–27 %). A CLA dose-dependent increase in the isoprostane 8-epi-$PGF_{2\alpha}$, a biomarker of lipid peroxidation, was observed in both cell lines following incubation with the three milk fats. Maximal stimulation of 8-epi-$PGF_{2\alpha}$ production by 73 and 92 % was observed in MCF-7 and SW480 cells respectively, following treatment with the FFR milk fat, which contained the highest CLA concentration. None of the milk-fat treatments significantly altered the production of 5-hydroperoxyeicosatetraenoate or PGD_2.

Effect of conjugated linoleic acid-enriched milk fat on apoptotic markers in SW480 cells

To determine if the cytotoxic effect of the milk-fat samples was executed via an induction of an apoptotic signalling pathway effects on cytosolic GSH content, membrane annexin V levels and bcl-2 expression were examined. The data demonstrate that treatment of SW480 cells with the milk-fat samples significantly ($P<0{\cdot}05$) reduced bcl-2 protein expression by 23–36 % ($P<0{\cdot}05$) in a milk-fat CLA concentration-dependent manner (Fig. 6). All milk-fat samples depleted cytosolic GSH by approximately 21–39 % in SW480 cells, with

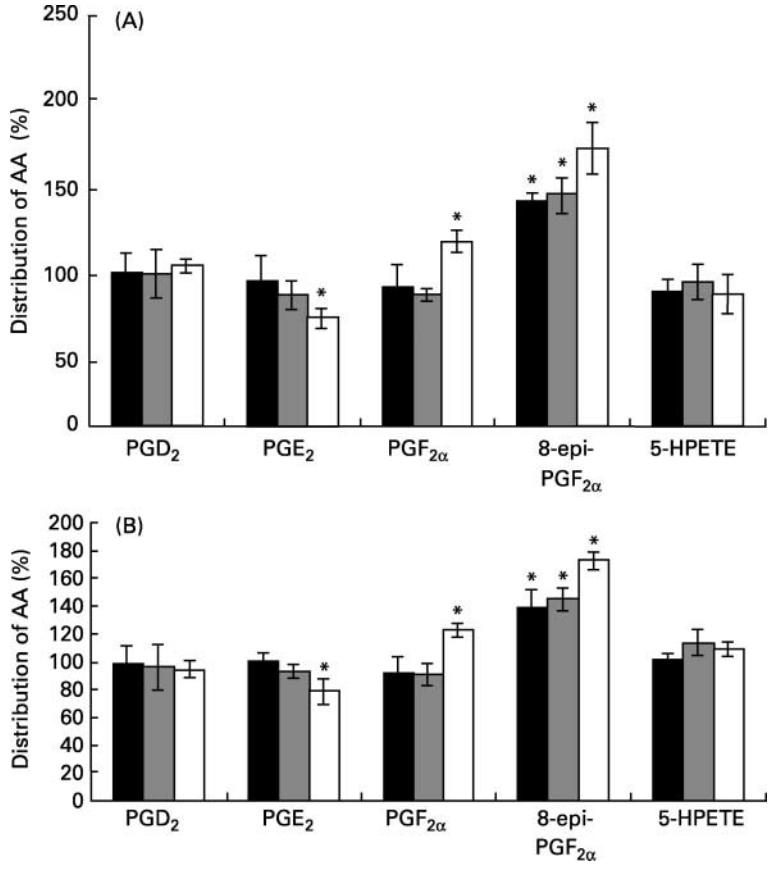

Fig. 5. Percentage distribution of arachidonic acid (AA) into eicosanoids following 24 h treatment of MCF-7 (A) and SW480 (B) cells with milk-fat samples (1 mg/ml) relative to control untreated cells (100 %). (■), Control fat; (▨), full-fat soyabean; (□), full-fat rapeseed; PG, prostaglandin; 5-HPETE, 5-hydroperoxyeicosatetraenoate. * Mean values were significantly different to those for the control cells ($P<0{\cdot}05$).

both FFS and FFR milk fats exerting a more potent effect ($P<0{\cdot}05$) than control milk fat (Fig. 7). All milk-fat treatments significantly ($P<0{\cdot}05$) increased levels of annexin V (29–32 %) in the cell membrane when compared with concentrations found in the membrane preparations of untreated SW480 cells (Fig. 7).

Effect of conjugated linoleic acid-enriched milk fat on ras expression

The effect of the milk-fat samples on total ras expression in the SW480 cell line, which overexpresses k *ras* (Geiser *et al.* 1989), was examined. Fig. 8 shows a representative example of a Western blot analysis of ras in cells treated with control fat, FFS or FFR milk fat for 4 d. Ras appeared as a doublet with the upper band representing farnesylated membrane-bound ras and the lower band representing non-lipid-modified ras p21. Incubation of SW480 cells with control, FFS and FFR milk-fat samples (at 1 mg/ml) decreased the amounts of total ras by 35, 45 and 52 %, respectively, relative to untreated cells.

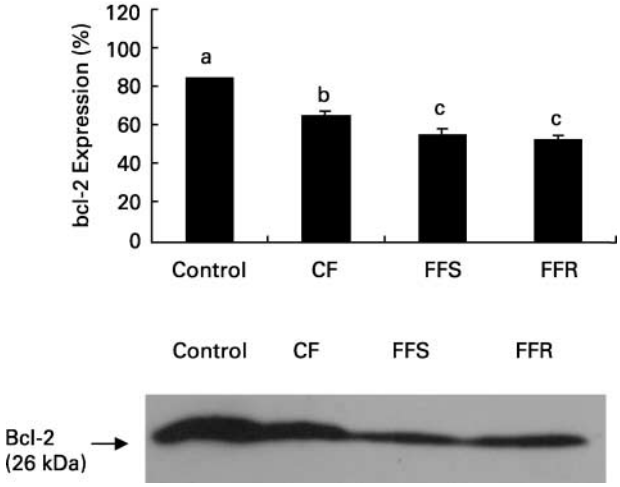

Fig. 6. Expression of bcl-2 in SW480 cells following 4 d treatment with milk-fat samples (1 mg/ml). CF, control fat; FFS, full-fat soyabean; FFR, full-fat rapeseed. [a,b,c] Mean values with unlike superscript letters were significantly different ($P<0{\cdot}05$).

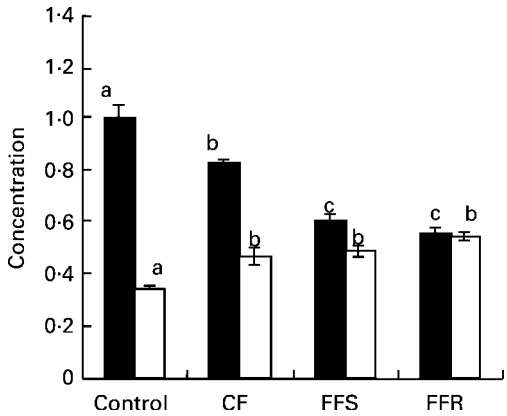

Fig. 7. Levels of cytosolic reduced glutathione (■; nmol/mg protein) and membrane annexin V (□; ng/mg protein) following 4 d treatment of SW480 cells with milk-fat samples (1 mg/ml). CF, control fat; FFS, full-fat soyabean; FFR, full-fat rapeseed. [a,b,c]Mean values within a variable (glutathione or annexin V) with unlike superscript letters were significantly different ($P<0{\cdot}05$).

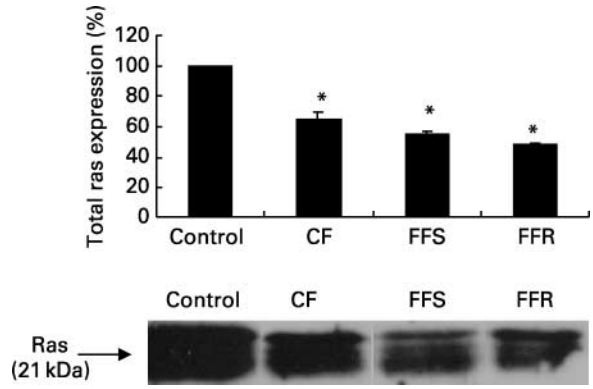

Fig. 8. Expression of ras in SW480 cells following 4 d treatment with milk-fat samples (1 mg/ml). CF, control fat; FFS, full-fat soyabean; FFR, full-fat rapeseed. * Mean values were significantly different to those for the control cells ($P<0{\cdot}05$).

Discussion

A prospective cohort study in Finland revealed that women who developed breast cancer had consumed less milk than cancer-free women and suggested that CLA may be the component in milk providing the protective effect (Knekt *et al.* 1996). Another study revealed an inverse association between dietary intake and serum CLA and the risk of breast cancer in post-menopausal women (Aro *et al.* 2000). Individuals with a high consumption of milk had a potentially reduced risk of colon cancer (Jarvinen *et al.* 2001). Using food duplicate methodology, the *c*9, *t*11-CLA intake in the USA was estimated to be approximately 193 and 140 mg/d for men and women, respectively (Ritzenthaler *et al.* 2001). The authors of the present study suggest that *c*9, *t*11-CLA intake must be increased approximately 3-fold to achieve consumption levels that can exhibit a cancer-protective effect (i.e. 0·1 g/100 g diet). A natural approach to enhancing CLA in dairy products is to increase the CLA content of milk fat by modifying the dietary regimen of the dairy cow. The FFR milk fat used in the present study contained 1·3-fold higher *c*9, *t*11-CLA concentration than the control fat.

The present study confirmed the cytotoxic effect of milk-fat CLA in MCF-7 cells previously reported after 8 d incubation (O'Shea *et al.* 2000) and also showed that the SW480 cell line is sensitive to the growth-inhibitory effects of milk-fat CLA. Triacylglycerol-bound milk-fat CLA was as effective an anticancer agent as the non-esterified fatty acid form of the *c*9, *t*11-CLA in the SW480 cells and even better in the MCF-7 cells, suggesting that lipolysis is an active process in these cells and that lipase activity may be rate limiting in the MCF-7 cell line.

Evidence is emerging that individual fatty acids, even within the same fatty acid type (for example, *c*9, *t*11-CLA and LA) may have different effects on carcinogenesis, tumour growth and metastasis (Zhou & Blackburn, 1999). The present study evaluated the individual effect of four

fatty acids, at concentrations similar to those found in the three milk-fat samples, on the cell growth of SW480 and MCF-7 human cancer cell lines. An inhibitory effect on cell growth was obtained following the incubation of both cell lines with the pure *c*9, *t*11-CLA isomer (at concentrations similar to those present in the three milk-fat samples), confirming the well-established cytotoxic effect of CLA in these two cell lines. When media were supplemented with synthetic TVA, at concentrations similar to those present in the three milk-fat samples, cell viability significantly decreased in both cell lines. It has been shown that TVA decreases cell growth and evidence has been provided to suggest that the growth-suppression responses of both cell lines to TVA are probably mediated by its desaturation to *c*9, *t*11-CLA (Miller *et al.* 2003). LA was either stimulatory or had no effect on cell growth when incubated with the cells at the lower concentrations found in the control (44·6 μM) and FFR (60·2 μM) milk fats. However, 152·5 μM-LA, the concentration present in the FFS milk fat, had a potent cytotoxic effect and was of similar magnitude to CLA on the growth of both cell lines. Oleic acid decreased the viability of SW480 cells when added at concentrations similar to those present in the three milk-fat samples but only decreased cell numbers in MCF-7 cells at the highest concentration of 952·9 μM. If the cytotoxic effects of these fatty acids in milk fat are additive, it would be expected that the accumulative growth-suppression effect by milk fats would be much greater than that observed and would vary as milk-fat content of the medium varied. The observation that final cell numbers were similar when the milk-fat content of the medium was varied to yield a final CLA milk-fat concentration of 71·3 μM suggests that the effects of these fatty acids when in triacylglycerol-bound form in milk fat are lessened. Yet, when cells were treated with milk fats containing increasing amounts of CLA they exhibited a dose-dependent decrease in cell number. These data support the earlier conclusion of Ip *et al.* (1996) that CLA is a unique fatty acid with anticancer properties acting independently of other fatty acids.

It has previously been reported that the growth-suppressive effect of the *c*9, *t*11-CLA isomer was associated with changes in AA distribution among cellular lipids and an altered PG profile (Miller *et al.* 2001). In the present study FFR milk fat increased [^{14}C]AA uptake into the MG fraction of MCF-7 cells but decreased [^{14}C]AA uptake into the phospholipid fraction. The FFR milk fat decreased [^{14}C]AA conversion to PGE_2 while increasing conversion to $PGF_{2\alpha}$. This altered pattern of eicosanoid production is similar to that previously observed following *c*9, *t*11-CLA treatment (Miller *et al.* 2001).

As observed with the pure *c*9, *t*11-CLA isomer (Miller *et al.* 2001), treatment of the two cell lines with all three milk fats increased the levels of 8-epi-$PGF_{2\alpha}$, a biomarker of lipid peroxidation. Studies suggest that oxidative stress, in general, and lipid peroxidation in particular are involved in both the initiation and mediation of apoptosis (Lopaczynski & Zeisel, 2001). The depletion of GSH furthers enhances oxidative stress within cells and has been associated with cytochrome c release (Tang *et al.* 1998). A balance between beneficial effects (such as apoptosis) and detrimental effects (for example, oxidative damage induced by the peroxidative process) may be controlled by factors such as the concentration or structure of specific antioxidants or by how they distribute intracellularly. The treatment of cells with all milk-fat samples depleted cytosolic GSH. The milk fat modulated bcl-2 protein levels, reducing its expression in a CLA concentration-dependent manner and increased levels of annexin V in cell membranes in a similar manner to the pure *c*9, *t*11-CLA previously reported (Miller *et al.* 2002). It can be concluded from these data that the incubation of SW480 cells with milk fats resulted in a cellular condition compatible with the induction of apoptosis. The treatment of SW480 cells with TVA and *c*9, *t*11-CLA was shown to have decreased total ras expression following 4 d of incubation (Miller *et al.* 2003). Ras is a central player in membrane-to-nucleus signal transduction and has several downstream targets, including the mitogen-activated protein kinase pathway, which is involved in cellular proliferation (Campbell *et al.* 1998). Mutations in the dominant oncogene ras represent the most commonly found gene mutations in human cancer cells (Gibbs *et al.* 1994). The present study now shows that CLA-enriched milk fats may also influence ras signalling by reducing its expression. Further comparative studies with other fatty acids are required to validate the use of bcl-2 and/or ras as specific biomarkers of CLA exposure.

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