Conjugated Linoleic Acid (CLA) Up-regulates the Estrogen-regulated Cancer Suppressor Gene, Protein Tyrosine Phosphatase γ (PTPγ), in Human Breast Cells

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Abstract. *Background: Conjugated linoleic acid (CLA), a naturally occurring compound found in ruminants products, has been shown to possess anticancer properties in vivo and in vitro. There are several CLA isomers in ruminant-produced foods, among which t10,c12-CLA and c9,t11-CLA are the most potent. Protein tyrosine phosphatase γ (PTPγ) has been implicated as a tumor suppressor gene in kidney and lung cancers. Our previous results indicated that estradiol-17β (E_2)-induced suppression of PTPγ may play a role in mammary tumorigenesis. Materials and Methods: The effects of t10,c12-CLA and c9,t11-CLA on PTPγ mRNA expression in human breast epithelial cells and stromal cells, isolated from surgical specimens of mammoplasty and breast cancer patients, were detected and quantified by RT-PCR. Results: The PTPγ mRNA expression was lower in cancer than in normal breast cells. Both t10,c12-CLA and c9,t11-CLA significantly ($p<0.05$) increased the PTPγ mRNA levels in primary cultured normal breast epithelial cells, normal breast stromal cells and breast cancer epithelial cells, but not in breast cancer stromal cells. t10,c12-CLA appeared to be the most active isomer in estrogen receptor α(ERα)-positive human breast cancer epithelial cells. Conclusion: The results indicate that dietary CLA might serve as a chemo-preventive and chemo-therapeutic agent in human breast cancers by up-regulating the estrogen-regulated tumor suppressor gene, PTPγ expression.*

CLA is produced by rumen fermentation of linoleic acid and is deposited in the subcutaneous fat (sub-Q) and intramuscular fat (IM or marbling) layer in beef cattle, and is also present in dairy milk fat (1). There are several CLA isomers in ruminant-produced foods, among which *c*9, *t*11-CLA and *t*10,*c*12-CLA are the most potent (2). CLA has been found to possess anti-carcinogenic, anti-diabetic, anti-atherogenic and anti-adipogenic activities in mouse and human cell lines, and in *in vivo* animal studies using mice (2). Human studies of CLA and breast cancer revealed that a diet composed of CLA-rich foods, particularly cheese, may protect against breast cancer in postmenopausal women (3). It was recently reported, in a large epidemiological study, that dietary CLA intake was associated with the regulation of estrogen receptor expression in human breast cancer patients. CLA has been proven to reduce the risk of estrogen receptor-negative tumor in premenopausal breast cancer (4). CLA studies in our laboratory (5, 6) also demonstrated the anti-mammary tumor effect of CLA on: (i) anti-angiogenesis by suppressing the predominant vascular endothelial growth factor (VEGF) isoforms, VEGF 121 and 165, mRNA expression in a human breast cancer cell line; (ii) decreasing estrogenic agent-induced breast cancer cell proliferation; (iii) regulating ERα and ERβ in human breast cancer.

Protein tyrosine phosphatases (PTPs) play an essential role in the regulation of cell activation, proliferation and differentiation, since they counterbalance the growth-promoting effects of the protein tyrosine kinases (PTKs) (7, 8). Therefore, alterations in PTPs activity might affect cell growth, neoplastic processes and transformation (8). Previous work suggests that PTPs may also be important in the growth of breast cancer and may be affected by estrogenic agonists and antagonists. Human breast tumors exhibit enhanced tyrosine kinase activity relative to benign breast disease or normal breast tissue (9). The growth rate of a large proportion of breast cancers is influenced by sex

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Key Words: Breast cancer cells, CLA, PTPγ.

steroid hormones, and both steroid hormone and protein tyrosine phosphorylation have been demonstrated to play important roles in cell proliferation (10). Protein tyrosine phosphatase γ (PTPγ) is a member of the receptor-like family of tyrosine-specific phosphatases, and the structure of the receptor-like PTPs include an extracellular, a transmembrane, and one or two tandemly repeated catalytic domains. This structure implies ligand-binding capability which may modulate enzymatic activity. The putative ligands for most of the PTPs with receptor-like structures have yet to be identified. According to mRNA analysis (11, 12), PTPγ is a broadly expressed enzyme that exists in many tissues, including human lung, stomach, esophagus, colon, liver, spleen and kidney (13). Based on the chromosomal location of the PTPγ gene (3p14.2) (11, 14) and studies showing loss of heterozygosity of the gene in kidney tumors (15), PTPγ has been implicated as a candidate tumor suppressor gene. More recently, PTPγ expression levels were shown to be reduced in ovarian and lung tumors (16). Our laboratory has recently demostrated that over-expression of PTPγ in human breast cancer cells leads to reducing anchorage-independent colonization and reducing cell proliferation (17). Over-expressed PTPγ induced binding ability of the transcriptional factor 9-cis-retinoic acid receptor (RXR), delayed cell cycle re-entry and elevated the protein level of the cell cycle checkpoint regulator, p21 (18). We also found aberrant methylation patterns in PTPγ promoter region in breast cancer cells, which may lead to its down-regulation. Moreover, over-expression of PTPγ can inhibit tumor formation in a mouse xenograft model (18).

Numerous growth factors, hormones and environmental agents regulate the signaling events involved in carcinogenesis. The probability of women developing breast cancer during their lifetime in the US is 1 in 7 (19). In 2005, The American Cancer Society estimated that breast cancer incidences would exceed that of all US cancer cases; breast cancer would represent one in three of all cancer cases and would be the second only behind to lung cancer in terms of mortality (20). Both age and the duration of exposure to endogenous and exogenous estrogens may be one of the best-defined risk factors linked to human breast cancer.

The local micro-environment of cancer is thought to be crucial for cancer progression, because cancer epithelial cells are surrounded by variable types of stromal cells (21). From experimental cancer models, the extracellular microenvironment has been demonstrated to influence tumor formation, the rate of cellular proliferation, the ability of the cancer cells to metastasize and the extent of invasiveness. In cancers, the influences of the microenvironment are mediated, in part, by paracrine signaling between epithelial cancer cells and the surrounding stromal cells (22, 23). It has been suggested that stromal cells may be serving as a local reservoir for CLA and, thus, may inhibit breast cancer cell progression (24). The aim of the study was to investigate whether CLA modulates paracrine signals capable of inducing the PTPγ tumor suppressor gene.

Materials and Methods

More than 98% pure *t*10,*c*12-CLA and *c*9,*t*11-CLA were purchased from Matreya, Inc. (PA, USA) and a CLA stock solution was prepared based on the paper published by Dr. Ip's group (25). The main difference in our CLA stock was that dextran-coated charcoal (DCC, Dextran T-70; Pharmacia; activated charcoal; Sigma)-treated fetal bovine serum (FBS, GibcoBRL, Bethesda, MD, USA) was added to pure *t*10,*c*12-CLA and *c*9,*t*11-CLA to form a CLA-serum protein complex.

Immortalized cell line. MCF-7 and MDA-MB-231 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Both the MCF-7 and MDA-MB-231 cells were placed in 75-cm^2 culture flasks in a humidified incubator (5% CO_2, 95% air, 37˚C) and cultured in phenol red-free high-calcium Dulbecco's Modified Eagle's Medium and Ham's F12 Medium (DMEM/F12, 1.05 mM $CaCl_2$) supplemented with 5% FBS. The medium was renewed every 2 days.

Isolation of epithelial and stromal cells from human breast tissues. Normal and cancer human breast tissues were obtained through the Tissue Procurement Program at The Ohio State University Hospital in Columbus, Ohio, USA. The tissues were placed in DMEM/F12 and stored at 4˚C. The isolation of epithelial and stromal cells from human breast tissues and their culture has been described in detail previously (26). Briefly, the tissues were minced and digested in 0.1% collagenase I (GibcoBRL) supplemented with 5% FBS and antibiotic-antimycotic (100 unit/ml penicillin G sodium, 100 mg/ml amphotericin B; GibcoBRL) in a 37˚C humidified incubator (5% CO_2 : 95% air) overnight. The digested mixture was centrifuged at 200 xg for 5 min at 25˚C. The cell pellet was re-suspended and allowed to settle by gravity 3 times. The stromal cells in the supernatant were then centrifuged at 200 xg for 5 min at 25˚C and the pelleted cells were re-suspended in phenol red-free high-calcium DMEM/F12 (1.05 mM $CaCl_2$) supplemented with 5% FBS. The cancer epithelial cells in the initial sedimented part were re-suspended in keratinocyte serum-free medium (Keratinocyte-SFM, 0.09 mM $CaCl_2$; GibcoBRL) supplemented with bovine pituitary extract (25 mg) and epidermal growth factor (10 μg). The normal epithelial cells in the initial sedimented part were re-suspended in low calcium DMEM/F12 (0.04 mM $CaCl_2$) supplemented with Chelex-100 (Bio-Rad Laboratories, Richmond, CA, USA)-treated FBS (10%).

Cell treatment and total RNA extraction. The treatments and total RNA extractions were performed on cells not propagated beyond the third passage, with the viabilities of each cell type exceeding 95%, as determined by the trypan blue dye exclusion method (27). Normal and cancer breast epithelial cells were plated in 6-well plate (3x10^4 viable cells/well) and cultured in phenol red-free low-calcium DMEM/F12 (0.04 mM $CaCl_2$) supplemented with DCC-stripped Chelex-100-treated FBS (10%). MCF-7, MDA-MB-231,

normal and cancer breast cells and stromal cells were plated in 6-well plate ($3x10^4$ viable cells/well) and cultured in phenol red-free high-calcium DMEM/F12 (1.05 mM $CaCl_2$) supplemented with DCC-stripped Chelex-100-treated FBS (5%). After 2 days, all the cultured cells were treated with 40 μM of *t*10,*c*12-CLA, 40 μM of *c*9,*t*11-CLA or vehicle as control in the same medium for 3 days. The total RNA from the treated and control cells was isolated in 1 ml TRIZOL® Reagent (GibcoBRL), according to the manufacturer's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was performed in a gradient mastercycler (Eppendorf®). The PCR conditions, primer sequences and length of the PCR products (PTPγ, ERβ, ERα and 36B4) have been described in detail elsewhere (26, 28). Briefly, for ERα, the conditions were as described before (26, 28), while the modified parts were: 0.5 mM $MgCl_2$, annealing at 60˚C for 45 sec and 29 cycles of amplification. For ERβ, the conditions were as described before (26, 28), with modifications of 1.25 mM $MgCl_2$, annealing at 56˚C for 1 min and 38 cycles of amplification. For PTPγ and 36B4, the conditions were as described before (26, 28) and only modified by a $MgCl_2$ concentration of 1.25 mM $MgCl_2$.

The primer sequences for ERα were 5'-TAC TGC ATC AGA TCC AAG GG-3' (sense) and 5'-ATC AAT GGT GCA CTG GTT GG-3' (antisense). For ERβ they were 5'-TGA AAA GGA AGG TTA GTG GGA ACC-3' (sense) and 5'-TGG TCA GGG ACA TCA TCA TGG-3' (antisense). For PTPγ they were 5'-GCG CAG CGA CTT TAG CCA GAC GA- 3' (sense) and 5'-GCT CCC GCT CCC CAT CCT CAC TC-3' (antisense). The primer sequences for 36B4 were 5'-AAA CTG CTG CCT CAT ATC CG-3' (sense) and 5'-TTG ATG ATA GAA TGG GGT ACT GAT G-3' (antisense). The final PCR products (10 μl), mixed with 1 μl 10x loading buffer, were separated on a 1.5% agarose gel containing ethidium bromide. The lengths of the PCR products were 650 bp for ERα, 528 bp for ERβ, 492 bp for PTPγ and 563 bp for 36B4. The specific bands were quantified by ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA). The results are presented as the ratio of PTPγ to 36B4.

Statistics. The data, from 3 replicated wells as 1 cell group, was presented as the mean±standard deviation (SD) and was analyzed using the StatView® ANOVA unpaired *t*-test. *P*-value less than 0.05 was considered to be statistically significant.

Results

Comparison of PTPγ mRNA expression levels in normal and human breast cancer epithelial cells and stromal cells. PTPγ expression has been previously documented in human lung, stomach, esophagus, colon, liver, spleen and kidney tissues (29). Also, previous work in our laboratory showed that the PTPγ mRNA expression levels were lower in mixed cell populations from human breast cancer tissues than in mixed cell populations from normal human breast tissues (30).

The purity of the human breast stromal and epithelial cell preparations had been examined by morphology and confirmed by immunohistochemical staining previously in our laboratory (26). The stromal cells exhibited a typical spindle-shaped morphology and the majority of stromal cells (>95%) were immunopositive for the presence of vimentin, although no expression of cytokeratin was detected, confirming the fibroblastic nature of the stromal cells. The epithelial cells tended to be characteristically rounded, and more than 95% of the cultured epithelial cells were positive for the presence of cytokeratin, with almost no expression of vimentin (26).

To compare the mRNA expression of PTPγ in normal and cancer human breast cells, PTPγ mRNA levels were determined by RT-PCR in specific cell types from 2 reduction mammoplasty patients (20–30 years old) and 2 breast cancer patients (40–50 years old). The experimental data demonstrated that the basal PTPγ mRNA expression levels in the human breast cancer cell lines MCF-7 and MDA-MB-231 were higher than those of the primary cultured human breast cancer epithelial cells isolated from 2 human breast cancer patients. Regardless of whether they were isolated from human normal or cancer breast tissues, the PTPγ mRNA expression levels were greater in isolated epithelial cells than in stromal cells (Figure 1), suggesting a cell-specific expression pattern. Also, normal breast epithelial cells expressed higher levels of PTPγ mRNA than breast cancer epithelial cells (Figure 1). The estrogen receptor (ER) status in different cell types is shown in Figure 1. Only breast cancer epithelial cells from 1 patient expressed ERα; ERβ expression was detected in most of the examined cells, though at very low levels in normal breast epithelial cells.

t10,c12-CLA and c9,t11-CLA up-regulated PTPγ mRNA in human normal and cancer breast epithelial cells, but not in human cancer breast stromal cells. To compare the effects of *t*10,*c*12-CLA and *c*9,*t*11-CLA on PTPγ mRNA expression in specific cell types from normal and cancer human breast tissues, the PTPγ mRNA levels were determined by RT-PCR in specific cell types after treatment with both CLA isomers for 3 days. Based on our previous study, the effective dose range to inhibit human breast cancerous epithelial and stromal cell proliferation is: 10-80 μM of *t*10,*c*12-CLA and 40-160 μM of *c*9,*t*11-CLA for 3 days treatment (data not shown). Therefore the effective doses of 40 μM *t*10,*c*12-CLA and 40 μM c9,t11-CLA, were chosen to investigate the effects of CLA on modulating estrogen-regulated PTPγ in human cancer or normal breast cells. The results are shown in Figures 2-6.

The experimental data demonstrated that *t*10,*c*12-CLA and *c*9,*t*11-CLA had a greater effect in up-regulating PTPγ mRNA expression on MCF-7 and MDA-MB-231 immortalized human breast cancer cell lines than in primary cultured human breast cancer epithelial cells (Figures 2 and 3). In contrast, CLA appeared to have no effects on PTPγ mRNA expression in human breast cancer stromal cells (Figure 4). These results suggested that, in breast cancer patients, CLA's anti-tumorigenetic effects were mainly

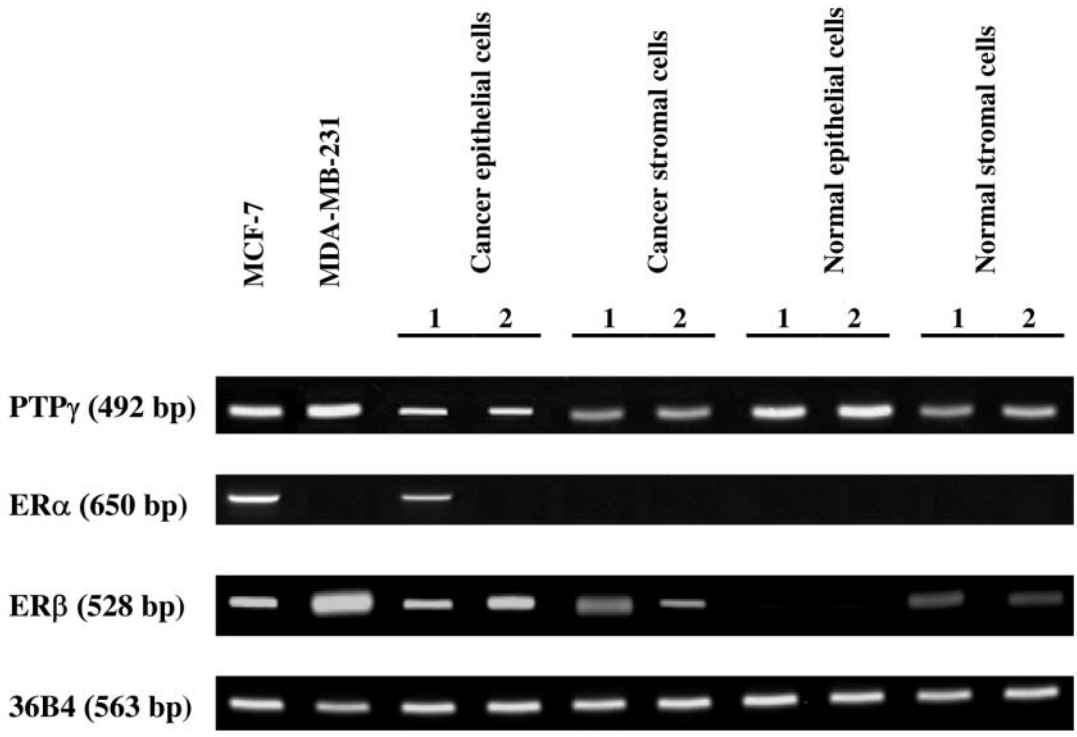

Figure 1. *PTPγ, ERα and ERβ basal mRNA level in human breast cells. 1 and 2 represent patients 1 and 2, respectively. Epithelial cells and stromal cells were isolated and cultured from either normal or cancer breast tissue. 36B4 was used as the loading control.*

exerted on epithelial cells. In normal human breast, both CLA isomers up-regulated PTPγ mRNA expression in normal breast epithelial cells and stromal cells to a similar magnitude (Figures 5 and 6), suggesting that CLA acted in both cells types in normal human breast.

t10,c12-CLA appeared to be the more active isomer in ERα+ human breast cancer epithelial cells. The effects of specific CLA isomers on specific cell types from human normal and cancer breast are shown in Figures 2-6. The experimental data demonstrated that *t*10,*c*12-CLA and *c*9,*t*11-CLA showed comparable effects in up-regulating *PTPγ* mRNA in normal breast epithelial cells, normal breast stromal cells, MDA-MB-231 (ERα–, ERβ+) and breast cancer epithelial cells (from patient 2, ERα–, ERβ+, Figure 1). Interestingly, *t*10,*c*12-CLA appeared to be the more active isomer in MCF-7 cells (ERα+, ERβ+), which can also be observed in the breast cancer epithelial cells isolated and cultured from patient 1 (ERα+, ERβ+, Figure 1). Both CLA isomers increased PTPγ mRNA in ERα+ breast cancer epithelial cells with a ratio of up-regulation *t*10,*c*12-CLA : c9,t11-CLA at approximately 2 : 1 (from patient 1, Figure 3). These results suggested the specific CLA isomer, *t*10,*c*12-CLA, may be more effective in ERα+ breast cancer patients.

Discussion

A fascinating link between PTPγ and cancer has been indicated by previous works. In renal and lung cancers, PTPγ acts as a tumor suppressor gene (11) and its expression is reduced in lung and ovarian tumors (16). Using cultured epithelial and stromal cells isolated from normal human breast tissues and breast cancer tissue, we showed that: (i) PTPγ mRNA levels are lower in breast cancer cells than normal breast cells; (ii) PTPγ mRNA levels are greater in epithelial cells than in stromal cells as determined by RT-PCR (Figure 1). This result is supported by our previous findings on the immunochemical staining of PTPγ protein expression and mRNA levels, indicating that PTPγ is less expressed in breast cancer tissues than tissues from normal breast patients; PTPγ was detected exclusively in the epithelial compartments in cultured human breast tissues (26, 30).

Our previous work showed that estrogenic down-regulation of PTPγ is associated with ERα, because the down-regulated PTPγ could be observed in ERα-transfected MDA-MB-231 cells, but not in mock-transfected cells (28). The anti-estrogenic agent, hydroxy-tamoxifen, increases the activity of a membrane-associated PTP(s) in ER-positive

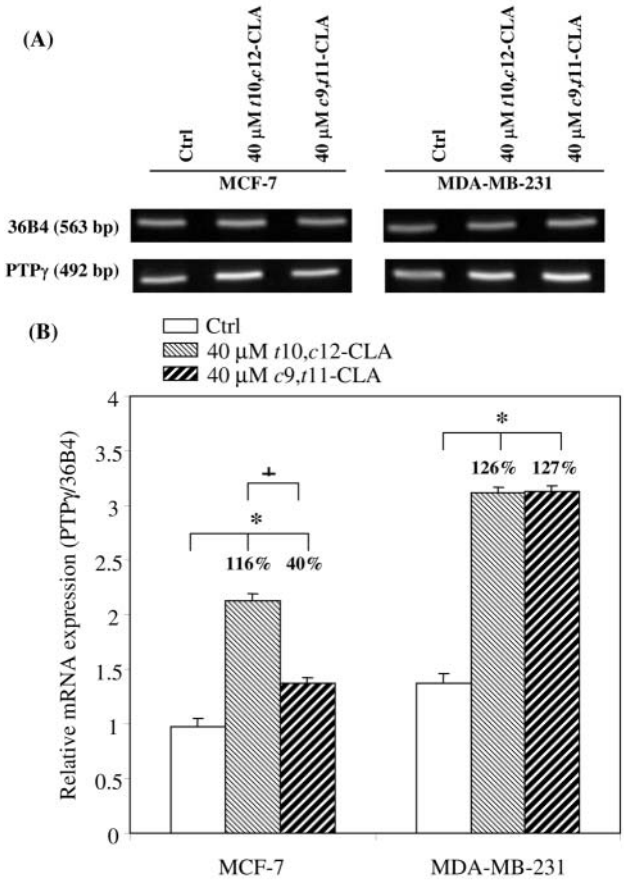

Figure 2. *CLA up-regulated PTPγ mRNA in the human breast cancer cell lines, MCF-7 and MDA-MB-231. (A) Ethidium bromide-stained PCR products separated in a 1.5% agarose gel. 1 and 2 represent patients 1 and 2, respectively. The cells were treated by 2 CLA isoforms and the total RNA was isolated from each group of cells separately. 36B4 was used as the loading control. (B) The mRNA ratio of PTPγ to 36B4 was measured by densitometry. Bars represent mean±SD, n=3. *p<0.05 stands for control versus CLA; +p<0.05 for 40 mM t10,c12-CLA versus 40 mM c9,t11-CLA.*

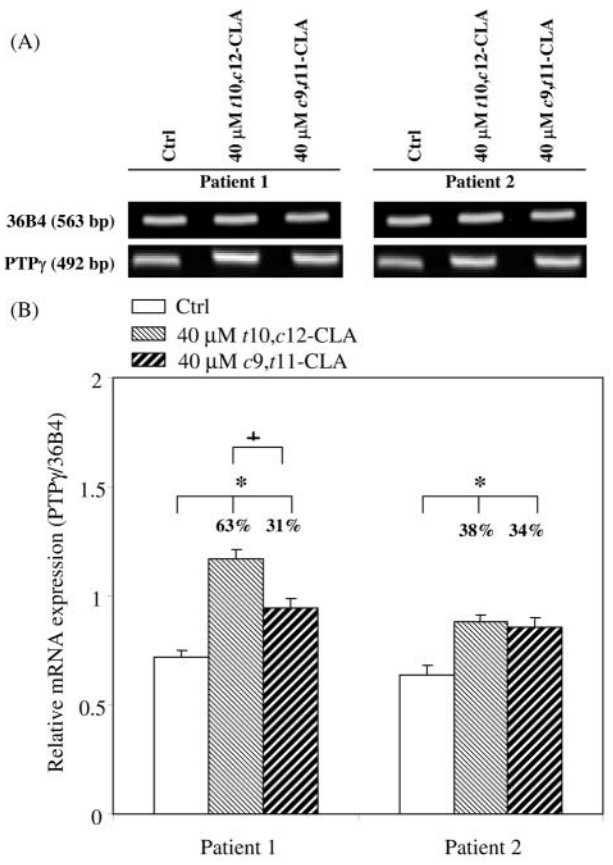

Figure 3. *CLA up-regulated PTPγ mRNA in primary cultured human breast cancer epithelial cells. (A) Ethidium bromide-stained PCR products separated in a 1.5% agarose gel. 1 and 2 represent patients 1 and 2, respectively. The cells were treated by 2 CLA isoforms and the total RNA was isolated from each group of cells separately. 36B4 was used as the loading control. (B) The mRNA ratio of PTPγ to 36B4 was measured by densitometry. Bars represent mean±SD, n=3. *p<0.05 stands for control versus CLA; +p<0.05 for 40 mM t10,c12-CLA versus 40 mM c9,t11-CLA*

breast cancer cell lines, but not in those that are ER-negative (31). *t*10,*c*12-CLA and *c*9,*t*11-CLA increased the PTPγ mRNA in both ERα-positive and -negative breast cancer epithelial cells (Figures 2 and 3). In accordance with our findings, we speculated that CLA may be beneficial to both ERα-positive and ER-negative breast cancer patients.

Growth factors have been implicated as autocrine/paracrine mediators of epithelial-stromal interactions (32). Human breast stromal cells secrete peptide growth factors, including insulin-like growth factor-I and -II (33) and transforming growth factor-α (34), to regulate breast epithelial cell functions. *t*10,*c*12-CLA and *c*9,*t*11-CLA both up-regulated PTPγ in human normal breast epithelial cells and stromal cells (Figures 3 and 4) suggest that CLA may modulate paracrine signals capable of inducing the PTPγ tumor suppressor gene which suppresses the characteristics of human breast neoplasms.

CLA has been shown to possess substantial bi-functional preventive and therapeutic effects in carcinogenesis; different tumor preventive and therapeutic effects of *t*10,*c*12-CLA and *c*9,*t*11-CLA have been well summarized in a review paper (24). In terms of chemoprevention, CLA was shown to inhibit dimethylbenz(*a*)anthracene (DMBA)- and *N*-nitroso-*N*-methylurea (NMU)-induced rat mammary carcinogenesis (35-39). In terms of chemotherapeutical, CLA was demonstrated to reduce tumor size and metastasis induced by human breast cancer cells in a mouse model (40). *t*10,*c*12-CLA was the more active isomer than *c*9,*t*11-CLA in inhibiting immortalized breast cancer epithelial cell growth (41). Our results

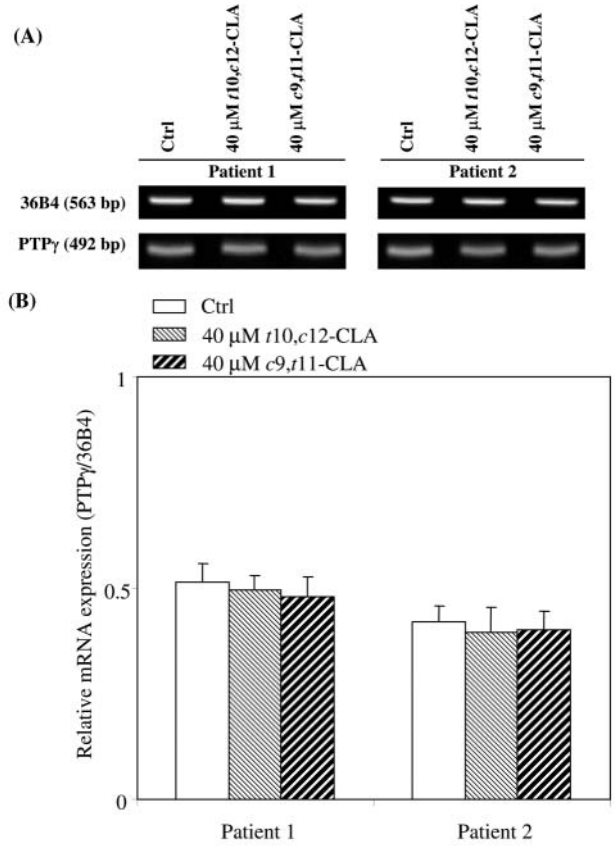

Figure 4. *Effects of CLA on PTPγ mRNA expression in primary cultured human breast cancer stromal cells. (A) Ethidium bromide-stained PCR products separated in a 1.5% agarose gel. 1 and 2 represent patients 1 and 2, respectively. The cells were treated by 2 CLA isoforms and the total RNA was isolated from each group of cells separately. 36B4 was used as the loading control. (B) The mRNA ratio of PTPγ to 36B4 was measured by densitometry. Bars represent mean±SD, n=3.*

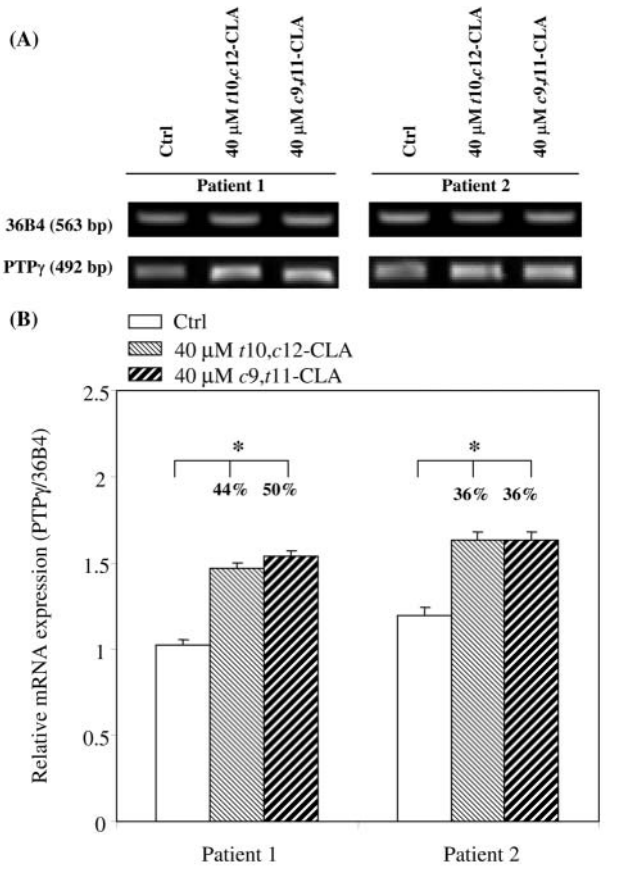

Figure 5. *CLA up-regulated PTPγ mRNA in primary cultured normal human breast epithelial cells. (A) Ethidium bromide-stained PCR products separated in a 1.5% agarose gel. 1 and 2 represent patients 1 and 2, respectively. The cells were treated by 2 CLA isoforms and the total RNA was isolated from each group of cells separately. 36B4 was used as the loading control. (B) The mRNA ratio of PTPγ to 36B4 was measured by densitometry. Bars represent mean±SD, n=3. *p<0.05 stands for control versus CLA.*

demonstrated that t10,c12-CLA and *c*9,*t*11-CLA showed similar activity in up-regulating PTPγ mRNA expression in human normal breast epithelial cells and normal breast stromal cells from reduction mammoplasty patients, suggesting that this might be one of the possible chemopreventive mechanisms of CLA. On the other hand, *t*10,*c*12-CLA appeared to be more active than *c*9,*t*11-CLA in up-regulating PTPγ mRNA expression in ERα-positive breast cancer epithelial cells, implying that its use could offer valuable therapeutic effects.

The term "functional food" is generally accepted as meaning that the levels of an effective naturally occurring food ingredient are enriched in that food (24). The consumption of functional foods produced from ruminant products containing CLA has been suggested to have a chemo-preventive or/and chemo-therapeutic influence (24). In addition it has been demonstrated that specially formulated ruminant feeds enhance the synthesis of high levels of CLA by ruminants (1). The experimental data described here yield new and potentially important information regarding the up-regulation of PTPγ, a potential estrogen-regulated tumor suppressor gene, by CLA in human normal and cancer breast cells. Future studies investigating the mechanisms by which CLA regulates PTPγ expression in ERα-positive and -negative breast cancer epithelial cells are ongoing.

Acknowledgements

This study was supported in part by NIH Grants CA94718 and CA95915 and DOD Breast Cancer Research Programs Grants DAMD 8140, 0319 and 9341. We appreciate Lihui Ou and Patricia A. Masso-Welch from Dr. Ip's group, and Dr. Yoon Jung Han for providing expertise in the CLA stock solution preparation.

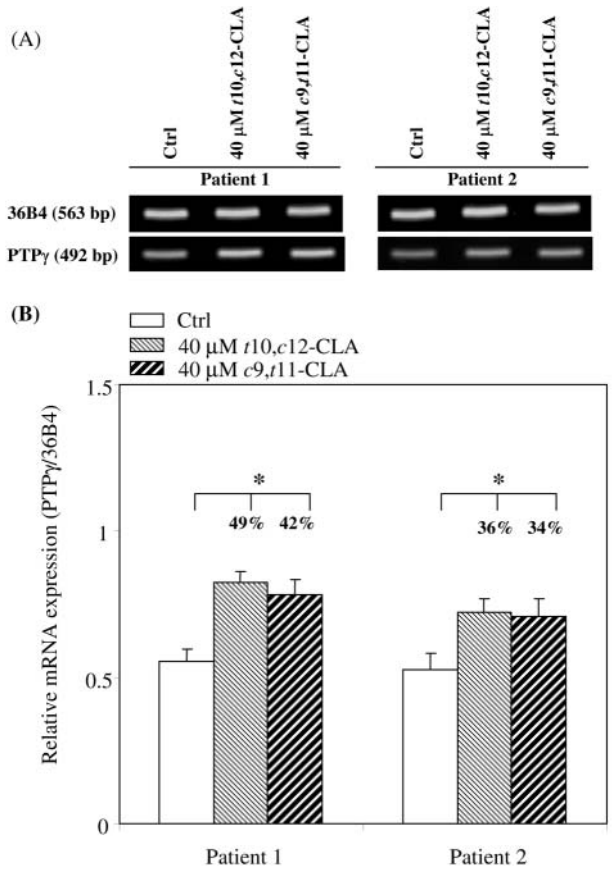

Figure 6. *CLA up-regulated PTPγ mRNA in primary cultured normal human breast stromal cells. (A) Ethidium bromide-stained PCR products separated in a 1.5% agarose gel. 1 and 2 represent patients 1 and 2, respectively. The cells were treated by 2 CLA isoforms and the total RNA was isolated from each group of cells separately. 36B4 was used as the loading control. (B) The mRNA ratio of PTPγ to 36B4 was measured by densitometry. Bars represent mean±SD, n=3. *p<0.05 stands for control versus CLA.*

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Received August 16, 2005
Accepted October 10, 2005