

Regulation of cell growth by fatty acid-CoA ligase 4 in human hepatocellular carcinoma cells

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Abbreviations: AA, arachidonic acid; FACL4, fatty acid-CoA ligase 4; HCC, hepatocellular carcinoma

Abstract

Fatty acid-CoA ligase 4 (FACL4) is a central enzyme controlling the unesterified free arachidonic acid (AA) level in cells and the free AA is known to induce apoptosis. We have recently reported that expression of FACL4 is upregulated in about 40% of human hepatocellular carcinoma (HCC) and 50% of HCC cell lines, suggesting that FACL4 may be involved in liver carcinogenesis. In this study, we investigated whether HCC cell growth is regulated by FACL4. Immunoblot analysis showed that SNU 398 cells express very low or no detectable level of FACL4. We, therefore, transfected the SNU 398 cells with FACL4 expression vector, and clones expressing FACL4 were pooled and analyzed. We found that forced expression of FACL4 in SNU 398 promotes the growth of cells. In addition, we observed that triacsin C, a FACL4 inhibitor, inhibits the growth of Hep 3B cell line which expresses high level of endogenous FACL4. We also found that the triacsin C-mediated growth inhibition in Hep 3B cells results from the induction of apoptosis with evidence of Bcl-2 reduction. Altogether, our data show that FACL4 affects HCC cell growth and suggest that modulation of FACL4 expression/activity is an approach for treatment of HCC.

Keywords: apoptosis; arachidonic acid; carcinoma, hepatocellular; long-chain-fatty-acid-CoA ligase; triacsin C

Introduction

Fatty acid-CoA ligase 4 (FACL4) is a central enzyme controlling the unesterified arachidonic acid (also called free AA) level in cells (Cao *et al.*, 1998). Free AA entering cells exogenously or released endogenously is rapidly converted to AA-CoA esters by the catalytic action of FACL, particularly by the AA-preferring FACL4 (Wilson *et al.*, 1982; Cao *et al.*, 1998). Several lines of evidence indicated that the level of free AA in cells regulate apoptosis (Reid *et al.*, 1991; Hayakawa *et al.*, 1993; Sapirstein *et al.*, 1996; Tang *et al.*, 1996; Chan *et al.*, 1998). Also, overexpression of FACL4 prevented AA-induced apoptosis by reducing the level of intracellular free AA (Cao *et al.*, 2000). These findings, together with overexpression of FACL4 in colon adenocarcinoma strongly suggest that the FACL4 pathway may be important in colon carcinogenesis (Cao *et al.*, 2001).

Hepatocellular carcinoma (HCC) is one of the most common tumors worldwide and is one of the leading causes of death among cancer patients in Korea. Identification of genes that are overexpressed in HCC is of importance to understand liver tumorigenesis and to develop diagnostic and therapeutic targets. Recently, we have shown for the first time that FACL4 is upregulated in HCC compared with adjacent normal liver tissues (Sung *et al.*, 2003b). We found that 14 out of 37 (37.8%) HCC express moderate to strong FACL4 in tumor cells, whereas both normal adult and fetal liver tissues showed very weak to no detectable expression by immunostaining. In addition, we found that 4 out of 8 (50%) human hepatoma cell lines express high levels of FACL4 by Northern blot analysis (Sung *et al.*, 2003b). These results show that FACL4 could be a new molecular marker for HCC and suggest that FACL4 pathway may be involved in liver carcinogenesis. In this study, we investigated whether HCC cell growth is regulated by FACL4. We found that forced expression of FACL4 promotes the growth of HCC cells. In addition, we observed that triacsin C, a FACL4 inhibitor, inhibits the growth of the Hep 3B cells by

induction of apoptosis with evidence of Bcl-2 reduction.

Materials and Methods

Cell lines

Cell lines derived from hepatocellular carcinomas (HCCs) were purchased from Korea Cell Line Bank (Seoul). Hep G2 and Hep 3B cells were grown in DMEM with 10% FBS. The SNU 354, SNU 368, SNU 398, SNU 423, SNU 449, and SNU 475 cells (Park *et al.*, 1995) were cultured in RPMI 1640 with 10% FBS.

Construction of FACL4 expression vector

Expression plasmid was constructed using pcDNA3.1/Myc-His(+) B vector (Invitrogen, Carlsbad, CA) as described (Farooq *et al.*, 2001) with minor modifications. Briefly, FACL4 cDNA was prepared by RT-PCR and it was subcloned into the *Bam*HI and *Xho*I sites of the pcDNA3.1/Myc-His(+) B vector, generating pFACL4/Myc-His. The orientation and accuracy of the sequences were confirmed by sequencing. The primers for PCR were 5'-CGGGA-TCCAACGCTATGGCAAAGAGAATA-3' and 5'-CC-GCTCGAGCGTTTGCCTCCATACATTCGT-3'. The *Bam*HI (GGATCC) and *Xho*I (CTCGAG) sites engineered for cloning are underlined.

Construction of stable cell line

The SNU 398 cells were grown to 50-80% confluence and then they were transfected with the control vector [pcDNA3.1/Myc-His(+) B vector] or with the pFACL4/Myc-His vector with using Lipofectamine. After 2 d, the cell cultures were diluted 1:10 in selection medium (RPMI 10% medium with 400 μ g/ml G418), and after 4 weeks, individual clones were isolated and examined for the expression of FACL4.

Immunoblot analysis

The total cell lysates were prepared and the protein concentration was measured using the BCA protein assay (Pierce, Rockford, IL). Protein samples (30 μ g/lane) were separated on 10% polyacrylamide gel and then transferred to nitrocellulose membranes. The membranes were blocked with 6% milk in PBS for 1 h, and they were probed with anti-human FACL4 antibody at a 1:2,000 dilution. HRP-conjugated donkey anti-rabbit Ig (Amersham, Buckinghamshire, UK) was used as the secondary antibody at a 1:5,000

dilution. Bands were visualized using ECL Plus (Amersham). Anti-human FACL4 antibody (a gift from Dr. Prescott in U. of Utah, USA) was raised in rabbit against a synthesized peptide (H2N-MAKRIKAKPTSDKPGC-amide) that corresponds to the deduced N-terminal sequence of human FACL4. For the detection of FACL4/ Myc-His fusion protein, cell lysates were also probed with mouse anti-c-myc antibody (Roche, Mannheim, Germany). HRP-conjugated sheep anti-mouse Ig (Amersham, Buckinghamshire, UK) was used as the secondary antibodies at a 1:5,000 dilution. For the detection of Bcl2, cells were treated with triacsin C for 24 and 48 h, and probed with mouse anti-Bcl-2 antibody (Roche, Germany).

Cell viability assay

Cells were plated overnight at a density of 5,000 cells/well in 96-well plates with 10% FBS-supplemented RPMI medium. The medium was then changed to serum-free medium with various concentrations of triacsin C (Biomol, Plymouth Meeting, PA), and the cells were incubated for the indicated times. The MTT assay was employed to check cell viability as previously described (Sung *et al.*, 2003a). Triacsin C was dissolved in DMSO. The final DMSO concentration in the media was 0.1%.

Analysis of the cell cycle distribution

The cells were collected and washed with PBS and then fixed in 70% ethanol at 4°C overnight. The cell cycle distribution was analyzed by flow cytometry as was previously described (Farooq *et al.*, 2003).

Apoptosis assay

This assay was performed using a Cell Death Elisa kit (Roche, Mannheim, Germany), following the manufacturer's instructions. Briefly, after treatment with 20 μ M triacsin C for 24 and 48 h, cytoplasmic extracts were prepared from 10,000 cells and DNA-histone complexes were measured by reading optical density at 405 nm using a microplate reader.

Statistical analysis

The *t*-test was used to determine the statistical significance of differences. Probability (*P*) values less than 0.05 were considered statistically significant.



Figure 1. FACL4 expression in the HCC cell lines. Expression of FACL4 protein was investigated in 8 human HCC cell lines using anti-human FACL4 antibody (top panel). Membranes were stripped and reprobed with mouse anti-actin antibody (bottom panel).

Results

Expression of FACL4 in the hepatocellular carcinoma cell lines

To investigate whether HCC cell growth is regulated by FACL4, we checked the FACL4 expression in 8 human HCC cell lines by performing immunoblot analysis using rabbit polyclonal anti-human FACL4 antibody. A major band with an approximate molecular weight of 75 KDa was detected (Figure 1). Similar to the previous Northern blot analysis (Sung *et al.*, 2003b), Hep G2, Hep 3B and SNU 423 cells expressed a high level of FACL4, whereas the SNU 354, SNU 368 and SNU 398 cells expressed very low or no detectable levels of FACL4.

The forced expression of FACL4 promotes the growth of SNU 398 cells

To assess whether FACL4 affects the growth of HCC cells, we transfected SNU 398 cells with a FACL4 expression vector (pFACL4/Myc-His). The individual G418 resistant clones were isolated after a few weeks and three clones expressing FACL4 were pooled and named as the 'Sense pool'. SNU 398 cells were also transfected with an empty vector [pcDNA3.1/Myc-His(+) B] and the G418 resistant clones were pooled and named as the 'Control pool'. Forced expression of FACL4/Myc-His fusion protein in the 'Sense pool' was confirmed by immunoblot analysis with using anti-human FACL4 antibody and anti-c-Myc antibody (Figure 2A). Measurements of the effect of FACL4 on cell growth revealed that cell growth was significantly promoted in the cells that expressed FACL4 (Figure 2B). We also found that forced expression of FACL4 in SNU368 cells, which express very low level of FACL4, promotes the cell growth by 40% (data not shown).

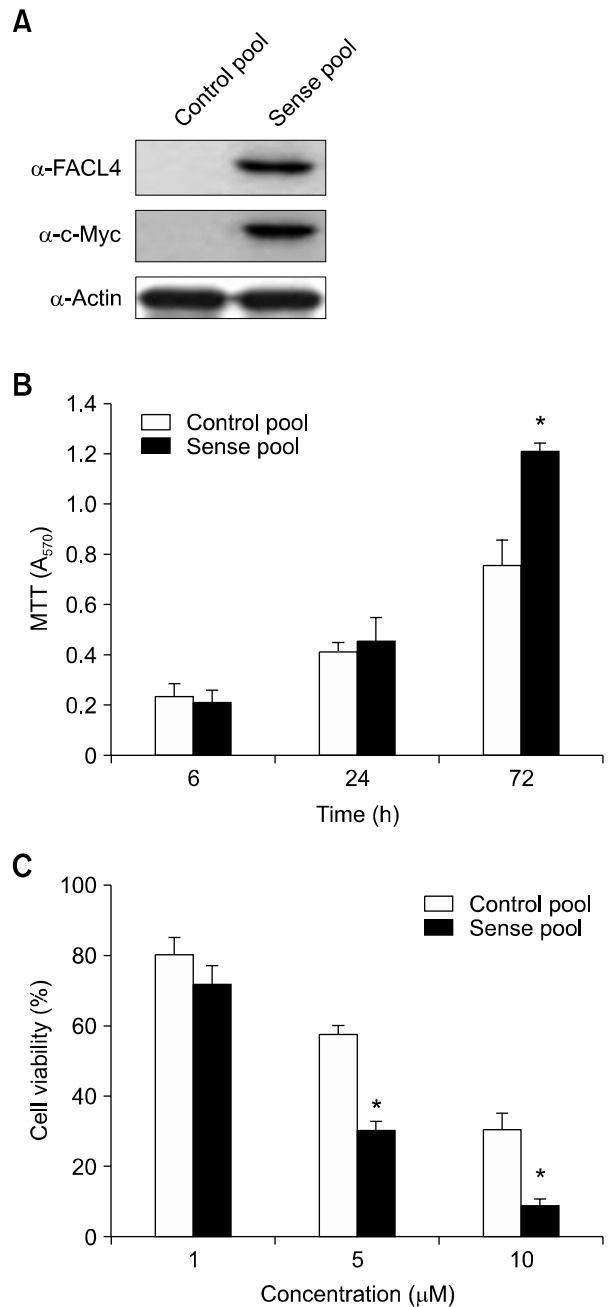


Figure 2. (A) Forced expression of FACL4 in SNU 398 cell line. Expression of FACL4/Myc-His fusion protein was investigated using anti-human FACL4 antibody (top panel) and c-myc antibody (middle panel). Three clones expressing FACL4 were pooled and named as 'Sense pool'. Also, SNU 398 cells were transfected with an empty vector and G418 resistant clones were pooled and named as 'Control pool'. (B) MTT assay showed increased cell proliferation in 'Sense pool'. Values represent the means \pm standard errors of 5 determinations per experiment from 3 independent experiments (* $P < 0.05$ compared to the control). (C) Comparison of cytotoxic effect of triacin C. Values represent the means \pm standard errors of 5 determinations per experiment from 3 independent experiments (* $P < 0.05$ compared to the control).

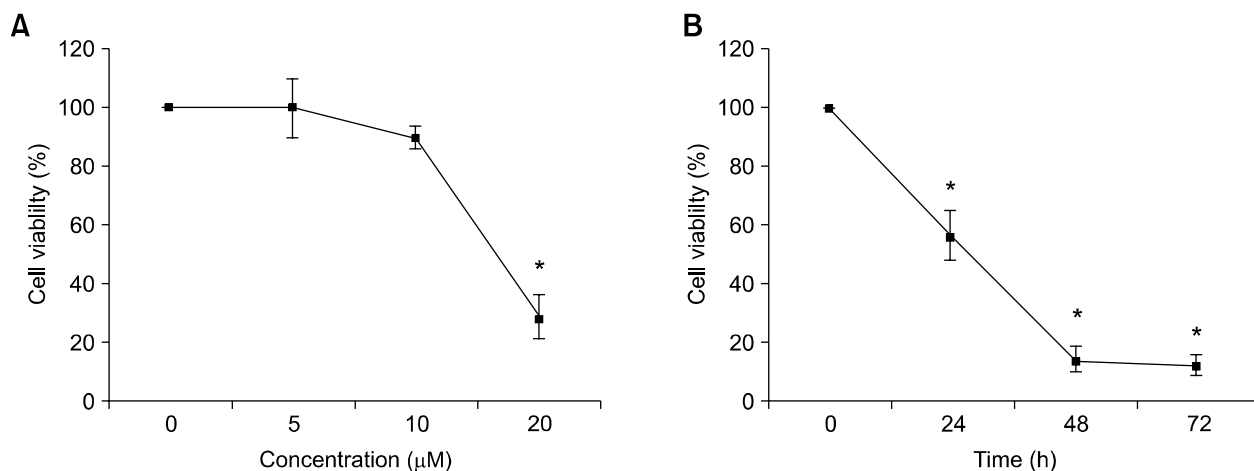


Figure 3. Growth inhibition of the Hep 3B cell line by triacsin C treatment . (A) Dose-dependent growth inhibition. Cells were treated with triacsin C for 72 h. (B) Time-dependent growth inhibition with 20 μM triacsin C. The MTT assay was employed to measure the cell viability in both cases. Data are the means ± standard errors of 5 determinations per experiment from 3 independent experiments (**P* < 0.05).

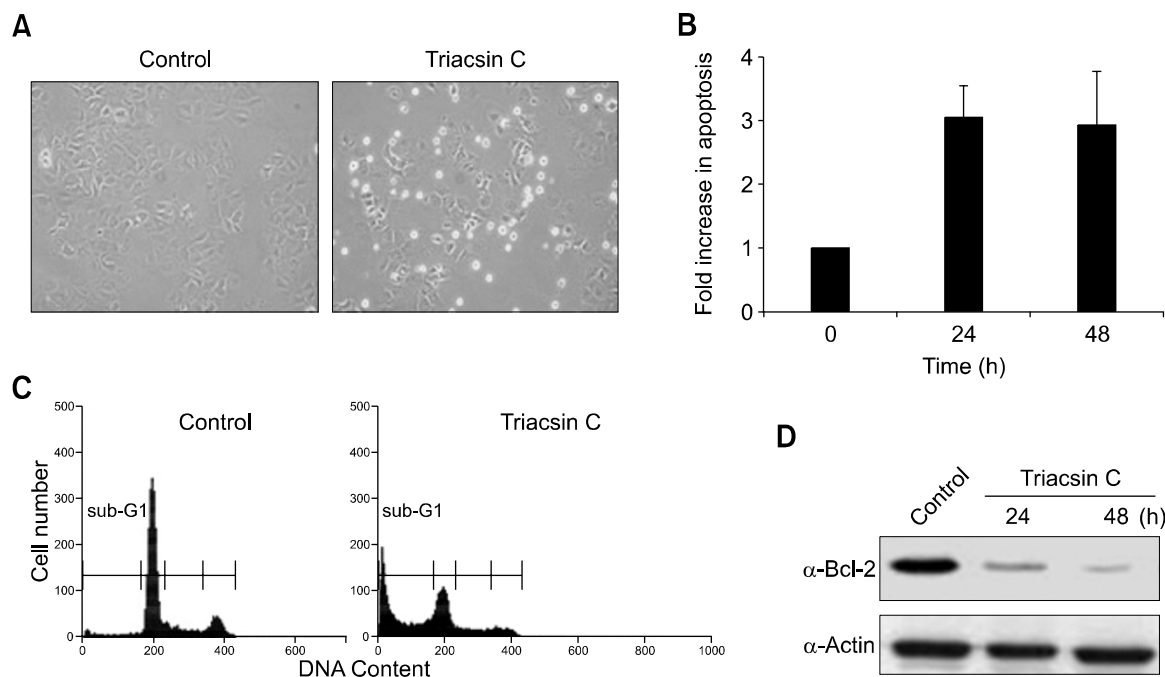


Figure 4. (A) Phase-contrast microscopy of morphological changes associated with apoptosis in Hep 3B cells treated with 20 μM triacsin C for 30 h. (B) Cell death ELISA assay. Cells were treated with 20 μM triacsin C for 24 and 48 h. The results are presented as the fold increase in apoptosis relative to the controls that received DMSO only. Data are the means ± standard errors of 2 determinations per experiment from 2 independent experiments. (C) Cell cycle analysis using flow cytometry. A representative DNA histogram is shown. Cells were exposed to either DMSO or 20 μM triacsin C for 48 h. (D) Immunoblot analysis of Bcl-2. Cells were treated with 20 μM triacsin C for 24 and 48 h.

Triacsin C inhibits the growth of Hep 3B cells

Triacsin C, a FACL4 inhibitor, treatment resulted in dose- and time-dependent growth inhibition in Hep 3B cells (Figure 3). Morphological changes were evident in cells cultured with triacsin C in both serum-free and 10% serum-containing medium: the cells rounded up and detached from the plate

(Figure 4A). We also quantitated the extent of apoptosis with the Cell Death Elisa Assay, which measures DNA fragments associated with cytoplasmic histone. Treatment for 24 and 48 h with 20 μM triacsin C produced about 3.0-fold increases in apoptosis (Figure 4B). Analysis of the cell cycle distribution by flow cytometry showed that the

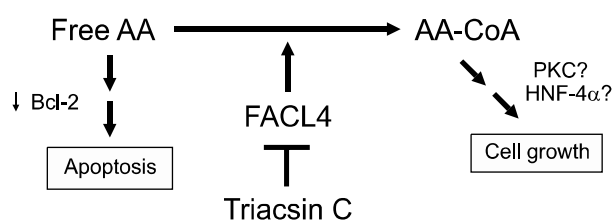


Figure 5. Simple schematic based on this study and previous reports from Tang *et al.* (1996), Faergeman and Knudsen (1997), Kluck *et al.* (1997), Hertz *et al.* (1998), Chan *et al.* (1998), Cao *et al.* (2000, 2001), Sung *et al.* (2003b), and Monjazeb *et al.* (2006).

population of cells in sub-G1 phase, representing apoptotic cells, increased significantly in response to triacsin C treatment (Figure 4C). We also observed that Bcl-2 level is significantly decreased as defined by immunoblot analysis (Figure 4D). Consistent with the above findings, we found that cytotoxic effect of triacsin C is significantly higher in SNU398 cells engineered to over-express FACL4 (Figure 2C).

Discussion

FACL4 is a central enzyme controlling the un-esterified free AA level in cells (Cao *et al.*, 1998). In this study, we have shown that forced expression of FACL4 promotes the growth of SNU 398 HCC cells. This result is in accord with the recent report of growth inhibition of Hep 3B cells by disrupting the expression of endogenous FACL4 using siRNA (Liang *et al.*, 2005). In addition, in a preliminary study, we found that antisense oligonucleotides directed against the translation start region of the FACL4 suppress Hep 3B cell growth (unpublished results). Altogether, these results show that FACL4 plays a growth-promoting role in hepatocellular carcinoma. The growth promotion by overexpression of FACL4 might be due to depletion of free AA, which is known to induce apoptosis via activation of caspase-3 (Chan *et al.*, 1998; Cao *et al.*, 2000; Monjazeb *et al.*, 2006). This scenario is in line with the reduction of AA-induced apoptosis by overexpression of FACL4 in human kidney epithelial 293 cells (Cao *et al.*, 2000). Overexpression of FACL4, thereby increasing the level of AA-CoA esters, might also promote cell proliferation and cell growth by regulating signaling molecules such as protein kinase C and binding to transcription factors such as hepatic nuclear factor-4 α , since fatty acyl-CoA esters have been shown to be involved in these processes (Faergeman and Knudsen, 1997, Hertz *et al.*, 1998). Further studies are needed to elucidate the

biochemical pathways involved in the FACL4-mediated growth promotion.

We also showed here that triacsin C inhibits the growth of the Hep 3B cells which express a high level of endogenous FACL4. Morphological changes, together with Cell Death Elisa Assay and cell cycle distribution analysis showed that triacsin C induces apoptosis in Hep 3B cells. Our data is consistent with the recent report of the induction of apoptosis in HT29 and HCT-116 human colon cancer cell lines by triacsin C (Cao *et al.*, 2000; Monjazeb *et al.*, 2006). In addition, in view of the fact that the free AA down-regulates Bcl-2 (Cao *et al.*, 2000), we anticipated that the level of Bcl-2 would be reduced during triacsin C-induced apoptosis in Hep 3B cells, and this has proved to be the case (Figure 4D). Therefore, triacsin C, by inhibition of FACL4 activity, is believed to induce accumulation of free AA, which in turn induces apoptosis. Our data also suggests that triacsin C-induced apoptosis in Hep 3B cells may be due to mitochondrial cytochrome c release leading to caspase-3 activation since Bcl-2 is known to prevent these processes (Kluck *et al.*, 1997; Yang *et al.*, 1997). Indeed, Monjazeb *et al.* (2006) very recently showed caspase-3 activation in colon cancer HCT-116 cells by triacsin C.

In conclusion, we, first time to our knowledge, show that overexpression of FACL4 promotes the growth of HCC cells. We also showed that triacsin C induces apoptosis of HCC cells via Bcl-2 reduction. Our data demonstrate that FACL4 is of importance in human hepatocarcinogenesis and suggest that modulation of FACL4 expression/activity is an approach for treatment of HCC.

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