Regulation of SCD1 expression by the *Polysiphonia morrowii* extract

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Regulation of SCD1 expression by the *Polysiphonia morrowii* extract

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SCD1 (Stearoyl-Coenzyme A desaturase 1) is the rate-limiting enzyme that catalyzes the synthesis of monounsaturated fatty acids from saturated fatty acids. The unsaturated fatty acids produced by

SCD1 are essential for the biosynthesis of triglycerides. Increased SCD1 expression and activity has been implicated in many disease conditions as in obesity, insulin resistance, cardiovascular diseases, and cancer. Recently, it has been suggest that inhibition of SCD1 is a modality to control lipogenesis and body weight, and to treat obesity and hyperglyceridemia.

In this study, the effect of the *Polysiphonia morrowii* extract on the plasma concentration of triglyceride was studied with regard to regulation of the SCD1 expression. Mice fed with a chow diet supplemented with the *P. morrowii* extract showed the lower plasma triglyceride level than that in control mice. Microarray analysis of mRNAs in livers revealed that the expression of *Scd1* was down-regulated by the extract significantly. The reduced expression of Scd1 was confirmed by quantitative real-time PCR and Western blot analysis in livers of mice. The treatment HepG2 cells with the *P. morrowii* extract reduced both SCD1 mRNA and protein in a-dose dependent manner. In addition, the extract reduced the expression of the Scd1 mRNA and protein in 3T3-L1 cells during the process of differentiation from pre-adipocyte to adipocyte. The *P. morrowii* extract was able to repress the transcription of *SCD1* regardless of mutations of conserved

regulatory elements, such as LXRE, C/EBP, and SRE in the *SCD1* promoter.

In conclusion, the extract *P. morrowii* down-regulates the expression of SCD1 at the transcriptional level mediated by unidentified transcriptional factors, and it is suggested that the *P. morrowii* extract has the therapeutic potential for treatment of hyperglyceridemia by down-regulating the expression of SCD1.

Key words: hyperglyceridemia, stearoyl-CoA desaturase, unsaturated fatty acid, cardiovascular disease, *Polysiphonia morrowii*, triglyceride, transcription

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I. INTRODUCTION

The increased triacylglycerol (TG) concentration in plasma is one of the important parameters related to the increased incidence of cardiovascular disease (CVD)[1,2]. Plasma TG exists as associated with other lipids in lipoproteins such as very low-density lipoprotein or low-density lipoprotein rather than as TG itself[3]. As the ratio of low density lipoprotein-cholesterol or total cholesterol versus high density

lipoprotein-cholesterol is considered to be the important predictor of CVD, the increased TG level with insulin resistance is a key metabolic abnormality in metabolic syndrome and future development of diabetes[4,5]. In general, it is strongly suggested that pharmacological reduction of the plasma TG level has beneficial health effects particularly in patient with obesity and diabetes[6]. The current therapeutic interventions for reduction of the plasma TG level in patients with obesity and type 2 diabetes are statins, intestinal cholesterol absorption inhibitors, fibrates, niacin, *n*-3 fatty acids, and glucagon-like peptide-1, with additional dietary and lifestyle modifications[7].

Currently, strategies to reduce the plasma TG level by inhibition of specific enzymes involved in *de novo* TG biosynthesis are under investigation very intensively[8,9]. For example, inhibitors of acyl-CoA:diacylglycerol acyltransferase 1 (DGAT1) are reported to improve

insulin sensitivity and hepatic steatosis[9]. Recently, stearoyl-CoA desaturase (SCD; EC 1.44.99.5) has also been elucidated as a potential target for the treatment of obesity and associated disorders[10].

SCD is an iron-containing enzyme catalyzing a rate limiting step in the synthesis of monounsaturated fatty acids from saturated fatty acids[11]. SCD introduces a double bond between carbon atoms 9 and 10 in palmitoyl- or stearoyl-CoA to produce palmitoleoyl- or oleoyl-CoA, respectively. This generation of monounsaturated fatty acids by SCD is important in biosynthesis of TG, membrane phospholipids and cholesterol ester[12,13], and in regulation of membrane fluidity[14], cellular differentiation, and apoptosis[15,16]. Four Scd isoforms, Scd1 through Scd4, have been identified in mouse[17], while two SCD isoforms (SCD1 and SCD5) have been identified in human[18-20]. SCD1 is a constituent of the lipid metabolism predominantly expressed in adipose tissues and liver[21], while SCD5 is a unique isoform expressed in brain and

pancreas[19,22]. The expression of SCD1 is regulated by several dietary and hormonal factors, such as high-carbohydrate diets[23-25], insulin and fructose[26], glucose[27], polyunsaturated fatty acids[25,28], cholesterol[28-30], retinoic acid[30,31], and fibrates[30,32,33]. The mechanism of all these regulations involves the activation of sterol regulatory element-binding protein-1c (SREBP-1c) which is a key transcription factor for the expression of several lipogenic genes[29,33-35].

The significance of SCD1 as a therapeutic target has been derived from the fact that the expression and activity of SCD1 are increased in several disorders, such as cancer[36,37], cardiovascular disease[38], obesity[10,13,39,40], steatosis[41], insulin resistance[42], metabolic syndrome[43], and diabetes[42]. In particular, studies in *Scd1* knockout mice elucidated how valuable the inhibition of SCD1 is in the control of obesity and related metabolic disorders. In *Scd1*$^{-/-}$ mice, fatty acid oxidation in mitochondria is increased in liver, skeletal muscle, and brown adipose

tissue, while lipogenesis is not induced by a diet high in saturated fat diet[44-46]. It has also been reported in *Scd1*$^{-/-}$ mice that signaling of insulin and AMP-activated protein kinase are enhanced in skeletal muscle and brown adipose tissue[45,47] while the lipid accumulation and adiposity are decreased[40,41]. The hepatic TG and cholesterol ester synthesis are greatly reduced in *Scd1*$^{-/-}$ mice, and the concentration of plasma TG decreases by 67% in these mice[48]. In addition to the loss of functional SCD1 in genetic models, pharmacological inhibition of SCD1 using SCD1-specific antisense oligonucleotide inhibitors reduce the synthesis and secretion of fatty acid, while they increase fatty acid oxidation in primary mouse hepatocytes, and prevent diet-induced obesity in mice[49].

In summary, the inhibition of SCD1 expression and activity is suggested to be a potential therapeutic target in the prevention of metabolic diseases. In this study, the extract of *Polysiphonia morrowii*

was evaluated *in vivo* and *in vitro* as a potential candidate of SCD1 inhibitors.

II. MATERIALS AND METHODS

1. General methods and supplies

Delipidated serum (DLPS) was prepared from fetal bovine serum (Invitrogen Brand, Life Technologies, Carlsbad, CA, USA) as described by Hannah et al.[50]. Protein concentrations were determined using a BCA kit (Pierce, Rockford, IL, USA). Cell culture medium and reagents were obtained from Invitrogen. Other reagents not specified were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2. Preparation of the *P. morrowii* extract

The *P. morrowii* was collected at the coast of Jeju Island, Korea. The dried *P. morrowii* was initially rehydrated with 60% methanol in 10 volumes to the dry weight of the seaweed at room temperature with vigorous stirring overnight. Upon removal of the solvent (denoted as the 60% extract), the wet seaweed was re-extracted with 7 volumes of 100% methanol four times for 10 to 14 hr per each extraction at room temperature (denoted as the 100% extract). The 60% and the 100% extract were pooled and evaporated at 52°C using a rotary evaporator

until the volume reaches the one tenth of the original volume. The extract was re-extracted once with the same volume of ethyl acetate. After complete evaporation of ethyl acetate, the dried extract were weighed and solubilized with 100% methanol at a concentration of 50 mg/ml and stored at -20°C, and used as the *P. morrowii* extract. The *P. morrowii* extract was further fractionated by hydrophobic interaction chromatography using octadecylsilyl (ODS) column (YMC Co. Ltd., Kyoto, Japan) with differential concentrations of methanol. The fractions were dried by evaporation, dissolved in 100% methanol, and stored at -20°C. For administration of the extract to mice and treatment of cells, each extract was dried completely to remove methanol and solubilized in DMSO just before use.

3. Cell culture

HepG2 (ATCC number HB-8065) cells were maintained in medium A (DMEM containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C under a humidified atmosphere of 5% CO_2. For induction of SCD1, cells were set up on day 0 (2×10^5cells/well in 12 well plate) in medium A supplemented with 10% FBS. On day 1, cells

were washed twice with PBS and changed to medium A supplemented with 10% DLPS in the presence or absence of the *P. morrowii* extract at indicated concentration. On day 2, cells were washed twice with PBS, harvested, and processed for immunoblot analyses.

Preadipocyte 3T3-L1 cells were maintained in medium A DMEM containing 8 μg/ml biotin, and supplemented with 10% (v/v) heat-inactivated calf serum at 37°C under a humidified atmosphere of 10% CO_2. To induce differentiation of 3T3 cells, on post-confluent day 2, cells were changed to medium A supplemented with 10% FBS and MDI (0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μg/ml insulin), then maintained for 2 days. Cells were then changed to DMEM medium containing 10% FBS and 1 μg/ml insulin. Two days after final medium change, cells were processed for oil-red O staining or harvested for immunoblot analyses.

4. Immunoblot analysis

After treatment, cells were washed with PBS and lysed with 100 μl of lysis buffer containing 0.33 M NaCl, 1.1 M urea, 1% Nonidet P-40, 25 mM HEPES (pH 7.6), and proteinase inhibitors (1 mM DTT, 10 μg/ml leupeptin, 5 μg/ml pepstatin A, 1 mM PMSF and 2 μg/ml

aprotinin) by adding directly onto the plate. Cell lysates were harvested and further vortexed at room temperature for 5 min for the complete liberation of proteins. Lysate were cleared by centrifugation at 20,000 g for 10 min at 4°C, and the supernatants were collected as whole cell lysate. After quantitation of proteins, aliquots of proteins were subjected to 10 % SDS-PAGE and transferred onto nitrocellulose ECL membranes (GE Healthcare, Piscataway, NJ, U.S.A.) and immunoblot analyses were performed using the SuperSignal West Pico Chemiluminescent Substrate System (Pierce). The following antibodies were used in the current studies: anti-SCD1 and anti-GAPDH from Cell Signaling Technology, Inc. Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce. The polyclonal antibodies against human FASN and ACLY were kindly provided by Kyung-sup Kim (Department of Biochemistry and Molecular Biology, Yonsei University College of Medicine, Seoul).

5. Microarray analysis and quantitative real-time PCR (RT-PCR)

Total RNA was prepared from HepG2 cells or livers of mice using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) or Trizol reagent (Invitrogen), respectively, according to the manufacturer's instruction.

The total RNAs from livers of mice were subjected to a microarray analysis using Illumina MouseRef-8 v2 expression BeadChip (Illumina, Inc., San Diego, CA). The bioinylated cRNA were prepared from 0.55 μg of total RNA using the Illumina TotalPrep RNA amplification Kit (Ambion, Austin, TX). Following fragmentation, 0.75 μg of cDNA were hybridized to the chip according to the protocols provide by the manufacturer. Array were scanned using the Illumina Bead Array Reader Confocal Scanner. The array data export processing and analysis were performed using Illumina GenomeStudio v.2011.1.

For RT-PCR, removal of DNA from RNA was achieved by a DNA-free kit (Ambion Brand, Life Technologies, Austin, TX) for RNA from tissues or with an RNase-Free DNase (Qiagen) for RNA from cells. cDNA was synthesized from 2 μg of DNase-treated total RNA using a High-Capacity cDNA Archive kit (Applied Biosystems Brand, Life Technologies, Foster City, CA, USA). Quantitative RT-PCR was performed using StepOne™ Real-Time PCR Systems (Applied Biosystems). All reactions were done in dulicate, and relative amounts of all mRNAs were calculated by the comparative cycle-time method.[51] *GAPDH* mRNA was used as the invariant control. The sequences of the specific primers for each gene are as follows:

Forward primer for *Scd1*: 5'-AGCGGTACTCACTGGCA-3'

Reverse primer for *Scd1*: 5'-CCTACGACAAGAACATTCAATCC-3'

Forward primer for *Elovl1*: 5'-ATCAGCTCGTGGTACAAGTTC-3'

Reverse primer for *Elovl1*: 5'-CCTGATCCCTTTGAACCC-TTC-3'

Forward primer for *Elovl6*: 5'-GTACAGCATG-TAAGCACCAGT-3'

Reverse primer for *Elovl6*: 5'-CAAGCGAGCCAAGTTTGAAC-3'

Forward primer for *Fasn*: 5'-GCTGCGGAAACTTCAGGAAAT-3'

Reverse primer for *Fasn*: 5'-AGAGACGTGTCA-CTCCTGGACTT-3'

Forward primer for *Acly*: 5'-GCCAGCGGGAGCACATC-3'

Reverse primer for *Acly*: 5'-CTTTGCAGGTGCCAC-TTCATC-3'

Forward primer for *Srebp-1c*: 5'-GCAGCCATGGATTGCACATT-3'

Reverse primer for *Srebp-1c*: 5'-GGCCCGGGAAGTCACTGT-3'

Forward primer for *Gapdh*: 5'-TGTGTCCGTCG-TGGATCTGA-3'

Reverse primer for *Gapdh*: 5'-CCTGCTTCACCACCTTCTTGAT-3'

Forward primer for *GAPDH*: 5'-GCCCCAGCGTCAAAGGT-3'

Reverse primer for *GAPDH*: 5'-GGCATCCTGGGCTACACTGA-3'

The primers for human SCD1 were purchased from Qiagen (Cat. No: QT00052381).

6. Cell cytotoxicity assay

Cytotoxic effect of the *P. morrowii* extract on the viability of HepG2 cells were determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay using the CellTiter 96® Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA). HepG2 cells were grown in 96-well plates at a density of 0.5 x 10^4 cells per well in 0.25 ml of medium A supplemented with 10% FBS on day 0. On day 1, cells were washed twice with PBS and changed to medium A supplemented with 10% DLPS in the presence indicated concentration of the *P. morrowii* extract. Cell survival (% of control) was calculated as the absorbance at 470 nm of extract-treated cells relative to those of DMSO-treated control cells.

7. Construction of the *SCD1* promoter-reporter plasmids, transient transfection, and reporter assay

The first base of the translated ATG in the human *SCD1* genomic sequence was arbitrarily annotated as +1. The *SCD1* promoter fragment[35] spanning from -1407 to -1 was amplified by PCR from human genomic DNA using the following primers: 5'-

ATGGTGTTTCTGGGCTCAAC-3' and 5'-ACCATCAAGCTTCTTG-GCTCTCGGATGCC-3'. The amplified DNA product was digested with *Kpn*I and *Hin*dIII and gel-purified using the Gel purification kit (Intron Biotechnology, Sungnam, Kyungkee-do, Korea). This digested fragment (-1295 to -1) was cloned into *Kpn*I and *Hin*dIII sites of pGL3-basic vector (Promega). Mutant clones for LXR response element (LXRE)[52], the binding site for CCAAT/enhancer-binding proteins (C/EBPs)[53] and sterol regulatory element (SRE)[35] were generated from the wild-type *SCD1* promoter construct using the QuickChange site-directed mutagenesis kit (Agilent Technologies) and the following forward primers: LXRE mutant, 5'-AGGAAATACCGGACACAATCACCCGTAACCAGCTCTAGCCTTTAAA-3'; C/EBPs mutant, 5'-CGGATAAAAGGGGGCCTAGAAG-ATACCGGACACGGTC-3'; SRE mutant, 5'-GGCGGCAGAGGGAACCGCC-GCTTGCGCCGAGCCAAT-3'. Locations of mutated nucleotides are underlined. The sequences of reverse primers are complementary to those of forward primers. The integrity of construct sequences was confirmed by DNA sequencing. HepG2 cells were transiently cotransfected in suspension state with promoter-reporter constructs and pTK-renilla control vector (Promega)

using Lipofectamine 2000 (Invitrogen) according to the method described by Jeong et al.[54] and luciferase activity was analyzed by the Dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instruction. Briefly, on day 0, HepG2 cells were transfected with plasmids, and cells were changed to medium A supplemented with 10% DLPS in the presence or absence the *P. morrowii* extract on day 1. On day 2, cells were washed twice with PBS, harvested, and cell lysate were prepared with passive lysis buffer (Promega) according to the manufacturer's instructions. The firefly luciferase activity driven by the *SCD1* promoter was normalized by the renilla luciferase activity and the amounts of protein in the lysate.

8. Animal experiments

Eight- to 10-week old male C57BL/6J mice purchased from Japan SLC, Inc. (Shizuoka Prefecture, Japan) were maintained on 12-h dark/12-h light cycles and fed a standard chow diet (LabDiet, St. Louis, MO, USA) *ad libitum* and had free access to tap water. All mice were sacrificed at the end of the dark cycle. The diet supplemented with the *P. morrowii* extract was prepared by adding the extract at a concentration of 40 mg% (w/v) into the milled chow diet. The powered diets were

reformed into pellets with the minimum amounts of distilled water. The mock diet contained the DMSO only as a vehicle. After treatment, livers were collected and stored at -80°C until later use. All animal experiments were performed with the approval of the Institutional Animal Care and Research Advisory Committee at Yonsei University College of Medicine.

III. RESULTS

1. The *P. morrowii* extract lowers the level of plasma TG

To evaluate the effect of the *P. morrowii* extract in mice, 8 to 10-week old male mice were fed a standard chow diet supplemented with 40 mg% (w/w) of the extract *ad libitum* for 82 day. Assuming that a mouse eats 5 g of diet a day, the concentration at 40 mg% of the extract in the diet was postulated to supply a mouse in 25 g of body weight with the extract in the amount of 80 mg/kg of body weight per day. Table 1 lists several relevant metabolic parameters of mice fed a diet with the *P. morrowii* extract. The plasma concentration of TG in mice fed with the *P. morrowii* extract was decreased significantly by 49% ($P < 0.01$) compared to that in control mice fed a chow diet supplemented with DMSO, the vehicle for the extract solubilization. The reduction of TG in plasma by the *P. morrowii* extract was observed consistently in 3 independent experiments. However, the other parameters remained unchanged by the *P. morrowii* extract.

Table 1. Metabolic parameters of mice fed with the *P. morrowii* extract

Parameter	DMSO	*P. morrowii*
No. & Sex	9 males	10 males
Body weight (g)	33.5 ± 1.2	33.5 ± 0.6
Liver weight (g)	1.63 ± 0.06	1.58 ± 0.34
Liver wt./body wt.	4.88 ± 0.10	4.72 ± 0.04
Plasma TG (mg/dl)	155 ± 10.4	79 ± 7.6*
Plasma cholesterol (mg/dl)	97 ± 4.81	79 ± 3.9

Male mice, 8-10 weeks of age, were fed a chow diet supplemented with 40 mg% (w/w) of the *P. morrowii* extract ad libitum for 82 days. Each value represents the mean ± S.E. of 9 or 10 values. Asterisks denote the level of statistical significance (Student's *t* test). *, $P < 0.05$. The similar results were observed in three independent experiments.

2. Expression of lipogenic genes in livers of mice was decreased by the *P. morrowii* extract

To determine the effect of the *P. morrowii* extract on the genes related to TG metabolism, the microarray analysis was performed using total RNA was isolated from pooled livers of mice described in the Table 1. Microarray analysis identified 201 genes up-regulated more than 30% in livers of mice fed a diet supplemented with the *P. morrowii* extract, and 288 genes down-regulated more than 30% by the *P. morrowii* extract. Among these affected genes, 12 lipogenic genes were focused for further evaluation in this study (Table 2). Table 3 shows the changes in transcripts of genes that play an important role in triacylglycerol metabolism.

Table 2. Number of genes whose transcripts changed by more than 30% in livers of mice by the *P. morrowii* extract analyzed by cDNA microarray

Gene cluster	Up-regulated	Down-regulated
Apoptosis	1	1
Amino acid metabolism	2	5
Carbohydrate metabolism	7	3
Cell cycle	1	6
Lipid, fatty acid metabolism	12	27

Each value represents the number of genes of which mRNAs were up- or down-regulated in livers of mice fed a chow diet supplemented with the *P. morrowii* extract as described in Table 1.

Table 3. Relative levels of gene transcripts which decreased significantly in livers of mice by the *P. morrowii* extract analyzed by cDNA microarray

Gene	Relative expression
Srebp-1	0.74
Fasn	0.70
Elovl1	0.66
Acly	0.61
Scd1	0.57
Elovl6	0.5

Each value represents the relative level of gene transcripts in livers of mice fed the *P. morrowii* extract compared to that in livers of control mice, which is arbitrarily defined as 1. *Srebp-1,* sterol regulatory element-binding protein-1; *Fasn,* fatty acid synthase; *Elovl1,* ELOVL family member 1, elongation of long chain fatty acids; *Acly,* ATP citrate lyase; *Scd1,* stearoyl-CoA desaturase-1; *Elovl6,* ELOVL family member 6, elongation of long chain fatty acids.

Changes in the expression of selected genes were further verified by RT-PCR using the total RNA isolated from pooled livers of mice used in the experiment for the Table 1 (Fig. 1). Amounts of *Fasn*, *Scd1, and Elovl6* mRNAs were decreased by the *P. morrowii* extract to the levels comparable to those analyzed by microarray, while amounts of *Srebp-1c and Acly* mRNAs has changed in a minimal range. RT-PCR quantification showed no significant change in the amount of Elovl1 mRNA by the extract. Immunoblot analysis showed that the amount of Scd1 protein was decreased most significantly in livers of mice fed a diet supplemented with the *P. morrowii* extract compared to that in control mice, while changes in amounts of Fasn and Acly proteins by the extract were relatively small (Fig. 2). These results suggest that expression of SCD1 is a potential target for reduction of the plasma concentration of triacylglycerol by the *P. morrowii* extract.

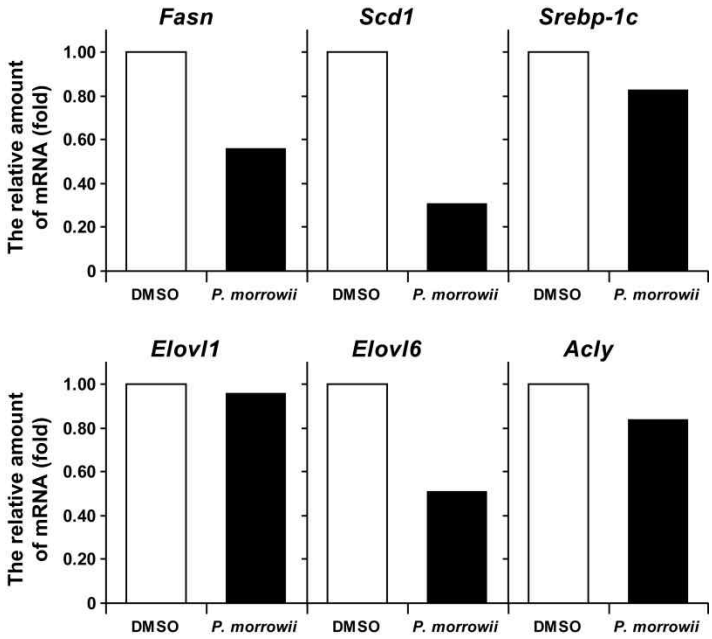


Figure 1. Effect of the *P. morrowii* extract on amounts of mRNAs for lipogenic genes in livers of mice analyzed by RT-PCR

Total RNAs from pooled livers of mice described in Table 1 were subjected to RT-PCR as described under "Materials and Methods". Each value represents the amount of mRNA in livers of mice fed the *P. morrowii* extract (*P. morrowii)* relative to that in livers of control mice (DMSO), which is arbitrarily defined as 1. The amount of mRNA was normalized by that of *Gapdh* mRNA. Values were measured in duplicate reactions. Similar results were obtained in two independent experiments.

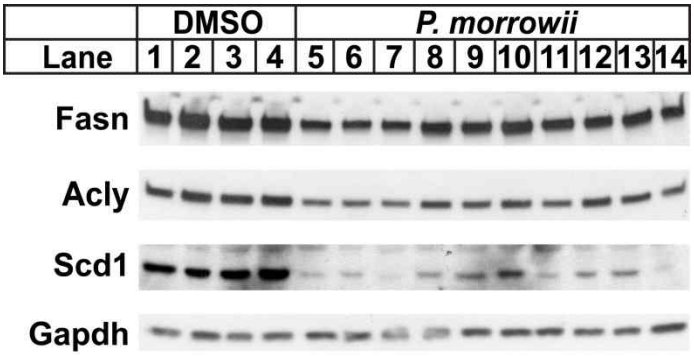


Figure 2. Effects of the *P. morrowii* extract on the expression of lipogenic enzymes in livers of mice analyzed by immunoblot

Lysates were prepared from pooled livers of two or three mice of the control group (DMSO) or from individual livers in the extract-fed group (*P. morrowii*). Mice used in this experiment are described in Table 1. The aliquotes of protein were subjected to SDS-PAGE and immunoblot analysis as described under "Materials and Methods". Fasn, fatty acid synthase; Acly, ATP citrate lyase; Scd1, stearoyl-CoA desaturase 1; Gapdh, glyceraldehyde dehydrogenase.

3. Determination of the highest concentration of the *P. morrowii* extract for treatment in HepG2 cells

For determination of the highest concentration of the *P. morrowii* extract applicable to an experiment *in vitro*, cell toxicity was evaluated by the MTT assay. The treatment of HepG2 cells with the extract at concentrations of up to 100 μg/ml showed a minimal cytotoxic effect both for 18hr (86% viability compared to vehicle treated cells, Fig. 3A) and for 3 days (82% viability compared to vehicle treated cells, Fig. 3B). The viability of HepG2 cells were dramatically decreased by treatment with the extract at a concentration 1 mg/ml for 18 hr (34% viability compared to vehicle treated cells). These results suggest that IC_{50} (half maximal inhibitory concentration) of the *P. morrowii* extract is between 300 μg/ml and 1 mg/ml when treated for 18 hr. According to this cytotoxicity evaluation, the concentration of the *P. morrowii* extract at 100 μg/ml or below was used in following studies *in vitro*.

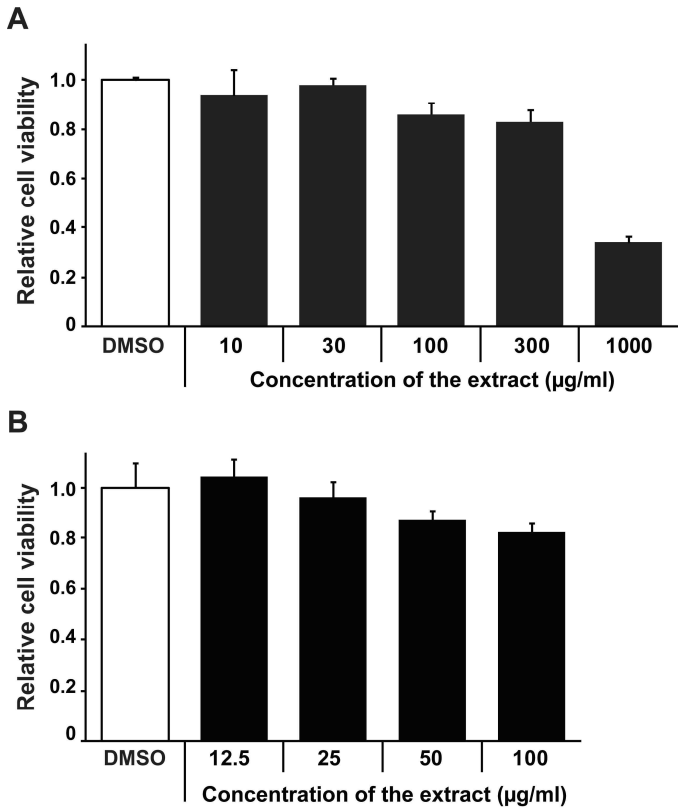


Figure 3. Cell viability assay of the *P. morrowii* extract in HepG2 cells

HepG2 cells were treated with indicated concentrations of *P. morrowii* extracts for 18 hr (A) or 3 days (B) in medium A supplemented with 10% FBS, and cell viability was determined by using MTT assay as described under "Materials and Methods". For 3-day treatment, fresh medium with the extract was changed at 48 hr after initial treatment. Values represent the cell viability relative to that in vehicle-treated cells (DMSO) which is arbitrarily defined as 1. Each value represents mean ± SD from triplicate reactions.

4. Expression of SCD1 is decreased by the *P. morrowii* extract in HepG2 cells

To determine whether SCD1 expression is decreased by the *P. morrowii* extract *in vitro*, HepG2 cells were setup in medium A supplemented with 10% FBS for 24h, then changed to medium A supplemented with 10% DLPS in the presence of indicated concentrations of the *P. morrowii* extract for 18 hr. The expression of SCD1 was evaluated by immunoblot analysis and RT-PCR. The *P. morrowii* extract decreased the amount of the SCD1 protein (Fig. 4A) and mRNA (Fig. 4B) in a dose-dependent manner.

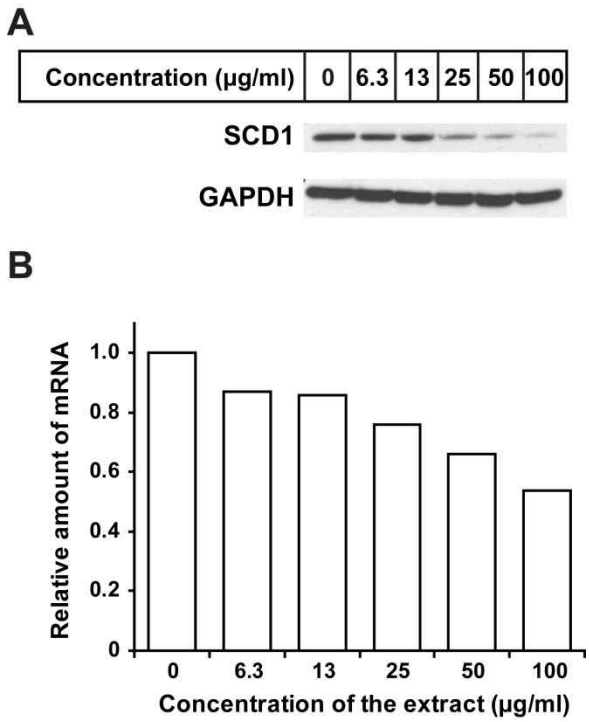


Figure 4. Regulation of SCD1 by the *P. morrowii* extract in HepG2 cells

(A) HepG2 cells were treated with the indicated concentration of the *P. morrowii* extract for 18 hr, and the whole cell lysates were subjected to immunoblot analysis for stearoyl-CoA desaturase-1 (SCD1). GAPDH protein was used as a loading control. (B) Total RNAs from the cells were prepared and subjected to quantitative RT-PCR as described under "Materials and Methods". The quantity of mRNA was normalized to that of *GAPDH* mRNA. Each value represents the relative amount of *SCD1* mRNA compared to that in vehicle-treated cells (the concentration of 0) in triplicate reactions. Similar results were obtained in three independent experiments.

5. Expression of SCD1 is decreased by the *P. morrowii* extract in 3T3-L1 preadipocytes

To determine whether the *P. morrowii* extract has the same effect in cells with more active fat metabolism than HepG2 cells, the expression of Scd1 in 3T3-L1 cells, a well-characterized model of adipogenesis[55], was also evaluated. 3T3-L1 cells were treated with the *P. morrowii* extract at indicated concentrations on the day of induction of differentiation by MDI (day 0; Fig. 5, D0), and the expression of Scd1 was determined at different time points after induction of differentiation. Fig. 5A shows that preadipocytes 3T3-L1 cells on day 0 barely express Scd1 protein in an undetectable level by immunoblot analysis with the antibody used in this study. On day 2, the expression of Scd1 protein and mRNA (Fig. 5B) in 3T3-L1 cells increased sharply, and continued to increase by day 4. The extract significantly blocked this induction of Scd1 in a dose-dependent manner. To evaluate the change in *Scd1* mRNA, a quantitative RT-PCR was carried out using the total RNA isolated from 3T3-L1 cells and analyzed by RT-PCR. Treatment of 3T3-L1 cells with the *P. morrowii* extract decreased the expression of *Scd1* mRNA significantly (Fig. 5B). On day 4, the mRNA level of *Scd1* was decreased by the extract approximately to 48% compared to control.

These data suggest that the *P. morrowii* extract effectively blocks expression of Scd1 in the course of differentiation of 3T3-L1 preadipocytes to adipocytes.

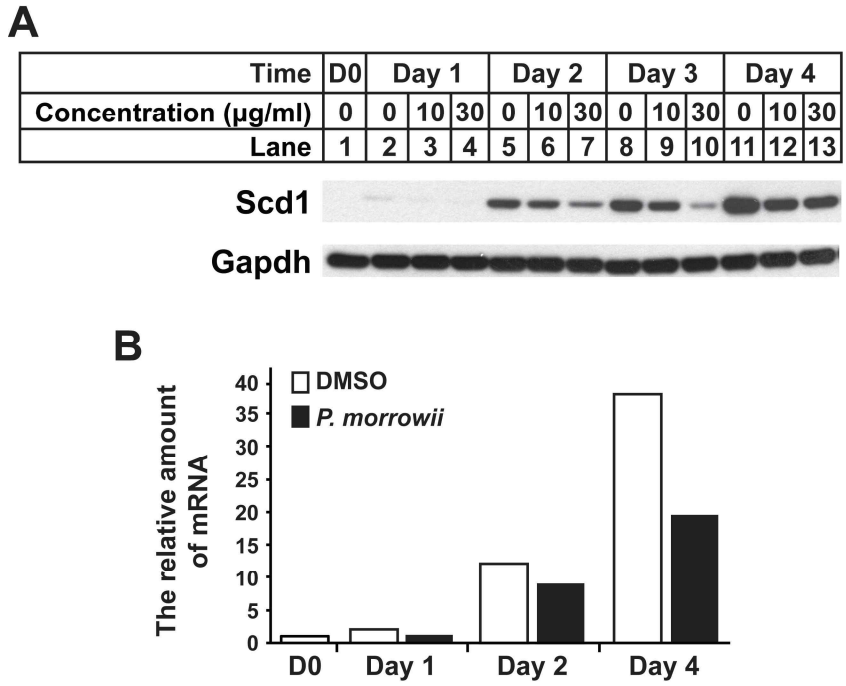


Figure 5. Effects of the *P. morrowii* extract on Scd1 expression in the process of adipocyte differentiation

Preadipocyte 3T3-L1 cells were induced for differentiation as described under "Materials and Methods", and effects of the *P. morrowii* extract on the expression of Scd1 in the process of 3T3-L1 differentiation were determined by immunoblot analysis (A) and by quantitative RT-PCR (B). D0 denotes the post-confluent cells before induction of differentiation by MDI. In RT-PCR experiment (B), the extract was treated to cells at a concentration of 30 μg/ml (*P. morrowii*). Each value in (B) represents the relative amount of mRNA compared to that in vehicle-treated cells (DMSO) on day 0 in duplicate reactions. Similar results were obtained in three independent experiments.

6. Regulation of transcriptional activity of the human *SCD1* promoter by the *P. morrowii* extract

To determine whether decrease in the SCD1 mRNA by the *P. morrowii* extract is caused by transcriptional regulation of the *SCD1* promoter, HepG2 cells were transiently transfected with *SCD1* promoter-reporter constructs and luciferase reporter assay was performed. The transcriptional activity of the *SCD1* promoter region (-1295 to -1) was decreased by the *P. morrowii* extract in a dose-dependent manner (Fig. 6). This result suggests that the expression of SCD1 is down-regulated by the *P. morrowii* extract at a transcriptional level.

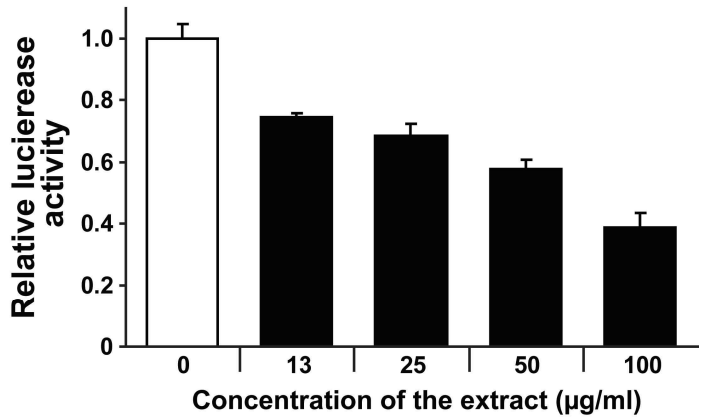


Figure 6. Down-regulation of the *SCD1* promoter activity by the *P. morrowii* extract in HepG2 cells

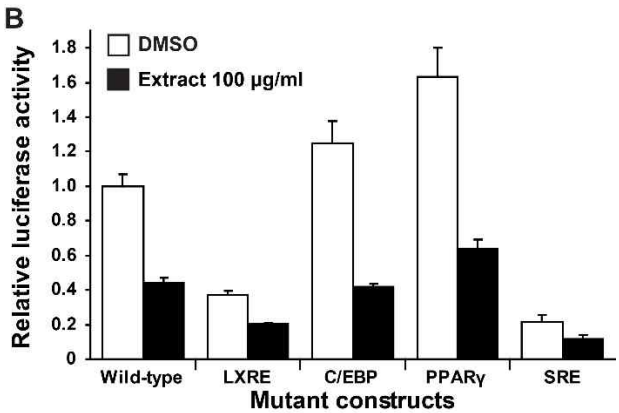
HepG2 cells were transiently cotransfected with the *SCD1* promoter-reporter constructs and pRL-RSV renilla vector, and luciferase activity was analyzed as described under "Materials and Methods". The firefly luciferase activity driven by the promoter was normalized to the renilla luciferase activity and the amounts of protein in the lysate. The relative luciferase activity denotes the promoter activity in cells treated with the extract at the indicated concentration of the extract relative to that in cells treated with DMSO (concentration at 0). Each value represents the mean ± SD of three independent experiments (each in duplicate reactions).

7. Regulation of transcription factors by the *P. morrowii* extract in the *SCD1* promoter

To determine the functionally important DNA elements that mediate the transcriptional regulation of the *SCD1* promoter by the *P. morrowii* extract, mutant clones for consensus sequences that are reported to be implicated in lipid metabolism were generated and the reporter assay was carried out (Fig. 7). Sequence analysis revealed that human *SCD1* promoter region spanning from -1295 to -1 contains several potential transcription factor-binding elements as follows: LXRE[52], the binding site for C/EBP[35], and SRE[35]. Quadrilaterals are locations of mutated nucleotides (Fig 7A). The *P. morrowii* extract reduced the transcriptional activity of the wild-type *SCD1* promoter (Fig 7B) as shown above in Fig. 6. Introduction of mutations in LXRE and SRE in the *SCD1* promoter resulted in reduced basal transcriptional activity compared to that of the wild-type construct, while mutations in C/EBP resulted in slight increase in basal transcriptional activity. Although these consensus elements are expected to play an important role for *SCD1* transcription, any mutations had no effect on the action of the *P. morrowii* extract to reduce *SCD1* transcription by 44% (SRE mutant) to 67% (C/EBP mutant). These results suggest that down-regulation of the

SCD1 transcription by the *P. morrowii* extract is achieved by the other mechanism than involving transcription factors reported previously, and that future elucidation of a new pathway mediating down-regulation of the SCD1 expression by the extract is required.


A
-1295 GGTACCCACCTCCCAGGGTCACAGAGAGGCTTACAGAAAACGATTCTTGTGAATTGGCTTGCAGTAATAATTCAATACCTGCCAGCTATTCTTATTCCAC
-1195 ATCCAAGCCCTTTCGCCTGCTGCTGGGTGAAAACACATGTCAGTGTTTCCTGACGGTTTCCACAAAGAAGATTCCAAAATTACAACCTGCCAGTCTGAAG
-1095 AATCTCCAAAACATCCCGCACGCATCCTGGAGGCGCGGGCTTGGGGATGGGACTGCCCGCCCGGGTCCTGAACAGGATGCGTGCGCGCAGGCACACACAC
-995 ACCAGCCAGCCTGTGTGTGCGGCCGGAGTCCGGTGCGGTCCCGGGTGAGCAGCGCGTGGCTGGTGGGCGGGGCAGAGCCATTGTTCGCAGGCGTACCGAG
-895 TCCCCCGCGCTCGCCCGGGAGGGAGGCGGGGCTTCCCGCGTCCCCCAAGCTCCAGATCCTGGGGTGGCTGCCACGTCTCCCTGCCACGCGCCTGGGGGGA
-795 CGGAAGACGGGACGGAGATGTTAGTGGTGGGCGCCCCCCGAGGGTTCACCACTGTTTCCTGAGAAACTTCCCCAGTGCCCACCCACCCGTTCTCCGTGTG
-695 CCCGAGGGCCGGTCCTGGGCTAGGCTCCGCGCCCCAGCCCCAAACCGGGTCCCCAGCCCCTTCCAGAGAGAAAGCTCCCGACGCGGGATGCCGGGCAGAG
-595 GCCCAGCGGCGGGTGGAAGAGAAGCTGAGAAGGAGAAACAGAGGGGAGGGGGAGCGAGGAGCTGGCGGCAGAGGGAACAGCAGATTGCGCCGAGCCAATG
SP1 SP1 SRE
-495 GCAACGGCAGGACGAGGTGGCACCAAATTCCCTTCGGCCAATGACGAGCCGGAGTTTACAGAAGCCTCATTAGCATTTCCCCAGAGGCAGGGGCAGGGGC
NF-1 NF-1 NF-1 NF-Y
-395 AGAGGCCGGGTGGTGTGGTGTCGGTGTCGGCAGCATCCCCGGCGCCCTGCTGCGGTCGCCGCGAGCCTCGGCCTCTGTCTCCTCCCCCTCCCGCCCTTAC
-295 CTCCACGCGGGACCGCCCGCGCCAGTCAACTCCTCGCACTTTGCCCCTGCTTGGCAGCGGATAAAAGGGGGCTGAGGAAATACCGGACACGGTCACCCGT
NF-1 C/EBP A T
-195 TGCCAGCTCTAGCCTTTAAATTCCCGGCTCGGGGACCTCCACGCACCGCGGCTAGCGCCGACAACCAGCTAGCGTGCAAGGCGCCGCGGCTCAGCGCGTA
G A TATA
LXRE
-95 CCGGCGGGCTTCGAAACCGCAGTCCTCCGGCGACCCCGAACTCCGCTCCGGAGCCTCAGCCCCCTGGAAAGTGATCCCGGCATCCGAGAGCCAAGATG


B
DMSO
Extract 100 µg/ml
Relative luciferase activity
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Wild-type
LXRE
C/EBP
PPARγ
SRE
Mutant constructs

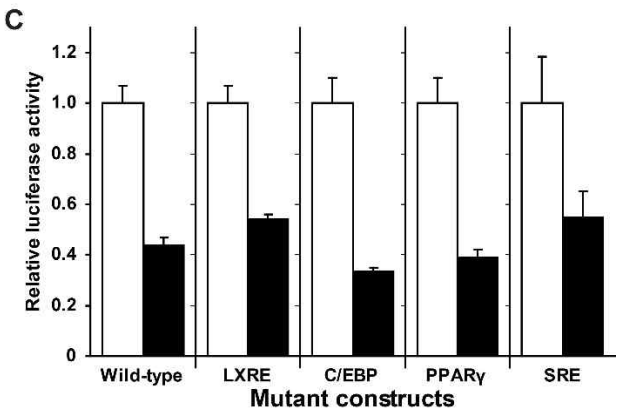

C
Relative luciferase activity
1.2
1.0
0.8
0.6
0.4
0.2
0
Wild-type
LXRE
C/EBP
PPARγ
SRE
Mutant constructs

Figure 7. Effects of mutations in transcription factor-binding sites on SCD1 transcription by the *P. morrowii* extract in HepG2 cells

(A) These nucleotides are the digested fragment (-1295 to -1) for the wild-type *SCD1* promoter construct. Mutant clones were generated from the wild-type *SCD1* promoter construct using the Quick Change site-directed mutagenesis kit as described under "Materials and Methods". (B) HepG2 cells were transfected with the wild-type or mutant constructs and luciferase-reporter assay was carried out after treatment of the *P. morrowii* extract at a concentration of 100 μg/ml. Each value represents the relative luciferase activity in cells compared to that in vehicle-treated cells which were transfected with the wild-type construct. (C) Values in panel A were recalculated to compare the effect of the extract for each mutant construct. Each value represents the relative luciferase activity in cells treated with the extract compared to that in cells transfected with the same construct and treated with vehicle (DMSO). Error bars denote the range for the two duplicate values. The similar results were obtained in two independent experiments.

Ⅳ. DISCUSSION

The reduction of the plasma TG level is an important management to reduce the incidence of CVDs particularly in patients with obesity and diabetes[1,2,6]. Although the proper management of hyperglyceridemia is required for prevention of obesity, metabolic syndrome, and coronary heart diseases, specific and effective medicines for hyperglyceridemia are not fully available. This unavailability has led global pharmaceutical companies continue to search and develop efficient drugs for this purpose.

Among several strategies of lowering the plasma TG concentration, strong support has been eluted from many researches for the inhibition of the expression and activity of SCD, the rate-limiting enzyme of *de novo* monounsaturated fatty acid synthesis, to be a potential therapeutic target in the prevention of metabolic diseases. The monounsaturated fatty acids synthesized by SCD1 are used as major substrates for TG and cholesteryl ester synthesis. Many studies have reported the possible link of SCD1 with treatment or prevention of obesity and dyslipidemic disorders[21]. For example, mice with disruption in the *Scd1* gene showed increased insulin sensitivity and energy expenditure, reduced body

adiposity, and resistance to diet-induced obesity[39]. In addition, the expression and activity of Scd1 was specifically repressed by leptin leading to lean and hypermetabolic condition in leptin-deficient *ob/ob* mice [56]. Most importantly, a disruption of the *Scd1* gene in mice showed decreased body adiposity, increased energy expenditure, increased sensitivity, and resistance to diet-induced obesity[39].

In this study, the effect of the *P. morrowii* extract on the regulation of the expression of SCD1 was elucidated for development of therapeutics to lower the plasma concentration of TG. *P. morrowii* is a red algae distributed widely on the coast of Korea, and the importance of seaweeds has been extensively reported as a therapeutic resource[57], however, only a few studies on *P. morrowii* has been reported inasmuch as therapeutic application is concerned. It has been reported that *P. morrowii* protects human keratinocytes from ultraviolet B radiation through the enhancement of antioxidant systems[58]. The other example is 3-bromo-4,5-dihydroxylbenzyl methyl ether and 3-bromo-4,5-dihydroxylbenzaldehyde isolated from *P. morrowii* to exhibit antiviral activity[59].

This study demonstrated that the extract of *P. morrowii* efficiently reduced the expression of lipogenic genes including *Fasn, Scd1, Acly,*

Srebp-1, *Elovl1*, and *Elovl6* in wild-type C57BL6/J mice. Although not all genes were evaluated for their protein expression, it is supposed that Scd1 is one of important mediators to lower the plasma TG concentration. The down-regulation of SCD1 *in vivo* and *in vitro* was accompanied with decreased in its mRNA, and reduced transcriptional activity of the SCD1 promoter in HepG2 cells.

For elucidation of transcriptional repression of the *SCD1* promoter by *P. morrowii*, mutational analysis of *cis*-elements on the promoter was carried out. SREBP-1 is a key transcription factor to govern the whole pathway of *de novo* synthesis of fatty acids and TG. The SRE, the binding site for SREBPs is present in the *SCD1* promoter and is important to the sterol-dependent transcription of the *SCD1* promoter[60]. C/EBP is one of the major components in transcriptional control of SCD1 of adipogenesis and insulin sensitivity[42 61]. SCD1 deficiency can protect against hypertriglyceridemia induced by LXR activation[11].

Unfortunately, the mutations of these conserved *cis*-elements had no effect on the transcriptional activity of the *SCD1* promoter by *P. morrowii*. This suggests that the other region or elements participate in regulation of transcription of *SCD1* by *P. morrowii*. As a result, the precise mechanism that mediates repression of transcriptional activity

of the SCD1 promoter by *P. morrowii* was not provided in this study and requires to be further elucidated.

In conclusion, this study provides strong support that the *P. morrowii* extract have potential to treat hyperglyceridemia and obesity by down-regulation of SCD1.

V. CONCLUSION

Elevated SCD1 activity has been implicated in a wide range of disorders including diabetes, atherosclerosis, and obesity. Therefore, modulation of SCD1 expression is one of the target to control hyperglyceridemia and obesity. This study indicates that expression of SCD1 protein and mRNA was efficiently repressed by *P. morrowii*, a red algae available in the coast of Korean, *in vivo* and *in vitro*. *In vivo* study showed decrease in the level of plasma TG by the *P. morrowii*. Although *cis*-elements that mediate the transcriptional repression of the *SCD1* promoter by *P. morrowii* have not fully elucidated, it is suggested that the inhibition of SCD1 expression by *P. morrowii* is a potential therapeutic modality to treat hyperglyceridemia.

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ABSTRACT (IN KOREAN)

모로우붉은실 추출물에 의한 SCD1 발현 조절 기전 규명

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이 나 래

SCD1 (Stearoyl-Coenzyme A desaturase 1)은 포화지방산으로부터 단일불포화지방산을 합성하는 효소로서 중성지방 (triglyceride, TG) 합성을 조절하는 주요 인자이다. SCD1은 기질인 stearoyl-CoA의 9번째 탄소에 이중결합을 형성하여 oleoyl-CoA를 합성한다. 혈중 중성지방의 증가는 고지혈증을 유발하고, 고콜레스테롤혈증과 함께 심혈 관계 질환 발병률을 증가시키며 인슐린 저항성을 유발시킨다. 고지혈증을 치료하는

여러 가지 방법이 임상적으로 사용되고 있으나 아직까지 중성지방에 특이적이며 효과적으로 중성지방 농도를 감소시키는 약물은 성공적이지 못하다. 최근 비만과 이상지질혈증의 치료 표적으로서 SCD1에 대한 연구가 활발히 진행되고 있다. 특히 *Scd1*이 결핍된 마우스는 비만 및 당뇨병 발생이 정상 마우스에 비하여 감소됨이 보고되었다. 그래서 이러한 근거로 본 연구에서는 고지혈증의 치료 약물을 개발하고자 해조류의 일종인 모로우붉은실 추출물이 SCD1 발현을 억제함으로써 혈중 중성지방 농도를 감소시키는 효과가 있음을 확인하였다. 이러한 작용은 마우스 및 HepG2 세포에서 SCD1 단백질 및 mRNA의 감소를 통하여 이루어짐을 밝혔으며, *SCD1* 프로모터의 전사 활성이 모로우붉은실에 의하여 감소됨을 밝혔다. *SCD1* 프로모터의 전사 억제 기전을 밝히고자 LXRE, C/EBP 및 SRE 부위의 돌연 변이 유도 실험을 진행하였으나 모로우붉은실의 전사 억제 작용은 큰 영향을 받지 않았다. 따라서 보다 구체적인 SCD1 발현 조절 기전을 향후 연구해서 밝힐 필요가 있다. 결론적으로 본 연구는

모로우붉은실이 SCD1 발현을 조절하여 고지혈증과 비만을 치료 혹은 예방할 수 있는 새로운 약물로서의 개발 가능성을 제시하였다.

핵심되는 말 : 고지혈증, 스테아로일코에이 디세츄레이즈, 불포화 지방산, 심혈관계 질환, 모로우붉은실, 중성 지방, 전사