Reduction of adipose tissue mass by the angiogenesis inhibitor ALS-L1023

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Abstract

Objective: Angiogenesis has been demonstrated to modulate adipogenesis and obesity. This study was undertaken to determine whether the botanical drug ALS-L1023 (ALS), which exhibits antiangiogenic activity, can regulate adipose tissue growth in high-fat-diet-induced obese mice.

Materials and Methods: The effects of ALS on angiogenesis and extracellular matrix remodeling were measured using *in vitro* assays. The effects of ALS on adipose tissue growth was investigated in nutritionally obese mice.

Results: ALS inhibited angiogenesis in a concentration-dependent manner in the tube formation assay and the VEGF-induced proliferation assay using human umbilical vein endothelial cells. ALS also suppressed matrix metalloproteinase (MMP) activity *in vitro*. ALS administration to high-fat-diet-induced obese mice significantly reduced body weight gain, adipose tissue mass, and adipocyte size compared with the findings in the controls. ALS treatment decreased blood vessel density and MMP activity in adipose tissues. ALS reduced the mRNA levels of angiogenic factors (VEGFs and FGF-2) and MMPs (MMP-2 and MMP-9), whereas it increased the mRNA levels of angiogenic inhibitors (TSP-1 and TIMPs) in adipose tissues.

Conclusion: These results suggest that *in vivo* and *in vitro* treatment with ALS, which has antiangiogenic and MMP inhibitory activities, reduces adipose tissue mass in nutritionally induced obese mice, providing evidence that adipose tissue growth can be regulated by angiogenesis inhibitors.

Keywords: central obesity; visceral adipose tissue; angiogenesis; MMP; VEGF inhibition

Introduction

Obesity is characterized by increased adipose tissue mass that results from both increased fat cell number (hyperplasia) and increased fat cell size (hypertrophy).[1] The development of obesity is associated with extensive modifications of adipose tissue involving adipogenesis, angiogenesis, and extracellular matrix (ECM) remodeling.[2] Similar to neoplastic tissue, adipose tissue is highly vascularized, and each adipocyte is nourished by an extensive capillary network.[2-4] Extensive changes in ECM remodeling have also been observed during adipose tissue growth.[6] The matrix metalloproteinase (MMP) system plays important roles in the development of adipose tissue and microvessel maturation via ECM modulation.[5-7] In most cases, MMPs are expressed at very low levels, but their expression is rapidly induced at times of active tissue remodeling associated with adipogenesis. Therefore, it is suggested that adipose tissue growth is dependent on angiogenesis and may be inhibited by angiogenesis inhibitors.

Activated adipocytes produce an array of vascular growth factors, including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), soluble VEGF receptor-2 (VEGFR2), hepatocyte growth factor, angiopoietin-2, and angiogenin. The serum concentrations of these growth factors are higher in overweight or obese individuals than in normal-weight individuals.[8] In addition to secreting vascular growth factors, adipocytes release several MMPs that modulate the ECM and allow matrix-bound vascular growth factors to induce angiogenesis. Thus, angiogenesis and MMP inhibitors are in development as targeted antiobesity therapeutics.[9]

We screened the antiangiogenic and MMP inhibitory activities of extracts of medicinal herbs and found that *Melissa officinalis* L.(Labiatae; lemon balm) exhibits antiangiogenic and MMP inhibitory activities.[10] Melissa has been used to as a medicinal plant to treat nervousness, insomnia, gastrointestinal disorders, herpes virus infection, and Alzheimer's disease.[11-16] An active fraction denominated ALS-L1023 (ALS) exhibited enhanced antiangiogenic and MMP inhibitory activities.

When high-fat-diet-induced obese mice were treated with ALS, adipose tissue mass and adipocyte size were significantly reduced in treated mice compared with that in control mice. The mRNA expression of angiogenic factors, MMPs, and their inhibitors were also modulated by ALS in obese mice. These studies suggest that antiangiogenic ALS can inhibit the growth of adipose tissue.

Materials and methods

Preparation of ALS

ALS was manufactured by activity-guided fractionation from the leaves of *Melissa officinalis* L. (Alfred Galke GmbH, Harz, Germany). The dried *Melissa* leaves were extracted with aqueous ethanol, and the extract was filtered and concentrated. The concentrated ethanol extract was further fractionated with ethyl acetate, after which it was concentrated and dried to obtain ALS in a dried powder form. The quality of ALS was controlled by standardization with reference compounds. ALS exhibited enhanced antiangiogenic and MMP inhibitory activities compared with that of water extract or ethanol extract of *Melissa* leaves.

In vitro human umbilical vein endothelial cell (HUVEC) tube formation assay

To perform the tube formation assay, HUVECs were purchased from Lonza (Basel, Switzerland) and cultured in M199 medium supplemented with 10% fetal bovine serum (FBS) and endothelial cell growth supplement (Sigma-Aldrich, St. Louis, MO, USA) at 50 μg/ml in a 37°C incubator with a humidified atmosphere containing 5% CO_2. Two hundred microliters of Matrigel (BD Biosciences, Bedford, MA, USA) were pipetted into the wells of a 48-well plate and allowed to solidify for 1 h at 37°C. HUVECs were plated on Matrigel-coated wells at a density of 4×10^4 cells/well and incubated for 16 h at 37°C with medium in the absence or presence of 25, 50, or 100 μg/ml ALS. The formation of capillary-like tubular networks was observed with an inverted microscope and photographed. The percentage of tubule area was quantified by image analysis using Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA).

VEGF-induced HUVEC proliferation assay

HUVECs were plated on 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h at 37°C with medium containing 10 ng/ml VEGF in the absence or presence of 25 or 50 μg/ml ALS dissolved in DMSO. HUVEC proliferation was measured by counting cells. After 48 h, the proliferation of HUVECs was measured by the XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide disodium salt) test using a Cell Proliferation Kit II (Roche, Cat. No. 1465015).

MMP assay

MMP activities were measured on an LS50B spectrofluorometer (Perkin-Elmer, Waltham, MA, USA) using 2,4-dinitrophenyl-Pro-Leu-Gly-Met-Trp-Ser-Arg (Calbiochem, San Diego, CA, USA) as a substrate for MMP-2 and MMP-9. Recombinant human MMP-2 and MMP-9 were purchased from R&D Systems (Minneapolis, MN, USA) and used after activation with 1 mM APMA (amino-phenyl mercuric acetate) before the assay. MMP (10 nM) and substrate (1 μM) were mixed in 2 ml of reaction buffer (50 mM Tricine, pH 7.5, 10 mM $CaCl_2$, 200 mM NaCl) in the presence or absence of ALS. Fluorescence intensity was measured at room temperature using a 280-nm excitation wavelength and a 360-nm emission wavelength.

Animal study

Eight-week-old male wild-type C57BL/6J mice (n=8/group) were randomly divided into four groups that exhibited uniformity in response to each treatment in the pilot study. Mice were fed for 8 weeks with a low-fat diet (13% kcal fat, CJ, Incheon, Korea), a high-fat diet (45% kcal fat, Research Diets, New Brunswick, NJ, USA), or the same high-fat diet supplemented with 0.4 or 0.8% (w/w) ALS. The body weight of each animal was measured daily by a person blinded to the treatments. Food

intake was determined by estimating the amount of food consumed by the mice throughout the treatment period. After a 12-h fast on the last day of the study, the animals were sacrificed by cervical dislocation. Blood was collected from the retroorbital sinus into tubes, and serum was separated and stored at −80°C until analysis. Visceral (VSC) and subcutaneous (SC) fat pads were removed, weighed, snap-frozen, in liquid nitrogen, and stored at −80°C until use. Portions were prepared for histology. All animal experiments were approved by the Institutional Animal Care and Use Committees of Mokwon University and were conducted according to National Research Council Guidelines.

Histological analysis

For hematoxylin and eosin (H&E) staining, adipose tissues were fixed in 10% phosphate-buffered formalin for 1 day and processed in a routine manner for paraffin sections. Tissue sections (5 μm) were cut and stained with H&E for microscopic examination. To quantify adipocyte size, the H&E-stained sections were analyzed using the Image-Pro Plus analysis system.

Blood vessel staining was performed using a blood vessel staining kit (Chemicon, Billerica, MA, USA). Adipose tissues were fixed in 10% phosphate-buffered formalin for 1 day and processed in a routine manner for paraffin sections. Sections (3-μm thick) were cut and irradiated in a microwave oven for epitope retrieval. Sections were incubated with a rabbit anti-von Willebrand Factor (vWF) antibody as a primary antibody, goat anti-rabbit antibody as a secondary antibody, and streptavidin-alkaline phosphatase solution. A freshly prepared chromogen reagent was added to sections for the visualization of blood vessel.

Zymography

.Adipose tissues were weighed and extracted with 10 mM sodium phosphate buffer (pH 7.2)

containing 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, and 0.2% NaN_3 at 4°C. Adipose tissue extracts were mixed with zymography sample buffer (63 mM Tris HCl, 10% glycerol, 2% SDS, 0.0025% bromophenol blue, pH 6.8) without heat denaturation. The HT1080 cell culture medium was used for the molecular weight markers for MMP. Electrophoresis was performed at 125 V on 10% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were incubated in renaturing buffer containing 0.25% Triton X-100 for 30 min at room temperature and equilibrated in developing buffer (50 mM Tris base, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, 0.2% Brij-35) for 30 min at room temperature. The gels were then incubated in developing buffer overnight at 37°C. The gels were stained with 0.1% Coomassie Blue R-250 and destained with 10% acetic acid in 40% methanol.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA from retroperitoneal and inguinal adipose tissues was prepared using Trizol reagent (Gibco-BRL, Grand Island, NY, USA). After 2 μg total RNA were reverse-transcribed using Moloney murine leukemia virus reverse transcriptase and an antisense primer, cDNA was generated. Synthesized cDNA fragments were amplified by PCR in an MJ Research Thermocycler (Waltham, MA, USA). The PCR primers used for gene expression analysis are shown in Table 1. The cDNA was mixed with PCR primers, *Taq* DNA polymerase (Solgent, Daejon, Korea), and a deoxyribonucleotide triphosphate mixture. The reaction consisted of 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 58°C, and elongation for 1 min at 72°C. PCR products were quantified from agarose gels using GeneGenius (Syngene, Cambridge, UK).

Statistics

Unless otherwise indicated, all values are expressed as mean ± standard deviation (SD). All data

were analyzed by the unpaired Student *t*-test to determine statistically significant differences between groups.

Results

Inhibition of angiogenesis by ALS

When ALS was added to the cell culture at a concentration of 25, 50, or 100 μg/ml in an *in vitro* HUVEC tube formation assay, total tubule area and length were decreased in ALS-treated cells compared to that in control cells (Fig. 1A). The capillary-like tube formation of HUVECs was inhibited in a concentration-dependent manner, demonstrating that ALS exerts antiangiogenic effects by inhibiting the differentiation and migration of endothelial cells.

Endothelial cell proliferation is considered one of the essential events of angiogenesis. VEGF plays important roles in pathogenic angiogenesis as an angiogenic stimulator by inducing endothelial cell proliferation. When ALS was added to the culture at a concentration of 25 or 50 μg/ml in VEGF-induced HUVEC proliferation assays, VEGF-induced endothelial cell proliferation was inhibited by 66 and 100%, respectively, compared with control cell proliferation ($p<0.05$; Fig. 1B). Thus, ALS exerts antiangiogenic effects by inhibiting endothelial cell proliferation.

Inhibition of MMP activity by ALS

MMP-2 and MMP-9 contribute to tissue remodeling by degrading the ECM in angiogenic processes as well as participate in adipocyte differentiation. The activities of MMP-2 and MMP-9 were inhibited by ALS in a concentration-dependent manner. The IC_{50} values were 17.7±1.0 μg/ml for MMP-2 and 12.3±1.4 μg/ml for MMP-9 (Fig. 2). Thus, ALS may exert inhibitory effects on both angiogenesis and adipocyte differentiation.

Effects of ALS on body weight and adipose tissue mass

To determine whether ALS reduces body weight gain and adipose tissue mass in obese mice, mice were fed a low-fat diet, a high-fat diet, or the same high-fat diet supplemented with 0.4 or 0.8% ALS for 8 weeks. After 8 weeks, the high-fat-diet-fed mice had 166% greater body weight gains compared to that of low-fat-diet-fed mice (17.0±1.49 g vs. 6.38±1.87 g, respectively, $p<0.05$). In contrast, mice fed a high-fat diet supplemented with 0.4 and 0.8% ALS had 70 (4.96±1.05 g) and 84% (2.72±0.60 g) lower body weights, respectively, than obese mice fed a high-fat diet alone ($p<0.05$; Fig. 3A). ALS was not toxic at either concentration.

Adipose tissue mass was also significantly decreased by ALS treatment in high-fat-diet-fed obese mice (Fig. 3B). As shown in Fig. 3C and D, the mass of both VSC and SC fat pads in ALS-treated mice was reduced in comparison to that of high-fat-diet-fed control mice. VSC and SC adipose tissue weights were decreased by 73 and 71% after 0.8% ALS administration, respectively. Compared with high-fat-diet-fed mice, 0.8% ALS-enriched high-fat-diet-fed mice had lower VSC (1.43±0.16 g vs. 0.37±0.06 g) and SC fat mass (4.24±0.73 g vs. 1.2±0.13 g). In addition, brown fat mass was decreased by 45% in 0.8% ALS-treated mice compared with that in obese mice, whereas liver, kidney, and heart weights were not changed by ALS treatment (data not shown). However, ALS treatment did not affect food consumption profiles throughout the study (Fig. 3E).

Effects of ALS on adipocyte size

Consistent with the effects of ALS on adipose tissue weight, the analysis of H&E-stained adipose tissue sections revealed that ALS markedly decreased the size of adipocytes. ALS at concentrations of 0.4 and 0.8% decreased the average size of VSC adipocytes by 47 and 48%, respectively (Fig. 4A). The average size of adipocytes was 5571±271 μm^2 in 0.4% ALS-treated mice and 5394±216 μm^2 in 0.8% ALS-treated mice, which were lower than that of the high-fat-diet-fed obese mice (10565±495 μm^2; $p<0.05$). ALS at concentrations of 0.4% and 0.8% also decreased the average size of SC adipocytes by 40 and 52%, respectively (Fig. 4B). The average size of adipocytes was 4475±420 μm^2

in 0.4% ALS-treated mice and 3585±446 μm^2 in 0.8% ALS-treated mice, which were lower than that of the high-fat-diet-fed mice (7544±1978 μm^2; $p<0.05$).

Effects of ALS on vascularization in adipose tissue

To determine whether the decrease of adipose tissue mass by ALS resulted from the inhibition of angiogenesis, we studied the effects of ALS on blood vessel density in both VSC and SC adipose tissue. Staining adipose tissue sections with an antibody against vWF, an endothelial cell marker, revealed that the blood vessel density of both VSC and SC adipose tissue from ALS-treated mice was lower than that of high-fat-diet-fed control mice (Fig. 5).

Effects of ALS on MMP activity in adipose tissue

MMP activity in adipose tissue extracts was examined using zymography on gelatin-containing gels. Gelatin zymography revealed that proMMP-2 activity was significantly reduced in the adipose tissue from ALS-treated mice compared with that from the control group, whereas proMMP-9 levels were not detectable. The proMMP-2 activity in VSC adipose tissue was reduced by 48 and 49% after the administration of 0.4 and 0.8% ALS, respectively (Fig. 6A). Similarly, proMMP-2 activity in SC adipose tissue was also decreased by 23 and 36% after treatment with 0.4 and 0.8% ALS, respectively (Fig. 6B).

Effects of ALS on mRNA expression of angiogenic factors, MMPs, and their inhibitors in adipose tissue

The expression patterns of genes involved in angiogenesis were investigated in VSC and SC adipose tissues from C57BL/6J mice fed a high-fat diet. The mRNA expression of angiogenic and

antiangiogenic factors was downregulated and upregulated, respectively, in ALS-treated mice compared with those in high-fat-diet-fed obese mice. In VSC fat tissue, the mRNA levels of angiogenic factors VEGF-A, -B, -C, and -D were decreased by 45 ($p<0.05$), 9 ($p<0.05$), 17, and 16%, respectively, whereas the mRNA levels of the antiangiogenic molecule TSP-1 were increased by 41% ($p<0.05$) in ALS-treated mice compared with those in obese mice ($p<0.05$; Fig. 7A). In SC fat tissue, the mRNA expression of FGF-2, VEGF-A, -B, -C, and -D was reduced by 29 ($p<0.05$), 83 ($p<0.05$), 11, 25, and 48% ($p<0.05$), respectively, whereas TSP-1 mRNA levels were elevated by 11% in ALS-treated mice compared with those in obese mice (Fig. 7B).

Similarly, MMP mRNA expression was inhibited significantly by ALS treatment. MMP-2 and MMP-9 levels were decreased by 19 ($p<0.05$) and 81% ($p<0.05$), respectively, in VSC adipose tissue and by 20 and 41% ($p<0.05$), respectively, in SC adipose tissue (Fig. 7A and B). In contrast, TIMP-1 and TIMP-2 mRNA levels were increased by 69 ($p<0.05$) and 31% ($p<0.05$), respectively, in VSC adipose tissue and by 122 ($p<0.05$) and 43% ($p<0.05$), respectively, in SC adipose tissue from ALS-treated mice compared with those in obese mice.

Discussion

Angiogenesis is a fundamental requirement for embryonic development wound healing, and female cyclical changes within the endometrium.[17] However, angiogenesis is also the underlying pathological process of all major diseases of the developed world. It is a prominent feature of cancer, atherosclerosis, diabetes, rheumatoid arthritis, and proliferative retinopathy.[18,19] Similarly, the growth and expansion of adipose tissue require the formation of new blood vessels to provide oxygen and nutrients to adipocytes. Recently, we demonstrated that the antiangiogenic herbal composition Ob-X reduces adipose tissue mass and suppresses obesity by inhibiting angiogenesis in nutritionally and genetically obese mice,[20,21] suggesting that antiangiogenic drugs provide a novel therapeutic option for preventing and treating human obesity. In the present study, we prepared the antiangiogenic botanical drug ALS and observed its effects in obese mice. Our results demonstrated that ALS inhibited adipose tissue growth by inhibiting angiogenesis and MMP activity.

We primarily examined the antiangiogenic activity of ALS by using HUVEC tube formation and VEGF-induced HUVEC proliferation assays. ALS inhibited the capillary-like tube formation of HUVECs in a concentration-dependent manner and markedly reduced VEGF-induced HUVEC proliferation, demonstrating that ALS exerts antiangiogenic effects by inhibiting the proliferation and migration of endothelial cells. Angiogenesis often precedes adipogenesis in the developing adipose tissue.[2] Newly formed adipose tissue depends on continued angiogenesis for further growth.[22] It has been demonstrated that different angiogenesis inhibitors significantly reduced body weight and adipose tissue mass,[23] strongly indicating an inhibitory role of ALS in adipose tissue growth.

In addition to angiogenesis, adipose tissue development is also known to be associated with MMPs, which play major roles in ECM remodeling. Moreover, adipocytes have to be extensively remodeled to allow the hypertrophic development observed in obesity.[24] Several studies suggest that MMPs play a role in the tissue remodeling events associated with adipogenesis. MMP-2 and MMP-9

can remodel the ECM of murine and human adipocytes to facilitate the adipogenic process[7,25] and regulate the bioavailability of adipocyte growth factors sequestered as inactive molecules in the matrix or blocked via interactions with their binding proteins.[26] ALS markedly inhibited the activities of two major MMPs (MMP-2 and MMP-9) in a concentration-dependent manner *in vitro*. These results strongly suggest that ALS, which can inhibit MMP activity as well as angiogenesis, can regulate adipose tissue growth.

We then treated high-fat-diet-induced obese mice with ALS. Consistent with our hypothesis that ALS can inhibit adipose tissue growth, ALS substantially reduced body weight gain and adipose tissue mass. Treatment with 0.4 and 0.8% ALS for 8 weeks decreased body weight gain by 70 and 84%, respectively. Both VSC and SC adipose tissue mass were also significantly decreased by 73 and 71% after ALS treatment, respectively, and this effect was higher than the 59 and 65% reductions in VSC and SC adipose tissue mass, respectively, after a 12-week administration of galardin, a broad spectrum inhibitor of MMPs.[5] Our data are also supported by other results indicating that several types of angiogenesis inhibitors, such as angiostatin, endostatin, and TNP-470 and its analog CKD-732 inhibit fat mass expansion in mice.[23,27-29] Similar to the effects of ALS on adipose tissue mass, the average size of adipocytes in both VSC and SC adipose tissues was substantially lower in ALS-treated mice than in untreated obese mice. The size of adipocytes was lower in ALS-treated mice, compared with those in control mice. These results indicate that ALS can reduce adipose tissue mass and inhibit adipocyte hypertrophy. VSC obesity due to adipocyte hypertrophy is known to be closely associated with metabolic syndromes including insulin resistance.[30-32] Given that obesity and obesity-associated disorders are associated with pathological angiogenesis,[33-35] modulating angiogenesis may be a novel therapeutic approach .[27,33]

During ALS-induced adipose tissue loss, food intake was not changed. Similarly, it was reported that the administration of angiogenesis inhibitors, such as endostatin, to ob/ob mice resulted in weight loss without appetite changes,[23] and that tolylsam, an MMP inhibitor with relative specificity for

gelatinases, reduced body and fat pad weights but did not affect food intake.[36] As vessels in adipose tissue may be maintained in a relatively immature state compared with those vessels in other organs that are weight-stable, and angiogenesis inhibitors target only growing or newly formed immature vessels,[22] ALS may selectively target adipose tissue and cause weight reduction. The weight loss induced by antiangiogenic ALS arose specifically from the loss of adipose tissue mass, as the weights of other organs such as the liver, kidneys, and heart were not reduced in ALS-treated obese mice. Thus, ALS can reduce adipose tissue mass by targeting only the growing adipose tissue without any side effects on weight-stable organs.

Consistent with the inhibitory effects of ALS on HUVEC *in vitro*, blood vessel staining revealed that the blood vessel density of both VSC and SC adipose tissues was reduced in ALS-treated mice compared with that in untreated obese mice. These data are supported by our previous results indicating that the antiangiogenic herbal composition Ob-X decreased the blood vessel density of VSC adipose tissue in nutritionally obese mice.[20] Our present results suggest that ALS is a very potent inhibitor of angiogenesis and controls adipose tissue growth via the regulation of angiogenesis.

As angiogenesis may represent a target for treating obesity, it is important to determine the expression profiles of genes involved in angiogenesis. The growing adipose tissue contains a diversity of cell populations, which determine the expression of several angiogenic modulators. Angiogenic factors, such as VEGFs and FGF-2, promote the proliferation, differentiation, migration of endothelial cells within fat tissue,[37-39] and moreover, VEGF-A and FGF-2 synergistically induce angiogenesis.[40] Blockage of the VEGFR2 signaling system by a neutralizing antibody inhibits both angiogenesis and preadipocyte differentiation, suggesting that VEGFs act on endothelial cells to regulate preadipocyte differentiation.[41] In addition, angiogenesis inhibitors, such as TNP-470 and VEGFR2-specific inhibitors, have been demonstrated to prevent the development of obesity in genetic mouse models and studies based on high-fat diets.[23,28,41,42] In contrast, TSP-1 inhibits angiogenesis *in vivo* and impairs the migration and proliferation of cultured microvascular endothelial cells.[43] Our RT-PCR

analysis revealed that ALS administration to obese mice decreased the mRNA expression of VEGF-A, -B, -C, and -D as well as FGF-2, whereas it increased the mRNA levels of the antiangiogenic TSP-1 in both VSC and SC adipose tissues.

During obesity, MMP expression is modulated in adipose tissue, and MMPs (e.g., MMP-2 and MMP-9) potentially affect adipocyte differentiation.[7,44,45] High MMP-2 expression was observed in the adipose tissue of mice with nutritionally induced obesity as well as in genetically obese mice.[26] Adipose tissue also secretes TIMPs, which act against all MMPs. Treatment with MMP inhibitors impairs adipose tissue development in mice fed a high-fat diet.[5,46] Furthermore, the secretion of MMP-2 and MMP-9 increases during adipocyte differentiation in both human adipocytes and mouse preadipocyte cell lines,[7,44,45] suggesting that MMP-2 and MMP-9 are important for adipocyte conversion. In this study, treating obese mice with ALS decreased MMP-2 and MMP-9 mRNA levels and increased TIMP-1 and TIMP-2 mRNA levels in both VSC and SC adipose tissues, indicating that ALS exerts a specific regulatory effect on genes involved in angiogenesis and the MMP system in adipose tissues. Our observations further indicate that the inhibition of adipose tissue growth by ALS may alter the expression of genes responsible for angiogenesis and the MMP system.

Zymography of VSC and SC adipose tissue extracts revealed that ALS reduced proMMP-2 activity, indicating that MMP-2 is inhibited. Conversely, proMMP-9 activity was not detectable. It is also reported that *in situ* zymography with gelatin-containing gels on cryosections of VSC and SC adipose tissue confirmed the lower MMP-2 and MMP-9 activities in the tissues of galardin-treated animals,[5] and treatment with MMP inhibitors impairs adipose tissue development in mice fed a high-fat diet.[46] Indeed, MMP-$2^{-/-}$ mice had reduced fat mass and smaller adipocyte size in both VSC and SC fat tissue compared with that in MMP-$2^{+/+}$ mice, suggesting a functional role of MMP-2 in adipose tissue growth.[47] Thus, these data demonstrate that the inhibition of MMP activity by ALS may lead to reduced adipose tissue mass in obese mice.

It was reported that MMPs play important roles in angiogenesis, and MMP-2 and MMP-9 activities indirectly facilitate angiogenesis[48,49] whereas MMP inhibitors, both synthetic and endogenous, inhibit angiogenic responses both *in vivo* and *in vitro*.[50-53] Moreover, MMP-deficient mice exhibit delayed or diminished angiogenic responses during development or in response to tumor xenograft.[54] However, it was also reported that MMP-based proteolysis of the ECM proteins releases anti-angiogenic cryptic fragments such as angiostatin and endostatin,,[55,56] showing that inhibiting MMP activity may decrease endogenous angiogenic inhibitors. On the other hand, studies demonstrated that MMPs have novel function of modulating adipocyte differentiation, which is independent of angiogenesis, and therefore, MMP inhibitors can block the adipocyte differentiation process.[7,44,45,57] Collectively, it seems that MMPs and their inhibitors play a pivotal role in controlling adipogenesis and adipose tissue growth.

In conclusion, our present findings demonstrate that ALS, which inhibits angiogenesis and MMP activity, suppresses the growth and development of adipose tissue growth in obese mice . These events may be mediated by changes in the expression of genes involved in angiogenesis, and the MMP system. Thus, antiangiogenic ALS provides a possible therapeutic approach for preventing and treating human obesity and its related disorders.

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Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Figure 1. Inhibitory effects of ALS on angiogenesis. (A) HUVEC tube formation. HUVECs were plated in Matrigel-coated wells with varying amounts of ALS (25, 50, and 100 μg/ml). After incubation, capillary-like tube formation was photographed (original magnification ×100). *$p<0.05$ compared with the control group. (B) VEGF-induced HUVEC proliferation. #$p<0.05$ compared with the vehicle group, *$p<0.05$ compared with the VEGF-treated control group.

Figure 2. Inhibitory effects of ALS on MMP activity. Inhibition of MMP-2 and MMP-9 activities by ALS was measured by spectrofluorometry, and the IC_{50} values were determined. * $p<0.05$ compared with the vehicle group.

Figure 3. Regulation of body weight gain, adipose tissue mass, and food consumption in high-fat-diet-fed obese mice. Adult male mice were fed a low-fat diet, a high-fat diet, or the same high-fat diet supplemented with 0.4 or 0.8% ALS for 8 weeks. Body weights were measured daily using a top-loading balance by a person blinded to the treatments. All values are expressed as the mean ± SD. (A) Body weight gains at the end of the treatment period are significantly different between the low-fat-diet group and the high-fat-diet group (#$p<0.05$) and between the high-fat-diet group and the groups fed a high-fat diet supplemented with 0.4 or 0.8% ALS (*$p<0.05$). (B) Photographs of VSC adipose tissue. At the end of the study, (C) VSC and (D) SC adipose tissue weights were measured. #$p<0.05$ compared with the low-fat-diet group, *$p<0.05$ compared with the high-fat-diet group. (E) Food intake was determined by estimating the amount of food consumed by the mice throughout the treatment period.

Figure 4. Light microscopic analysis of the size of adipocytes in adipose tissue. Adult male mice were fed a low-fat diet, a high-fat diet, or the same high-fat diet supplemented with 0.4 or 0.8% ALS for 8 weeks. Representative H&E-stained sections (5-μm thick) of (A) epididymal VSC and (B) inguinal SC adipose tissues are shown (original magnification ×100). Adipocyte size in the high-fat-diet plus ALS groups was smaller than that in the high-fat-diet groups. The size of adipocytes in a fixed area (1,000,000 μm^2) was measured. All values are expressed as the mean ± SD. #$p<0.05$ compared with the low-fat-diet group, *$p<0.05$ compared with the high-fat-diet group.

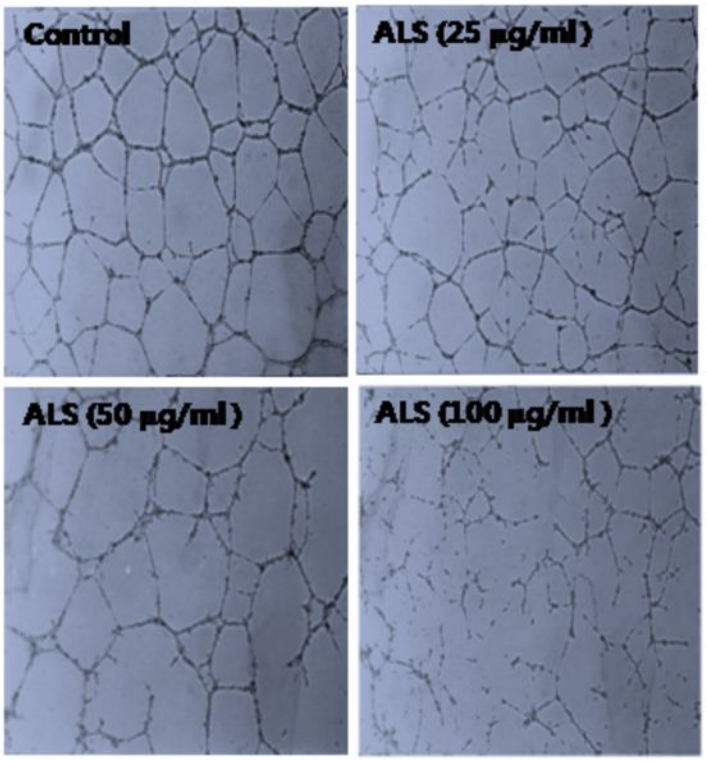
Figure 5. Histological analysis of the blood vessels in adipose tissue stained with an antibody against vWF. The blood vessels of (A) epididymal VSC and (B) inguinal SC adipose tissues derived from mice fed a high-fat diet or a high-fat diet supplemented with 0.8% ALS for 8 weeks were stained and analyzed (original magnification ×100).

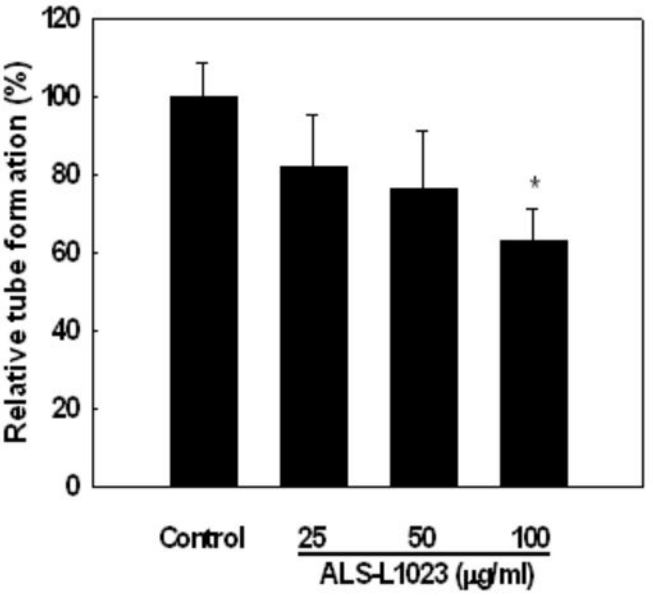
Figure 6. Zymographic analysis of adipose tissue. Extracts from (A) epididymal VSC and (B) inguinal SC adipose tissues obtained from mice fed a high-fat diet or a high-fat diet supplemented with 0.4 and 0.8% ALS for 8 weeks were applied to a gelatin-containing gel. Gelatinolytic activity was measured by zymography.

Figure 7. Effects of ALS on the mRNA expression of angiogenic factors, MMPs, and their inhibitors in (A) retroperitoneal VSC and (B) inguinal SC adipose tissues of diet-induced obese mice. Adult male mice were fed a high-fat diet or a high-fat diet supplemented with 0.8% ALS for 8 weeks. Representative PCR bands from one of three independent experiments are shown.

Fig. 1

(A)


Control
ALS (25 μg/ml)
ALS (50 μg/ml)
ALS (100 μg/ml)


Relative tube formation (%)
120
100
80
60
40
20
0
*
Control
25
50
100
ALS-L1023 (μg/ml)

(B)

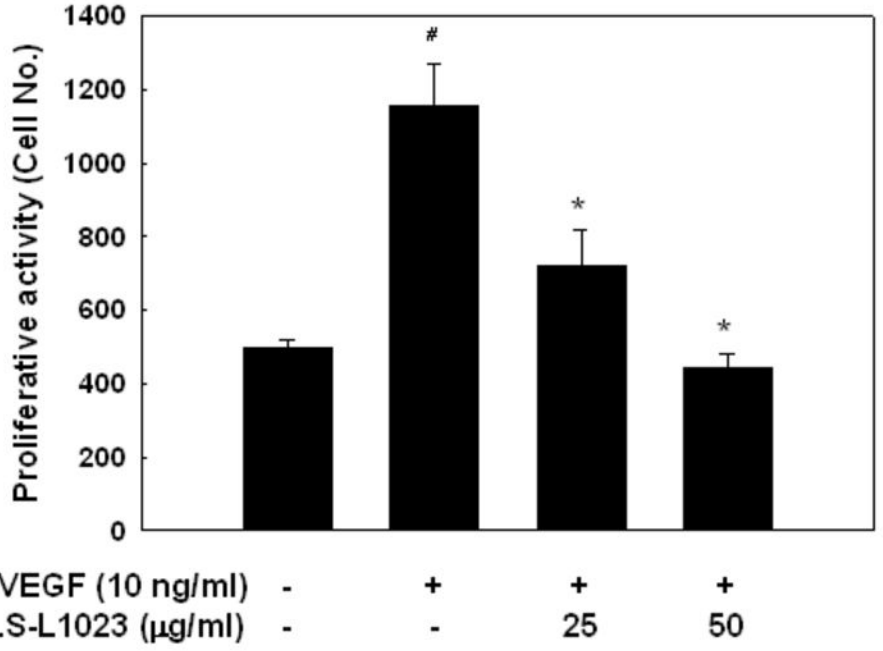

Proliferative activity (Cell No.)
1400
1200
1000
800
600
400
200
0
#
*
*
VEGF (10 ng/ml) - + + +
ALS-L1023 (μg/ml) - - 25 50

Fig. 2

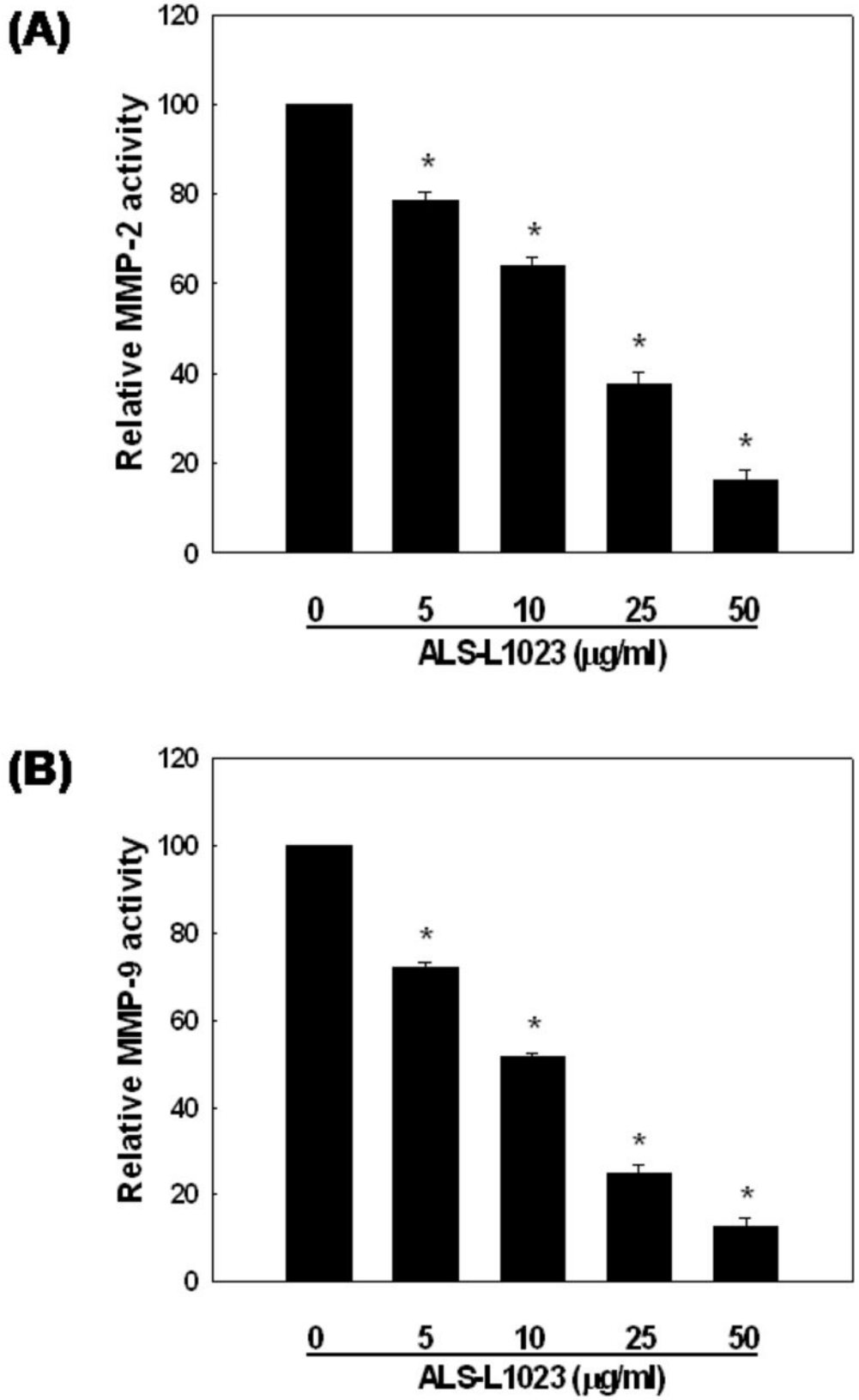
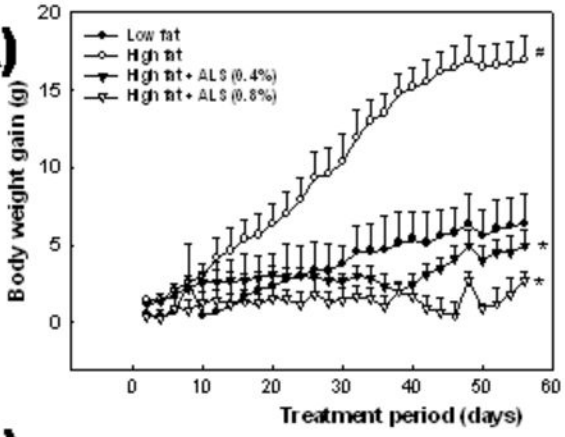
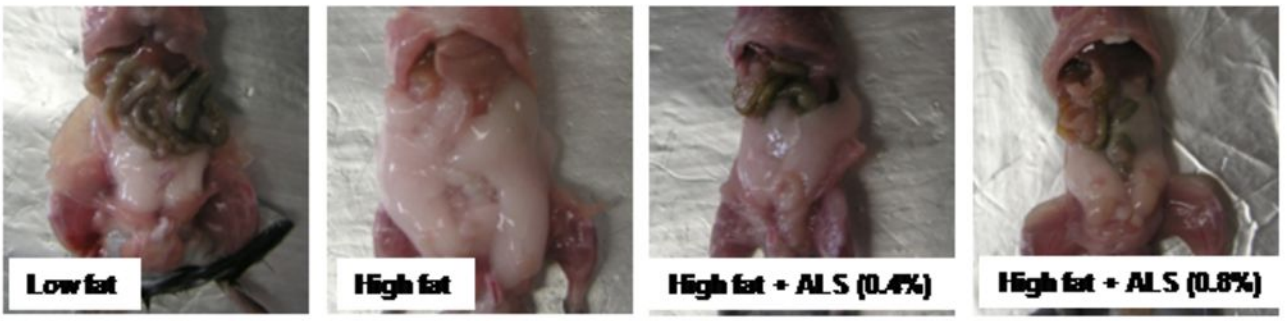

(A)
Relative MMP-2 activity
120
100
80
60
40
20
0
*
*
*
*
0
5
10
25
50
ALS-L1023 (μg/ml)
(B)
Relative MMP-9 activity
120
100
80
60
40
20
0
*
*
*
*
0
5
10
25
50
ALS-L1023 (μg/ml)

Fig. 3

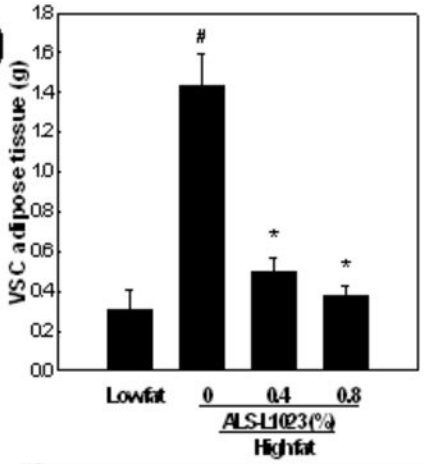
(A)


Low fat
High fat
High fat + ALS (0.4%)
High fat + ALS (0.8%)
Body weight gain (g)
Treatment period (days)
#
*
*

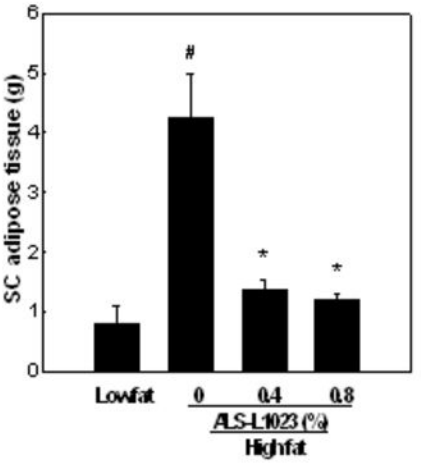
(B)


Low fat
High fat
High fat + ALS (0.4%)
High fat + ALS (0.8%)

(C)


VSC adipose tissue (g)
#
*
*
Lowfat
0
0.4
0.8
ALS-L1023(%)
Highfat

(D)


SC adipose tissue (g)
#
*
*
Lowfat
0
0.4
0.8
ALS-L1023(%)
Highfat

(E)

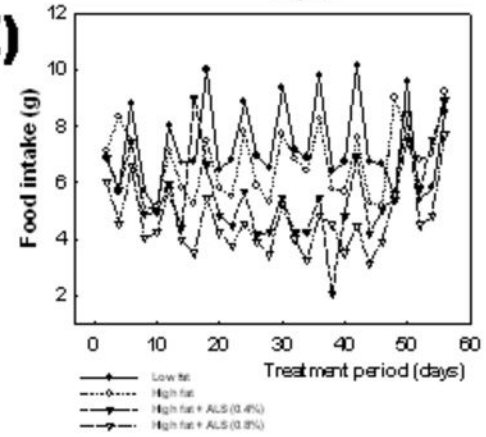
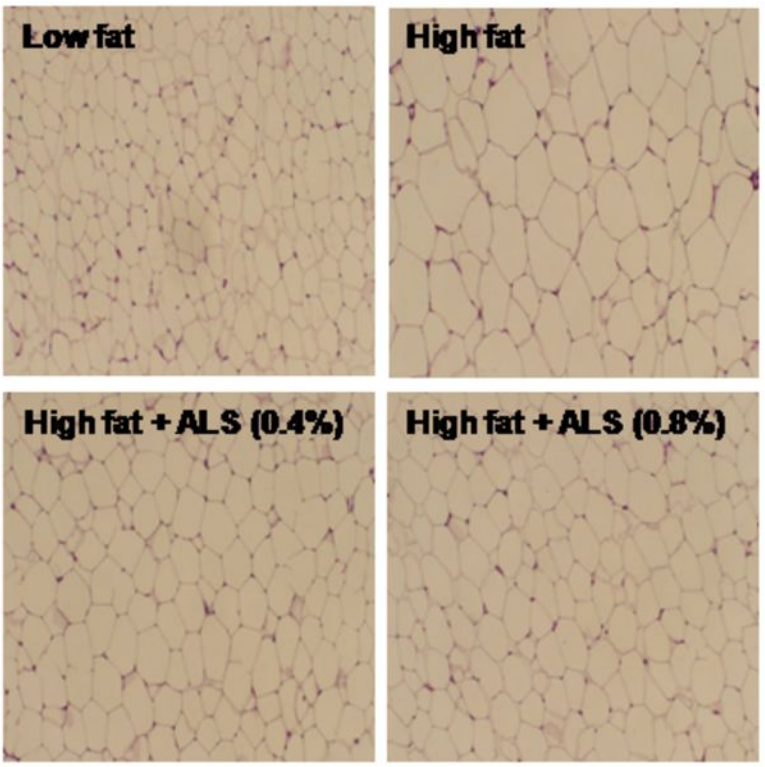
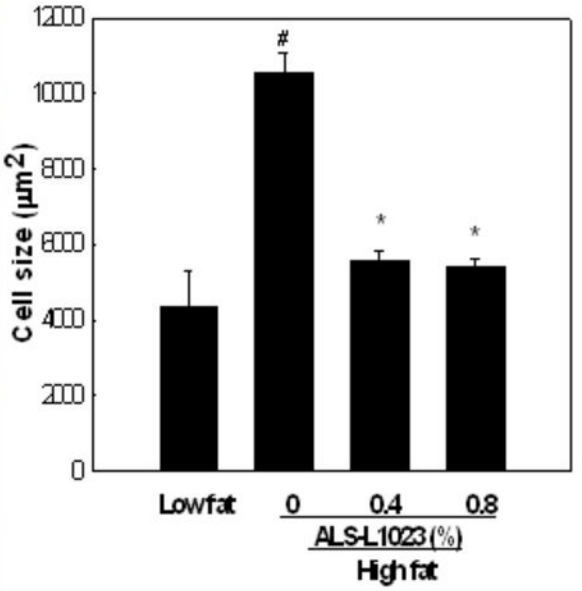

Food intake (g)
Treatment period (days)
Low fat
High fat
High fat + ALS (0.4%)
High fat + ALS (0.8%)

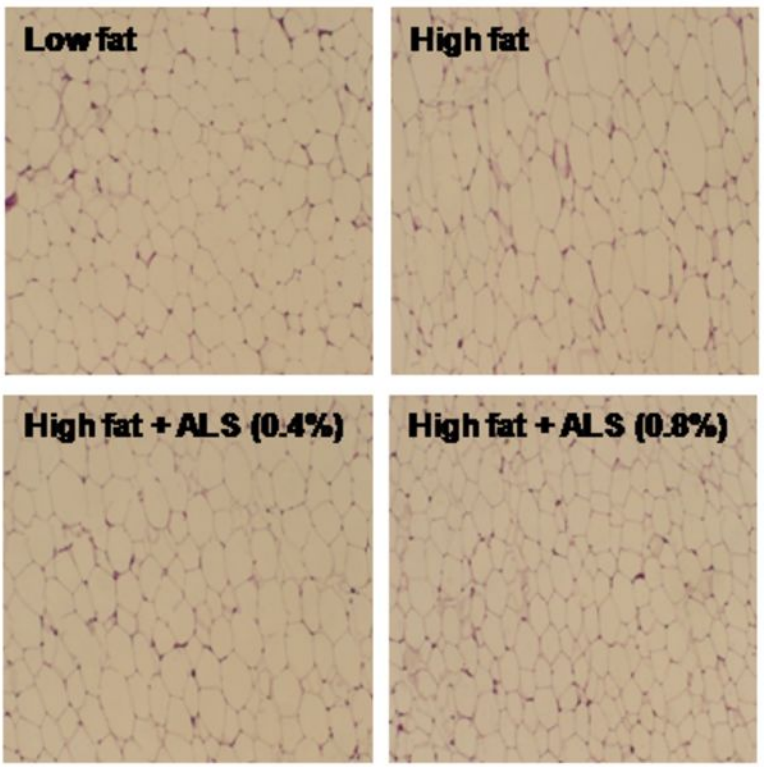
Fig. 4

(A)


Low fat
High fat
High fat + ALS (0.4%)
High fat + ALS (0.8%)


Cell size (μm2)
12000
10000
8000
6000
4000
2000
0
#
*
*
Lowfat
0
0.4
0.8
ALS-L1023 (%)
High fat

(B)


Low fat
High fat
High fat + ALS (0.4%)
High fat + ALS (0.8%)

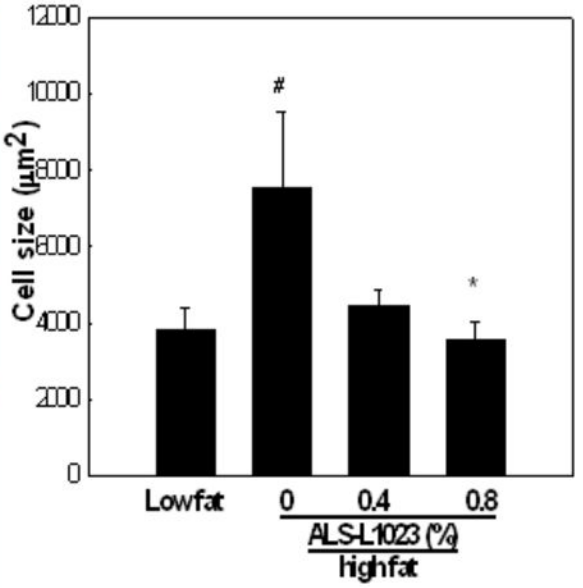
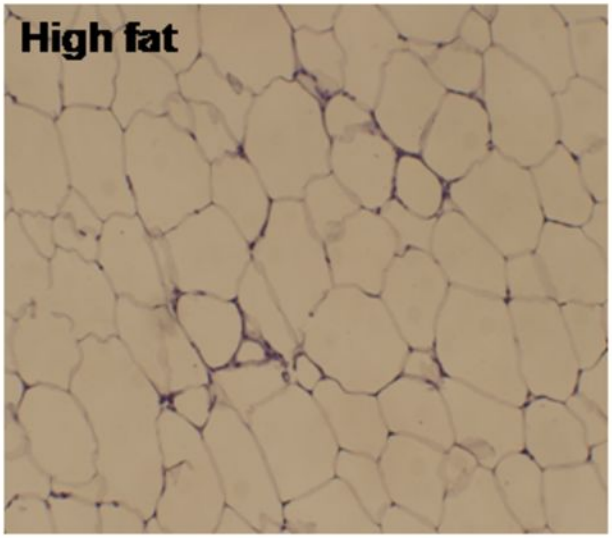
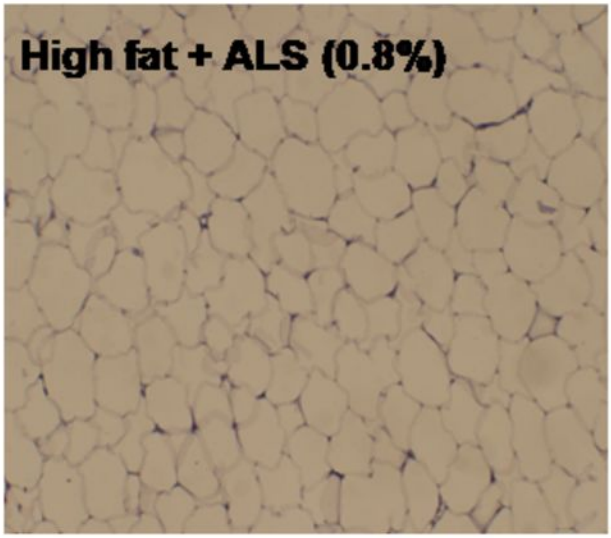

Cell size (μm2)
12000
10000
8000
6000
4000
2000
0
#
*
Lowfat
0
0.4
0.8
ALS-L1023 (%)
highfat

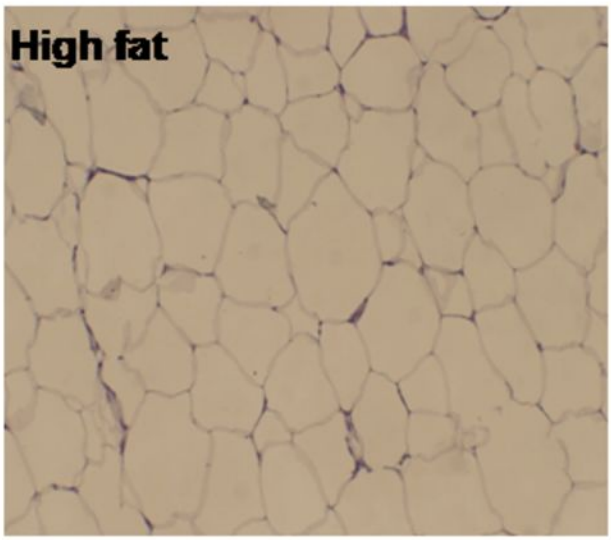
Fig. 5

(A)





(B)



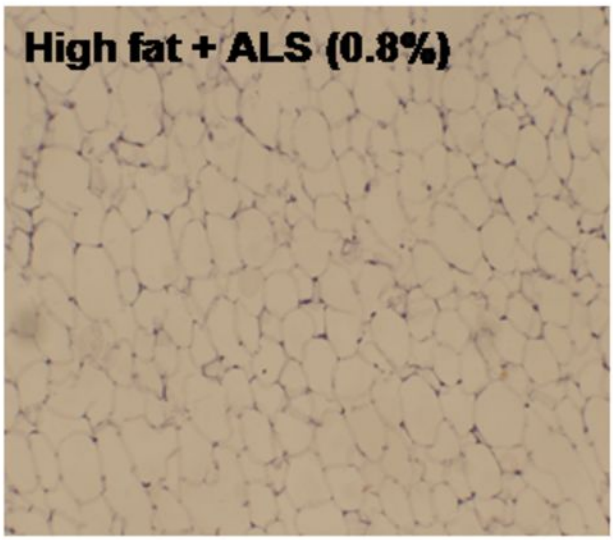
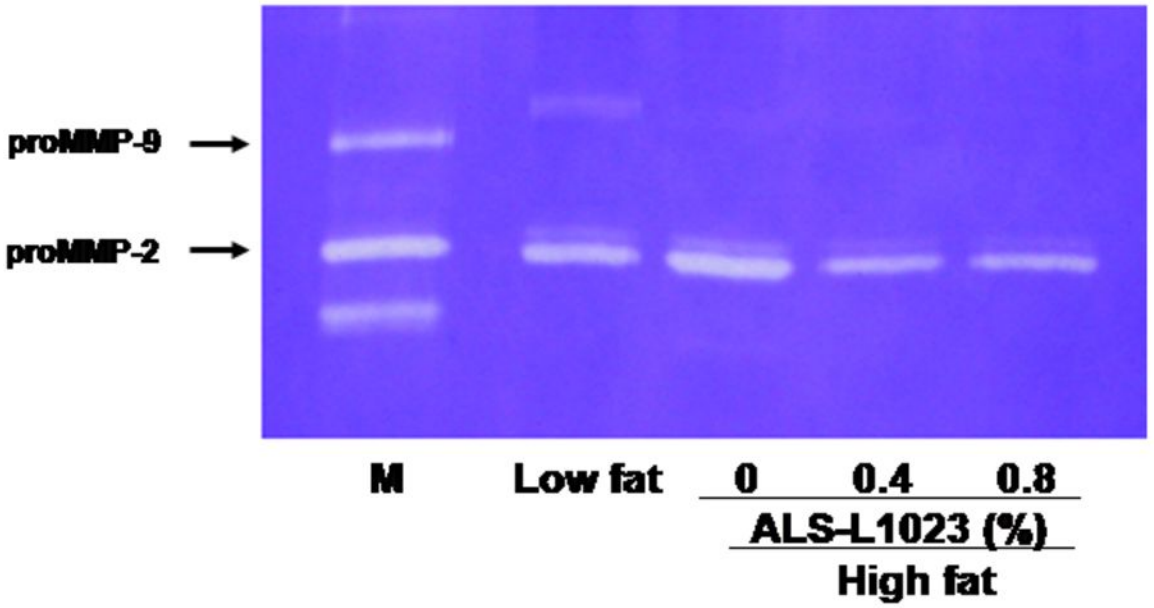


Fig. 6

(A)


proMMP-9
proMMP-2
M
Low fat
0
0.4
0.8
ALS-L1023 (%)
High fat

(B)

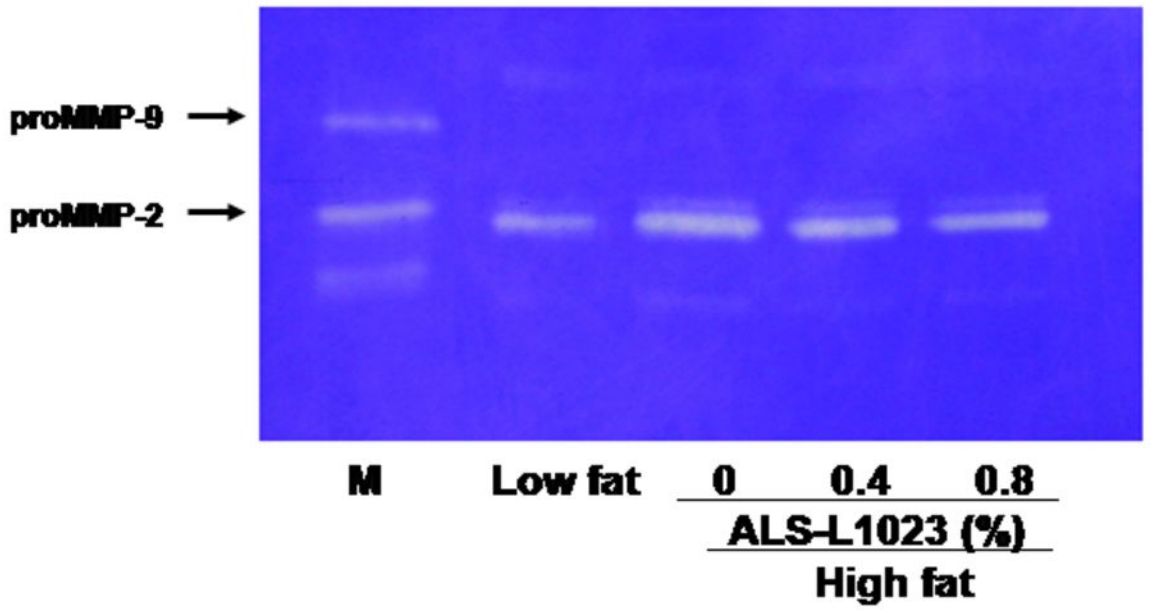

proMMP-9
proMMP-2
M
Low fat
0
0.4
0.8
ALS-L1023 (%)
High fat

Fig. 7

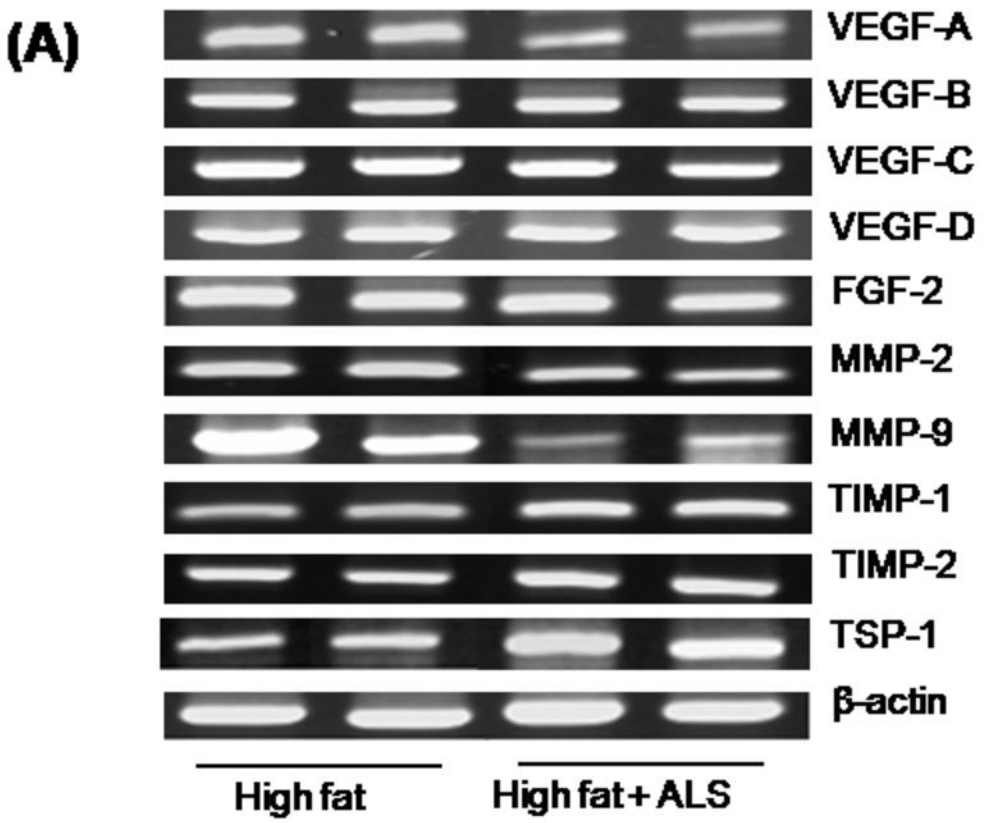

(A)
VEGF-A
VEGF-B
VEGF-C
VEGF-D
FGF-2
MMP-2
MMP-9
TIMP-1
TIMP-2
TSP-1
β-actin
High fat
High fat + ALS
(B)
VEGF-A
VEGF-B
VEGF-C
VEGF-D
FGF-2
MMP-2
MMP-9
TIMP-1
TIMP-2
TSP-1
β-actin
High fat
High fat + ALS

Table 1. PCR primers used for cDNA synthesis by RT-PCR.

Gene	Gene Bank	Primer sequence	cDNA size (bp)
VEGF-A	NM009505	Forward: 5´-gctctcttgggtgcactgga-3' Reverse: 5'–caccgccttggcttgtcaca-3'	561
VEGF-B	NM_011697	Forward: 5´-gtcaaacaactagtgcccag-3' Reverse: 5´-tgtctgggttgagctctaag-3'	407
VEGF-C	NM_053653	Forward: 5´-ccaaaccagtaacaatcag-3' Reverse: 5´-attcacaggcacattttc-3'	461
VEGF-D	D89628	Forward: 5´-acctcctacatctccaaac-3' Reverse: 5´-tccagactttctttgcac-3'	385
FGF-2	NM174056	Forward: 5´-aactacaacttcaagcagaagagaga-3' Reverse: 5' - ttaagatcagctcttagcagacat-3'	293
TSP-1	M62470	Forward: 5´-cctcatttgttgtgtgactgagtaa-3' Reverse: 5'-ttgttgttccttgtacataagaaac-3'	556
MMP-2	M84324	Forward: 5´-agatcttcttcttcaaggaccggtt-3' Reverse: 5´-ggctggtcagtggcttggggta-3'	224
MMP-9	NM_013599	Forward: 5´-tgcgaccacatcgaacttcg-3' Reverse: 5´-gagaagaagaaaaccctcttgg-3'	683
TIMP-1	NM_001044384	Forward: 5´-ggcatcctcttgttgctatcactg-3' Reverse: 5'-gtcatcttgatctcataacgctgg-3'	170
TIMP-2	NM_021989	Forward: 5´-gagatcaagcagataaagatg-3' Reverse: 5'-gacccagtccatccagaggc-3'	320
β-actin	NM_00793	Forward: 5´-tggaatcctgtggcatccatgaaac-3´ Reverse: 5´-taaaacgcagctcagtaacagtccg-3´	348