Evaluation of the inhibition of the differentiation of pre-adipocytes into matures adipocytes

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Summary

Up until today, the key to contouring has been resumed in these two alternatives, either limiting the adipocyte storing capacity by modulating lipogenesis, or by stimulating lipolysis to eliminate adipocyte lipid content. Another interesting way could be the regulation of adipocyte differentiation. In this work, we have evaluated the effect of a brown algal extract of *Sphacelaria scoparia (SSE)* on the differentiation of pre-adipocytes into adipocytes. A pre-adipocyte line (3T3-L1) was used. The differentiation was evaluated by the measure of produced lipids thanks to red oil coloration and spectrophotometry, and also by the expression of adipocyte differentiation markers: enzymes such as fatty acid synthase (FAS) and stearoyl CoA desaturase (SCD), or membrane proteins such as glucose transporters (GLUT-4) and fatty acid transporters (FAT) expressed on the surface of human adipocytes. These genes are under control of two transcription factors: CAAT-enhancer binding protein (c/EBP alpha) and sterol response element binding protein (SREBP1). All these markers were analysed at different stages of differentiation by RT-PCR. Sphacelaria extract (SSE) inhibits pre-adipocytes differentiating into adipocytes following a dose-dependant relation, using a kinetics similar to retinoic acid. It decreases the expression of mRNA specific to FAS, FAT, GLUT-4, SCD1, c/EBP alpha and SREBP1. Moreover, SSE regulated on collagen 1 and collagen 4 expression. A stimulation of collagen 1 was also measured in human skin fibroblasts. Thus, SSE performs as a genuine differentiation inhibitor and not only as a lipogenesis inhibitor, and could be used in slimming products.

Introduction

Numerous works have demonstrated that certain substances can have an effect on hypodermal fat cells (adipocytes), particularly by stimulating intra-cellular triglyceride lysis and the extra-cellular release of fatty acids and glycerol (lipolysis) or by inhibiting the formation of new triglycerides (lipogenesis). Plant extracts that have this type of effect have been used in the composition of cosmetic and/or pharmaceutical products for body contouring. In the present document, we have evaluated another way of action: the control of formation of fat cells (mature adipocytes) originating from precursor adipocytes (pre-adipocytes). This transformation is called "cell differentiation".

The adipocyte is a fat storing cells, normally located in the hypodermis and specialized in the synthesis and the storing of energy in the form of triglycerides. Its an extensible, spherical cell of a about 50 to 150 µm in diameter. One of the particularities of adipose tissue is its large plasticity, because the numerous adipose cells increase throughout their life span in function of environmental conditions. There is therefore a phase of pre-adipocyte increase (growth step) followed by a phase of lipid accumulation in the new adipose cells (differentiation). The adipocyte comes from a pluripotent mesenchymatous stem cell. This cell presents the faculty of differentiating itself into different cellular types: adipocytes (fatty tissue), or chondrocytes (connective tissue), osteoblasts (bone tissue), or myocytes (muscular tissue). Differentiation into a given cell type necessitates the expression of specific transcription factors. By differentiation, these stem cells transform first of all into adipoblasts, non-differentiated cells, then into pre-adipocytes, cells that do not yet have the capacity to accumulate triglycerides. The pre-adipocyte expresses precocious genes, amongst which are CCAAT-Enhancer Binding Protein (c/EBP) and Peroxisome Proliferator Activated Receptors (PPAR). Finally, during terminal differentiation, the cell acquires the characteristic functions of adipose cells, i.e. the capacity to synthesize and store triglycerides and to hydrolyse them in a response to the hormonal environment.

This last stage corresponds to the induction of c/EBP and PPAR type transcription factors. Once the adipocytes have differentiated, they can store triglycerides.

We have used a method to study the differentiation of pre-adipocytes into mature adipocytes. The cells used in culture for this test belong to the 3T3-L1 line of pre-adipocytes. These cells are already heading towards adipogenesis. They have been widely studied in this type of test. Adipocytes obtained in this way express differentiation markers on their surface and in their cytoplasma [1, 2].
The studied parameters were the cellular morphology, the lipid content measured by red oil coloration, the expression of differentiation markers such as the two transcription factors CCAAT/ enhancer binding protein alpha (c/EBP alpha) and sterol-response-element-binding protein 1 (SREBP1), the two enzymes fatty acid synthase (FAS) and stearoyl CoA desaturase (SCD1), and the two membrane receptors glucose transporter (GLUT-4) and fatty acid translocase (FAT).
We have tested the effects of a hydroglycolic extract obtained from the brown alga *Sphacelaria scoparia* (SSE).

Material and Methods

Materials

The 3T3-L1 line cells were obtained from the European Collection of Cell Cultures (ECACC, UK). Human skin fibroblasts were obtained from cosmetic surgery. Dubelco's modified Eagle's medium (DMEM), Hank's balanced salt solution (HBSS), calf serum and foetal calf serum (FCS) were from Gibco Life Technology (Grand Island, NY, USA). Insulin (IS), 3-isobutyl-1-methylxanthine (IBMX), dexamethasone (DEX), all-trans retinoic acid (ATRA) and ascorbic acid (vitamin C), transforming growth factor beta 1 (TGF-beta1) and antibody anti-collagen 1 (COL1) were from Sigma Chemical (St. Louis, MO, USA).

Sphacelaria scoparia extract (SSE) preparation

Sphacelaria scoparia extract (SSE) was prepared by a short-time extraction of the brown seaweed *Sphacelaria scoparia* in demineralised water and dipropylene glycol (ratio 70/30) at room temperature, followed by filtration with a 0.22 µm sterilised filter for the adipocyte differentiation study.

Cell culture and adipocyte differentiation

The 3T3-L1 cells were cultured at 37°C in DMEM supplemented with 10% calf serum, 2 mM glutamine, 4,5 g/l glucose, 0.11 g/l sodium pyruvate, 50 U/ml penicillin, and 50 mg/ml streptomycin in a 5 % CO_2 cell incubator. Pre-adipocytes were grown in 24-well plates until 2 day post-confluence. The differentiation was induced as previously described [3] with addition of 10 µg/ml IS, 0.25 mM IBMX and 0.25 µM DEX (MID mixture) in DMEM supplemented with 10% FCS. Two days after induction, the MID-containing medium was replaced with fresh culture medium (DMEM with 10 % FCS) containing only 1 µg/ml IS. This medium was replaced each two days. To determine the effect of *Sphacelaria extract* in adipocyte differentiation, SSE was tested at 3 non-cytotoxic concentrations (0.4 %, 1.0 % and 2.5 %) in the medium containing MID either at the beginning of the induction (day 0), or at different stages of the induction (day 2, 4, or 6) and renewed every two days with the culture medium. ATRA was tested at 10 µM and used as a positive indicator. It was tested under the same conditions as SE. The differently induced cells were analysed 7 days after the initiation of induction.

Microscopic observation and red oil coloration

The degree of differentiation, with and without the tested products, was evaluated by microscopic observation of the lipid accumulation. At 7 days after the initiation, cells were washed twice with HBSS and then fixed for 20 minutes with 3.7 % formaldehyde. Cells were washed twice in HBSS and the lipidic content of the cells is evaluated by the red oil coloration method. Briefly, cells were stained for 10 minutes using a 0.5 % Red Oil in isopropanol / water at room temperature. Then the stain was solubilized in pure isopropanol, and the absorbance was measured at 540 nm on a spectrophotometer (Perkin Elmer, USA). The absorbency is proportional to the amount of stainable lipids accumulated in the adipocytes. Experiments were made in triplicate. Results are expressed as percentage of the not induced cells +/- SD. Statistical analysis was made using Student T test. An effect was considered as significant for $p<0.05$.

RNA isolation and semi-quantitative RT-PCR

After 7 days of incubation, cells were harvested and pelleted. Total RNA was extracted using a total RNA isolation kit (Tri-Reagent). DNA was eliminated using DNA-free Sytem (Ambion). The RNA quality was evaluated on agarose gel. Reverse transcription (RT) was made with oligo(dT) and Superscript II enzyme (Gibco Life Technology, USA). The obtained cDNA was quantify by fluorimetry and the solution was adjusted at 20 ng/ml. Polymerase chain reaction (PCR) was performed with Light cycler System (Roche Molecular Systems Inc.). The incorporation of fluorescence in amplified DNA was continually measured during the PCR cycles in order to give a relative expression value for each tested marker.

Detection of collagen 1 by immunocytochemistry

Human skin fibroblasts were cultivated for 48 hours in presence or absence of SSE at 0.5 % and 1 %. In parallel, a mixture composed of 2 ng/ml TGF-beta1 and 2 µg/ml vitamin C was used as a positive indicator. The collagen 1 synthesis was analysed on cells by immunocytochemistry thanks to an anti-collagen 1 antibody (COL1) and an alkaline phosphatase (AP)-conjugated anti-IgG as previously described [4]. The intracellular staining was evaluated using an image analyser. Results were expressed in function of untreated cells.

Results

SSE decreased the lipid accumulation induced during differentiation

In absence of MID, the cells remained pre-adipocytes in very high majority, i.e. with a fibroblast-like morphology (figure 1A). In presence of MID for 2 days, the cells contained a multitude of red oil coloured lipidic droplets in their vast majority (figure 1B). After incubation for 7 days with both MID and 10 µM ATRA, cells did not differentiate into adipocytes. ATRA at 10 µM inhibited by 82 ± 21 % (mean and SD of 5 independent experiments) the differentiation. There was almost no lipidic droplets. The cells conserved their pre-adipocyte morphology (figure 1C). After incubation with both MID and SSE at different concentrations, the appearance of lipidic droplets was also limited (figure 1D). SSE, tested at 0.4 % and 1.0 % (v/v), inhibits by 28 ± 2 % and 78 ± 11 % respectively the differentiation of pre-adipocytes into adipocytes, (figure 2). Tested at 2.5 %, SSE totally inhibits the differentiation. This effect is dose dependant and statistically significant ($p<0.05$).

SSE blocked adipocyte differentiation as soon as induction takes place

When pre-adipocytes were incubated with 1 % SSE after differentiation induction (from day 2 to day 7), the inhibiting effect was lower than the effect observed when the same concentration of SSE was incubated at the beginning of the induction (from day 0 to day 7): only 15 % instead of 72 % (figure 3, left). But when it was incubated for the two first days (from day 0 to day 2), SSE at 1 % continued to inhibit by 43 % the adipocyte differentiation (figure 3). ATRA at 10 µM was also very effective when it was incubated during the two first days, but totally ineffective when it was added after the two first days (figure 3, right).

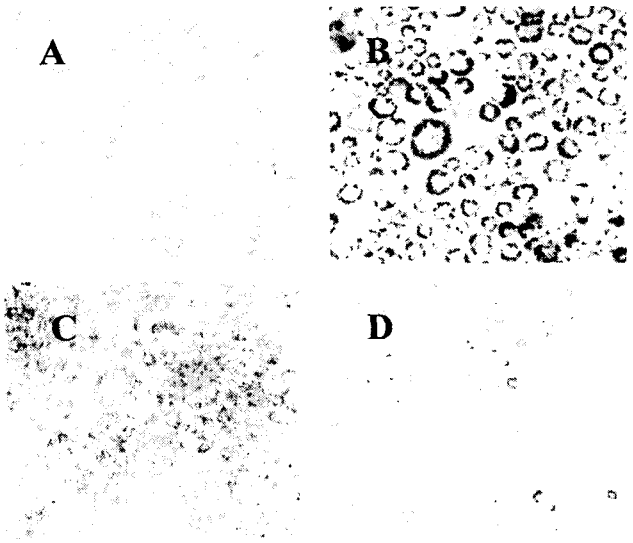

Figure 1 : Microscopic observation of undifferentiated pre-adipocytes (A), MID-differentiated adipocytes (B), adipocytes cultivated with MID and 10 μM ATRA (C) or with MID and 1 % SSE (D)

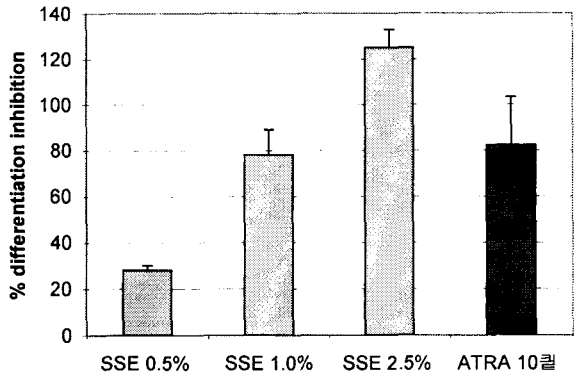

Figure 2 : Effect of SSE and ATRA on lipid content measured by red oil coloration

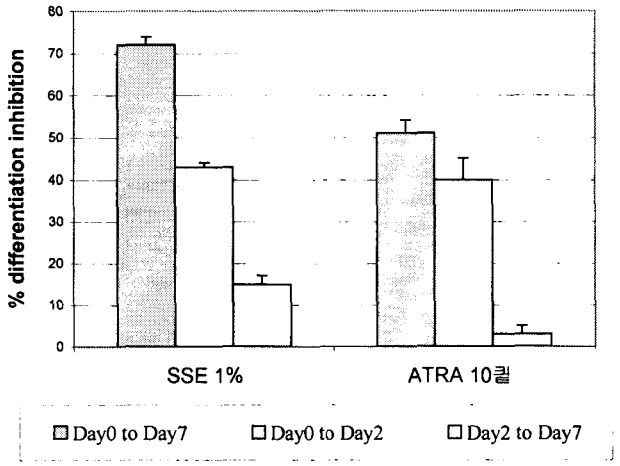

Figure 3 : Effect of SSE and ATRA on lipid content in function of incubation periods

SSE decreased the expression of adipocyte differentiation markers

In absence of stimulation, the 3T3-L1 line cells expressed a low level of adipocyte specific markers. During differentiation by MID, cells expressed an elevated level of mRNA coding for all the studied markers: the relative level increased by 2.7 for SREBP1, 8.3 for FAS, 27.7 for c/EBP alpha, 87 for FAT, 567 for SCD1, and 3,079 for GLUT-4 (figure 4). In all case, incubation with 1 % SSE decreased the mRNA level for these markers as compared to the level measured in adipocytes differentiated without SSE: SREBP1 mRNA expression decreased by 31 %, FAS mRNA expression decreased by 58 %, c/EBP alpha mRNA expression decreased by 37 % (figure 4), SCD1 mRNA expression decreased by 27 %, FAT mRNA expression decreased by 56 %, GLUT-4 mRNA expression decreased by 23 % (figure 4).

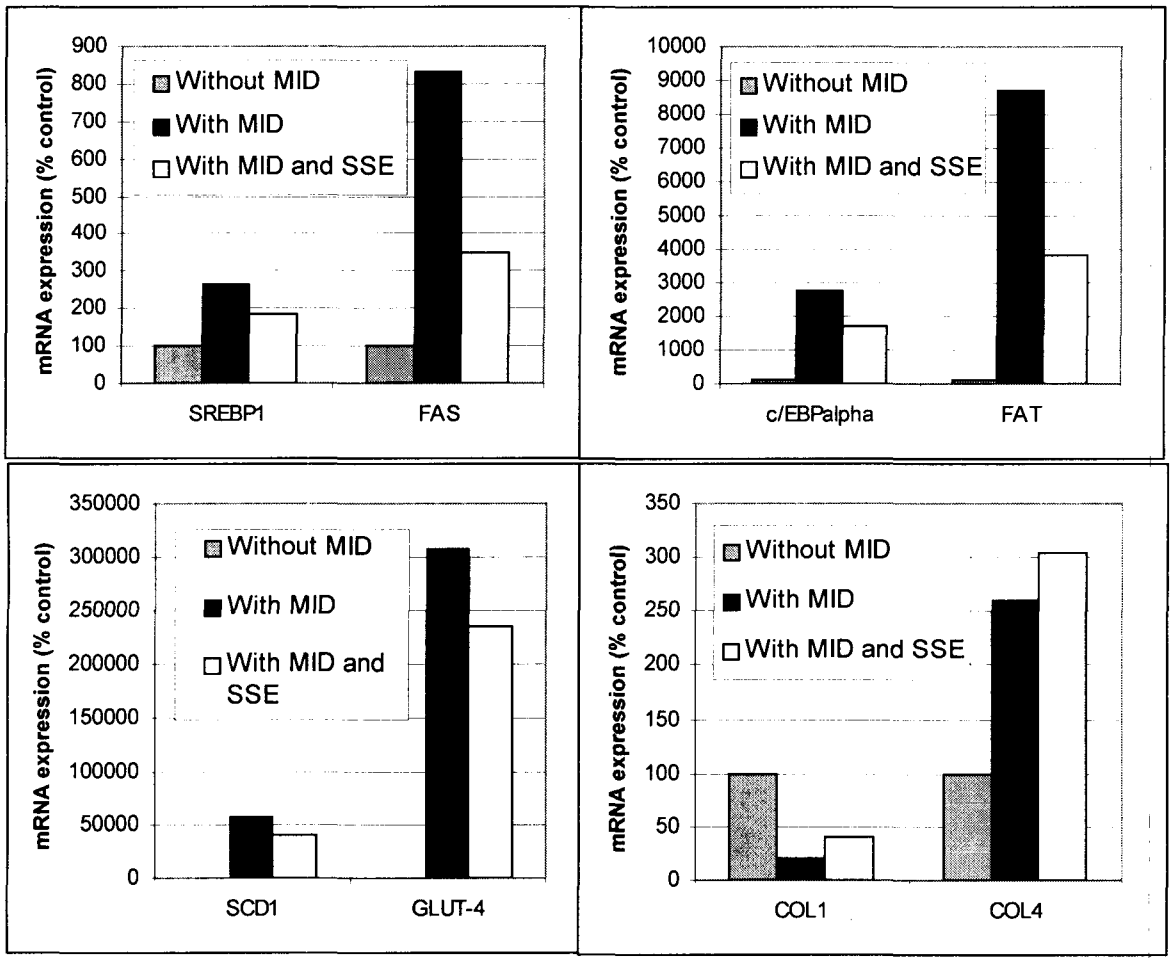

Figure 4 : Effect of SSE on mRNA expression coding for severail adipocyte differentiation markers: SREBP1, FAS, c/EBP alpha, FAT, SCD1, GLUT-4, COL-1 and COL-4

SSE maintained the collagen expression in pre-adipocytes

Type 1 collagen was highly expressed in 3T3-L1 cells. Its expression was diminished by 80 % in differentiated adipocytes after 7 days. The presence of SSE maintained a high level of collagen 1 expression: SSE at 1.0 % increased by a factor 2 the collagen I expression in comparison with the cells cultivated with MID alone (figure 4).

By contrast with type 1 collagen, the type 4 collagen expression was increased in differentiated adipocytes (by a factor 2.6 after 7 days). Even though it limited differentiation, SSE did not limit collagen 4 expression, but it increased it slightly by 17 % (figure 4).

SSE increased the expression of collagen 1 in human skin fibroblasts

After an incubation of 48 hours with human skin fibroblasts, SSE at 1.0 % increased by 61 % the collagen 1 synthesis (figure 5). This effect was statistically significant ($p<0.02$).

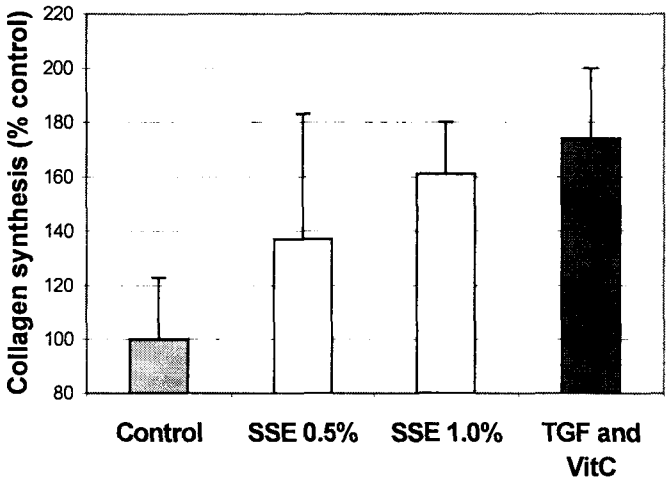

Figure 5 : Stimulation by SSE of collagen synthesis by human skin fibroblasts

Discussion

The 3T3-L1 cell line is a well-known line usually used as a model for adipocyte differentiation studies [5]. After 2 days of culture after confluence, cells could be induced to become adipocytes with an adipogenic cocktail containing a phosphodiesterase inhibitor (that increases the intracellular cAMP level), an anti-inflammatory molecule (such as dexamethasone), and insulin.
It was previously described that this antigenic cocktail increased lipid production and the more visible modification was the accumulation of lipid droplets easily observed by microscopy. In the same time, it increased the expression of mRNA coding adipocyte specific markers: cells expressed mRNA for fatty acid binding proteins [6]. Other markers are known to be expressed earlier such as the transcription factors c/EBP alpha, beta and gamma [7], and the sterol response element binding protein (SREBP1) [8]. c/EBP alpha controls the expression of stearoyl CoA desaturase (SCD1) and the glucose receptor (GLUT-4) [9]. Others adipocyte specific markers were studied as the key enzyme important for fatty acid synthesis (FAS) and fatty acid translocation (FAT). The expression of these genes follows a chronology during the adipocyte differentiation: it was initially studied thanks to mRNA measures by Northern blotting: the confluence induces an increase in lipoprotein lipase (LPL) expression and adipogenic cocktail stimulates a fast expression of transcription factors such as c-myc, c-fos and c-jun, then c/EBP beta, c/EBP delta and PPAR gamma [10]. It was also noted that the c/EBP alpha expression starts after c/EBP beta and c/EBP delta (that appeared after only a few hours), but just before the expression of GLUT-4, SCD1, FAS and FAT, and the appearance of lipid droplets. After 7 days, only c/EBP alpha continue to be detectable [6]. Gene expression in 3T3-L1 was more recently studied thanks to DNA micro-array method [11, 12]. It is clear now that almost of these genes are implicated into the metabolism disorders that drive to an excess of production and storage of fat in adipose tissue [13].

In this work, lipid production was measured using red oil coloration, and mRNA level was measured using semi-quantitative RT-PCR in the 3T3-L1 cells cultivated for 7 days with and without *Sphacelaria extract* (SE). Our experiments have shown that this seaweed extract can work as a blocking adipocyte differentiation as soon as induction takes place. It acts as a veritable differentiation inhibitor and not a lipidic synthesis (lipogenesis) inhibitor. Tested at 1 %, this extract has the same level of action than ATRA tested at 10 µM. This could be explained by the fact it decreased the expression of c/EBP alpha. This latter belongs to the CCAAT / enhancer binding proteins transcription factor family that plays a role in the later stages of adipocyte differentiation by maintaining the expression of PPAR gamma precocious nuclear transcription factors associated with retinoid X receptor (RXR). The retinoic acid was known for inhibiting the differentiation of pre-adipocytes into adipocytes [14]. The c/EBP alpha is

also known to regulate GLUT-4 and SCD1 coding gene transcription, but also the expression of fatty acid binding protein (FABP4) and leptin [15]. FABP4 is specific to adipocyte, and it is product in 3T3-L1 after MID induction [16]. It belongs to the cellular retinoic acid binding protein (CRABP) family. It is recently showed that it is the aP2 gene product [17].

SSE regulates the glucose receptor expresion. The glucose penetrates the adipocyte by diffusion facilitated thanks to transmembrane proteins that serve as specific transporters : GLUT-4 is present in the adipocyte, associated with intracellular vesicles. In presence of insulin, these vesicles quickly migrate from their perinuclear position to the plasmic membrane and fusion with it. GLUT-4 is then expressed on the adipocyte surface (this phenomenon is called translocation). It was previously described that the GLUT-4 expression increases in the 3T3-L1 cells by MID [18].

SSE decreased the stearoyl-CoA desaturase (SCD1). It is an enzyme that converts saturated fatty acids into unsaturated fatty acids by the insertion of a double binder; palmitate and stearate are the principle subtracts [10]. Inactivated 3T3-L1 cells do not express the SCD1 gene. Its expression is increased by factor 100 after induction with the MID mixture, by contrast to SCD2 expression that is few modified [10]. SCD1 expression is known to be increased after 3 days and is maximal after 5 days. It is inhibited by thiazolidinediones such as troglitazone.

SSE decreased Sterol Response Element Binding Protein 1 (SREBP1) that is induced precociously during adipocyte differentiation. Also named Adipocyte Differentiation and Determination Factor 1 (ADDF1) [8] ; it regulates PPAR gamma implicated in adipocyte differentiation and insulin sensitivity [19]. This regulation could be linked to PPARg-1 et PPARg-3 promoters that have type E-box sequence. its activity is dependant to cholesterol level. SREBP1 is also known as a FAS expression regulator [20] and is related to a promoter sequence of the FAS gene called SRE (Sterol Response Element).

SSE decreased the fatty acid synthase (FAS). This is a key enzyme in lipogenesis because it participates in the synthesis of fatty acids. It is regulated by the SREBP transcription factor and is induced by nutrition and insulin. The FAS activity is weak in non-induced 3T3-L1, and it becomes strong (x 20) after 48 hours of treatment with MID [21]. Insulin alone increases FAS gene expression (x 3) by 3T3-L1 cells but also by human adipocytes [22]. The FAS activity is negatively regulated by unsaturatted fatty acids such as eicosapentanoic acid (EPA).

SSE decreased the fatty acid translocase expression. FAT is a fatty acid membrane transporter, expressed on the surface of adipocytes. It could be detectable using an anti-CD36 antibody.

By contrast, SSE maintained the expression of type 1 collagen expression. These fibres are produce by fibroblasts but also by chondrocytes and osteoblasts. These cells have the same precursor as fibroblasts and adipocytes. Collagen 1 was visualised between the adipocytes, and are probably important to preserve the adipose tissue structure. But during differentiation, adipocytes lost their ability to produce type 1 collagen, and mature adipocytes produce other types of collagen, as type 4 collagen. This collagen has not fibre structure but it is a constituent of basal membranes, specially the membrane between dermis and epidermis. This location allows to think it has a filtration function.

Conclusion

Sphacelaria scoparia extract (SSE) inhibits the differentiation of pre-adipocytes into mature adipocytes by diminishing the expression of the main adipocyte differentiation markers: it decreases the expression of sterol response receptor (SREBP 1) that controls the fatty acid synthesis enzyme (FAS); it decreases the expression of c/EBP alpha transcription factor that controls the enzyme for conversion of saturated fatty acids into mono-unsaturated (SCD1) and the glucose transporter (GLUT-4). Moreover, it decreases the expression of fatty acid membrane receptor (FAT). By contrast, it stimulates the expression of both collagen 1 and 4, and reinforces the properties for dermis support. In addition to its action on adipocyte differentiation, SSE contributes to deeply restructuring the skin, particularly skin distended during cellulite. This adipogenesis inhibitor could be integrated in contouring, anti-cellulite ranges, either alone, or combined with lipolytic ingredients and lipogenesis inhibiting ingredients.

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