Effect of *Foilum mori* on Adipocyte Differentiation

Geun Won Lee and Sang Yo Byun*

School of Chemical Engineering and Biotechnology, Ajou University, Gyunggi 442-749, Korea

Abstract Several natural products were tested to control the differentiation of cultured human mesenchymal stem cell into adipocyte. The extent of the inhibitory effect on the conversion of adipose was measured using Oil red O staining, which stains accumulated lipid droplets in the cytoplasm of adipocyte. Of the various natural product extracts, the adipocyte differentiation was inhibited by an extract from *Folium mori* in the concentration range 1×10^{-4} ~5×10^{-2} g/mL. These results suggest that *Folium mori* has an inhibitory activity toward the adipose conversion, which is a major cause of obesity.

Keywords: adipocyte, *Folium mori*, adipose conversion

Adult bone marrow contains mesenchymal stem cells (hMSCs), which can be induced to exclusively differentiate into adipocytic, chondrocytic or osteocytic lineages. Human MSCs are present in adult marrow, which can replicate undifferentiated cells. These cells display a stable phenotype, and remain as a monolayer *in vitro*. Adipocytic induction is apparent by the accumulation of lipid-rich vacuoles within the cells. Multiple induction treatments result in more than 95% of the cells committing to this lineage, and the lipid vacuoles continue to develop over time, coalesce and eventually fill the cells [1].

Adipose tissue allows for the storage of triacylglycerol in periods of energy excess, and the subsequent use of these stores during energy deprivation [2]. Until recently, adipose tissue was considered to act as a strong deposit for fat, causing obesity. However, adipocytes are actively involved in maintaining the energy balance in the body. The presence of adipose cells in a cell culture can be monitored using methods such as, staining with lipophilic dyes, RT-PCR detection of marker genes or biochemical measurement of the enzymic activities [3-5]. Intracytoplasmic lipid accumulation seems to be directly proportional to the extent of differentiation. This relationship has been commonly used as a qualitative marker of adipose conversion after staining with Oil red O, a lipid soluble dye, which is suitable for the histochemical staining of neutral fats and cholesteryl esters [6,7].

Throughout studies on the adipogenesis of 3T3 cells, a number of negative regulators of adipocyte differentiation have been identified. Negative regulators of adipogenesis include, retinoic acid, transforming growth factor β, vitamin E and fibroblast growth factor [8-11]. It is a question of great interest that a number of the negative regulators of adipocyte differentiation are growth factors [12]. We have undertaken a systemic approach to investigate the effects of extracts from *Folium mori*, *Liriope platyphylla*, dried orange peel and green tea, on the adipocyte differentiation. Our studies focused on the inhibitory activities of several natural products on the differentiation of hMSC into adipose cells, which could be determined by the extent of adipose conversion, using Oil red O staining.

Human MSCs, marrow-derived stem cells, were cultivated, in the undifferentiated state, on gelatin-coated plates. The cells were inoculated into T75-cm^2 tissue culture dishes, at a density of 6×10^5 cells per dish, in 20 mL of Dulbecco and Vögt-modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS). For some experiments, the growing cells were fed every three days with the medium, supplemented with 10% FBS and antibiotics. The cells were cultured as monolayers, and grown to confluence. The growing cells were inoculated into 12-well plates, at a 4×10^4 cells per well, in 0.7 mL of DMEM, supplemented with 10% FBS. After confluence, an adipogenic induction (MDI + I) medium, containing 1 μM dexamethasone, 0.5 mM methyl-isobutylxanthine, 10 μg/mL insulin, 100 μM indomethacin and 10% FBS in DMEM, was added. The hMSCs were incubated in this medium for 72 h, and then the medium was changed to adipogenic maintenance(AM) medium, containing 10 μg/mL insulin, 10 μg/mL biotin and 10% FBS in DMEM, for 24 h. The lipid vacuoles were then first detectable within the cells. The cells were then re-treated with MDI + I for the second or third treatments. The cultures were then maintained in the AM medium for another week prior to fixation [1]. The adipocyte differentiation was determined by the accumulation of lipid vacuoles, as shown in Fig. 1.

The cultured cells were fixed for at least 1 h, with 10% formalin in phosphate buffered saline, washed with water, fixed again overnight, stained for 2 h by complete immer-

***Corresponding author**
Tel: +82-31-219-2451 Fax: +82-31-219-1612
e-mail: sybyun@ajou.ac.kr

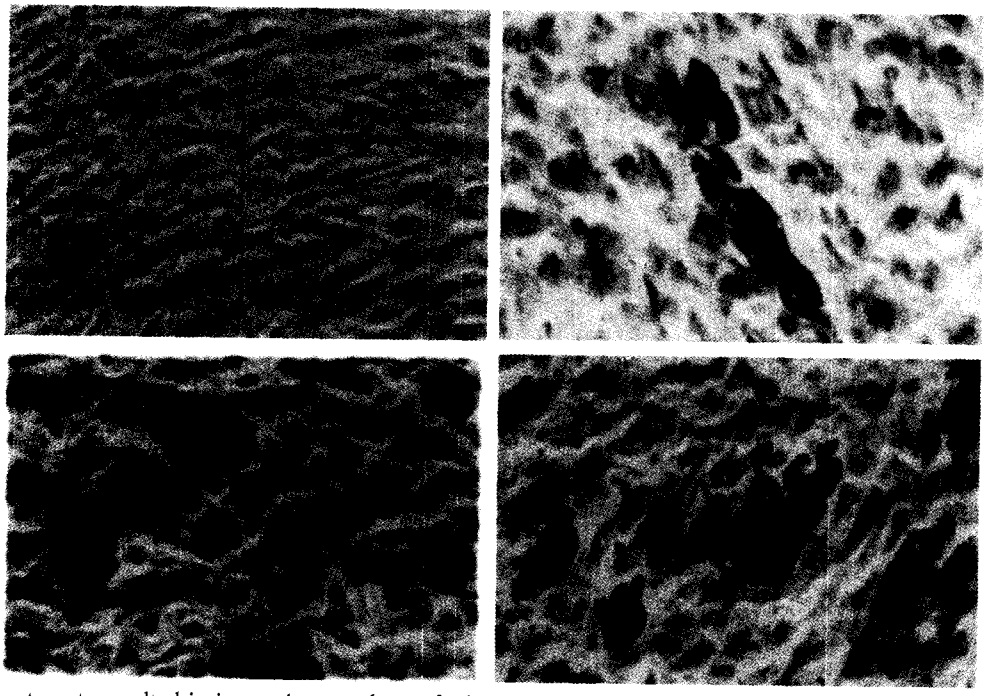

Fig. 1. Multiple treatments resulted in increasing numbers of adipocytes, as shown by Oil red O staining: (a) the confluent stage of hMSCs, (b) the cells with MDI + I for the first treatment, (c) the cells with MDI + I for the second treatment and (d) the cells with MDI + I for the third treatment.

sion in a working solution of Oil red O and washed with water. Excess water was dried from the stained cells at room temperature. In order to determine the extent of adipose conversion, 200 μL of isopropyl alcohol was added to the stained culture dish. The extracted dye was taken immediately and its absorbance monitored, using a Digiscan, at 495 nm in a 96-well plate. The Oil red O working solution was prepared by dissolving 4.2 g of Oil red O in 1,200 mL of absolute isopropanol, which was left to stand overnight. 900 mL of distilled water was the added [3]. The number of absorbance units obtained from the extraction of Oil red O, at 495 nm, was used to calculate the triglyceride content, according to a triolein calibration curve, as shown in Fig. 2. Triolein was dissolved in hexane, at a concentration of 25 mg/mL, and stored at -20°C. Aliquots of the triolein solution were placed in microtubes, and the solvent evaporated with N_2. The tubes were stained with Oil red O, as described above, for the adipose conversion assay.

Water soluble extracts, from the *Folium mori*, *Liriope platyphylla*, dried orange peel and green tea, were prepared. 100 grams of dried *Folium mori* leaves were ground into powder using a homogenizer. The extraction was carried out by boiling the powder with 1 litre of water for 3 h. The extract was filtered, and concentrated to dryness using a rotary evaporator. For other extracts, the same procedure was applied to ground powders of the *Liriope platyphylla*, dried orange peel and green tea leaves. The effects of the water extracts, from the *Folium mori*, *Liriope platyphylla*, dried orange peel, and green tea, on the adipocyte differentiation were tested. The extracts

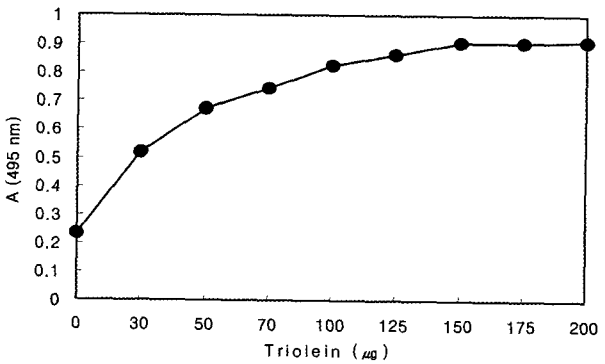

Fig. 2. Standard triolein calibration curve. The data represent average values from five experiments.

were to human MSCs dosed three times in MDI + I medium. After the third treatment, the differentiated cells were maintained in adipogenic maintenance (AM) medium for one week, and the degree of cell differentiation measured using the Oil red O staining assay. *Folium mori* inhibited the adipose conversion in the concentration range $1 \times 10^{-4} \sim 5 \times 10^{-2}$ g/mL. The triglyceride accumulated in the control cells was 133.2 μg. In the *Folium mori* treated cells, the triglycerides decreased to 33.2 ~ 43.7, as shown in Fig. 3. The *Folium mori* extract reduced the triglyceride accumulation by up to a 1/4, indicating less MSCs differentiation to adipose cells. When the *Folium mori* extract was dosed at the higher concentration of 5×10^{-2} g/mL, cell death and lysis were observed.

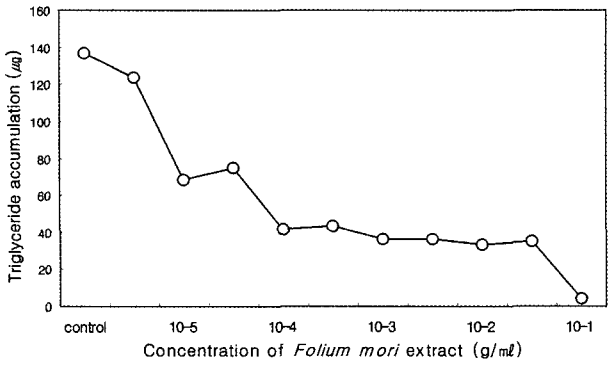

Fig. 3. Determination of the intracytoplasmic triglyceride content of the adipocytes in *Folium mori* treated cells.

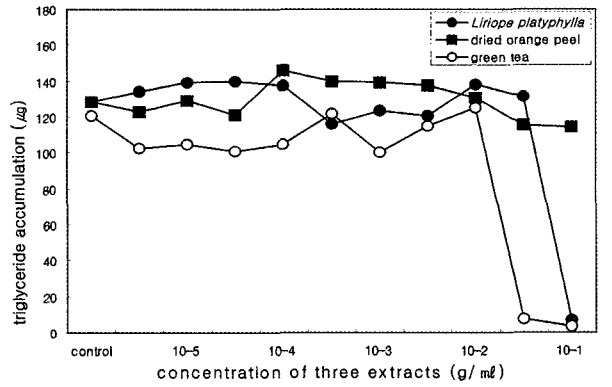

Fig. 4. Determination of the intracytoplasmic triglyceride content of the adipocytes in *Liriope platyphylla*, dried orange peel and green tea treated cells.

When preadipocytes grow to confluence, they convert, with a certain frequency, to adipose cells. In the course of this conversion, they undergo many changes, both morphological and enzymic. They develop an increased activity of the enzymes for fatty acid synthesis. The differentiation was defined by a change in the shape of the cells and increases in the activity of enzymes important in triglyceride synthesis [6]. However, the three extracts, *Liriope platyphylla*, dried orange peel and green tea, did not affect the adipose conversion, as shown in Fig. 4.

The physiological activities of *Folium mori,* have until now been reported to effect the depression of blood sugar, by deoxynojirimycin type, depression of blood pressure, by rutin, antibacterial function, by moracin type, and antiphlogistic function by umbelliferone [13]. *Folium mori* has been recorded to have an effect on diabetes mellitus, in "Class and Order of Medical Herbs", a Chinese medical book. It has been reported that *Folium mori* extract are potent inhibitors of intestinal α-glycosidase, and inhibit the digestion of starch and sucrose in the small intestine [14]. Because white mulberry, root bark of the mulberry tree, has been used as a fatness inhibitor in folk remedies, it was anticipated that *Folium mori* would have this activity. Recently, the inhibition of cholesterol synthesis and acceleration of cholesterol catabolism in the liver have been reported as the hypolipidemic effects of *Folium mori* on rats [15]. In connection with this, our study clearly showed the inhibitory affect of *Folium mori* on the accumulation of triglyceride, *in vitro,* which is related to adipocyte differentiation, a factor in obesity. Obesity, an unhealthy excess of body fat, carries with it substantial health risks. The casual relationship between diabetes and obesity is not fully understood, but upper body obesity is clearly a strong risk factor for the development of diabetes. A probable common link between diabetes and obesity is the adipocytes, which store excess energy, in the form of triglyceride, and release free fatty acids in response to energy requirements, such as in times of fasting.

Acknowledgement This research work was supported by a grant from the Engineering Research Center for Advanced Bioseparation Technology, KOSEF.

REFERENCES

[1] Pittenger, M. F. and D. R. Marshak (1999), Multilineage potential of adult Human mesenchymal stem cells. *Science* 284: 143-147.

[2] Ailhaud, G., P. Grimaldi, and R. Negrel (1992) Cellular and molecular aspects of adipose tissue development. *Annu. Rev. Nutr.* 12: 207-233.

[3] Ramirez-Zacarias, J. L. and W. Kuri-Harcuch (1992) Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Biochemistry* 97: 493-497.

[4] Sottile, V. and K. Seuwen, (2000) Bone morphogenetic protein-2 stimulates adipogenic differentiation of mesenchymal precursor cells in synergy with BRL 49653 (rosiglitazone). *FEBS Lett.* 475: 201-204.

[5] Wise, L. S. and H. Green (1979) Participation of one isozyme of cytosolic glycerophosphate dehydrogenase in the adipose conversion of 3T3 cells. *J. Biol. Chem.* 254: 273-275.

[6] Kuri-Harcuch, W. and H. Green (1978) Interruption of the adipose conversion of 3T3 cells by biotin deficiency : differentiation without triglyceride accumulation. *Cell* 14: 53-59.

[7] Kasturi R. and V. C. Joshi (1982) Hormonal regulation of stearoyl coenzyme A desaturase activity and lipogenesis during adipose conversion of 3T3-L1 cells. *J. Biol. Chem.* 257: 12224-12230.

[8] Murray, T. and T. R. Russell (1980) Inhibition of adipose conversion in 3T3-L2 cells by retionoic acid. *J. Supramole. Structure* 14: 255-266.

[9] Ignotz, R. A. and J. Massague (1985) Typeβ transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts. *Proc. Nat. Acad. Sci. USA* 82: 8530-8534.

[10] Kawada, T., N. Aoki, Y. Kamei, and E. Sugimoto (1990) Comparative investigation of vitamins and their analogues on terminal differentiation, from preadipocytes to adipocytes, of 3T3-L1 cells. *Comp. Biochem. Physiol.* 96A: 323-

326.
[11] Hayashi, I., T. Nixon, M. Morikawa, and H. Green (1981) Adipogenic and anti-adipogenic factors in the pituitary and other organs. *Proc. Nat. Acad. Sci. USA* 6: 3969-3972.
[12] Miriam J. S., L. S. Rodney, and W. Walker (1993) Proadipocyte cell lines: Models of cellular proliferation and differentiation. *J. Cell Sci.* 106: 1-9.
[13] Ahn M. Y. and Y. K. Lee (2001) Cytotoxicity of the ethanol extracts of mulberry leaves, branches and silkworm feces. *Korean J. Seric. Sci.* 43: 26-28.
[14] Lee K. H. and S. H. Chung (2000) Antidiabetic effect and mechanism of Mori Folium on streptozotocin-induced diabetic mouse. *Bull. K. H. Pharma. Sci.* 28: 87-99.
[15] Pascot, A. (2001) Deterioration of the metabolic risk profile in women: Respective contributions of impaired glucose tolerance and visceral fat accumulation. *Diabetes Care* 24: 902-908.

[Received May 12, 2003; accepted September 20, 2003]