Expression of APAF1 gene during 3T3-L1 preadipocyte differentiation and regulative role of tumor necrosis factor-alpha

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Original article

Background: **The size and number of adipocytes are believed to play a key role in the pathophysiology of obesity. The former mainly refers to the differentiation of adipocytes and the latter principally refers to the proliferation and apoptosis of adipocytes. Our cDNA microarray assay had, compared to normal rats, suggested that apoptotic protease activating factor 1 (APAF1) gene expression level was lower in the adipose tissue of diet-induced obese (DIO) rats. This study was undertaken to investigate the changes of APAF1 gene expression during the differentiation of 3T3-L1 preadipocytes (0-10 days), and to analyze the regulative role of tumor necrosis factor-alpha (TNF-α) on APAF1 gene expression in matured 3T3-L1 adipocytes.**

Methods: **3T3-L1 preadipocytes were cultured *in vitro* and differentiated into matured adipocytes by insulin, 3-isobutyl-1-methyxanthine (MIX), and dexamethasone (DEX). Oil red O dyeing was used to identify the differentiation of 3T3-L1 preadipocytes. To further evaluate the linkage between the APAF1 gene and 3T3-L1 cells differentiation/de-differentiation, TNF-α which can promote the matured adipocytes to de-differentiation was incubated with 3T3-L1 matured adipocytes. TNF-alpha (1.0 ng/ml) was added into the culture medium of differentiated 3T3-L1 cells (day 10) for various periods (2, 6, 12, 24 hours). Total RNA and protein of these cells were then extracted. The level of APAF1 gene mRNA was evaluated by RT-PCR. Western blot was used to detect the protein level of APAF1.**

Results: **During the differentiation of 3T3-L1 preadipocytes, the down-regulation of APAF1 gene expression was significant ($P<0.05$). TNF-α treatment significantly up-regulated the expression of the APAF1 gene in adipocytes in a time-dependent way.**

Conclusions: **The APAF1 gene may play a role in the differentiation of preadipocytes and TNF-α related adipocytes and this finding highlights that the APAF1 gene has a potential role in the pathophysiology of obesity.**

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Key words: **APAF1; preadipocyte; differentiation; TNF-α**

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Introduction

The World Health Organization estimates that around one billion people throughout the world are overweight and that over 300 million of these are obese and if current trends continue, the number of overweight persons will increase to 1.5 billion by 2015.[1] The prevalence of obesity in children has increased over the last two decades and is becoming a major public health concern in many countries.[2,3] Currently, 16% of children are overweight and 34% are at risk of overweight.[4] Obesity is biologically characterized at the cellular level by an increase in the number and size of adipocytes differentiated from preadipocytes in the adipose tissue.[5] The proliferation and differentiation of preadipocytes are related to cell apoptosis.[6]

Apoptotic protease activating factor 1 (APAF1), a novel 130 kD protein acting as a key regulator of apoptosis, has been confirmed in APAF-1$^{-/-}$ mice.[7-10] APAF1-deficient mice exhibited reduced apoptosis in the brain and striking craniofacial abnormalities with hyperproliferation of neuronal cells. APAF1-deficient mice were resistant to a variety of apoptotic stimuli and its phenotypes included severe craniofacial

malformations, brain overgrowth, persistence of the interdigital webs, and dramatic alterations of the lens and retina. Inactivation of APAF1 in human melanoma may impair the mitochondrial pathway of apoptosis induced by chemotherapeutic drugs.[11] *In vivo*, loss of expression of APAF1 is associated with tumor progression, suggesting that APAF1 inactivation may provide a selective survival advantage to neoplastic cells.[10-13] Our previous studies[14] suggested that APAF1 gene expression was down-regulated in diet-induced obese (DIO) rat adipose tissue and this highlighted that the potential role of the APAF1 gene in the pathophysiology of obesity.

To further study the APAF1 gene effects on obesity, we examined the changes of APAF1 expression during 3T3-L1 preadipocyte differentiation and analyzed the regulative role of tumor necrosis factor-alpha (TNF-α) in APAF1 gene expression in matured 3T3-L1 adipocytes.

Methods

Differentiation and TNF-alpha treatment of 3T3-L1 cells

The mouse preadipocyte fibroblast line 3T3-L1 (ATCC, Manassas, VA, USA) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (Gibco BRL), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Amresco, USA) in 5% CO_2 at 37°C. To induce preadipocyte differentiation, 3T3-L1 preadipocytes were grown to confluence for 2 days. Two days later, cells about 85% confluence were induced to differentiate (day 0) with the addition of the standard differentiation-inducing mixture. Insulin (100 nM, Sigma), dexamethasone (0.25 μM, Sigma) and isobutylmethylxanthine (0.5 mM, Sigma) were added to the medium for the first 48 hours. From day 2 to 4, the full medium was supplemented with 100 nM insulin only. The cells were then switched back to DMEM containing only 10% FBS for the remaining days (day 4-10). Cultures were replenished every 2 days.[14]

On day 10 differentiation, TNF-alpha (1.0 ng/ml, 0-24 hour) was added to the culture medium and cultured for various periods (2, 6, 12, 24 hours).

Oil red O staining

3T3-L1 preadipocytes were washed three times with phosphate-buffered saline (PBS) and fixed with 4% formalin in phosphate buffer for 30 minutes at room temperature. After fixation, the cells were washed twice with PBS and stained with 0.6% (w/v) filtered oil red O solution (60% isopropanol, 40% water) for 60 minutes at room temperature. The cells were then washed again with water to remove unbound dye, visualized by light microscopy and photographed.[15]

RNA preparation and amplification by RT-PCR

Total RNA was isolated from cultured 3T3-L1 cells using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacture. Single strand cDNA synthesis was performed as follows: the reverse transcription mixture contained 1 μg total RNA, 0.5 μg oligo d(T) primer, 4 μl 5 × RT buffer, 0.5 mM deoxynucleotides, 50 U RNase inhibitor, and 200 U reverse transcriptase (Promega, Madison, WI, USA) in a total volume of 20 μl. The reaction was carried out at 42°C for 1 hour followed by heat inactivation at 95°C for 5 minutes. An aliquot of the cDNA was amplified with the primers listed in Table 1. The number of cycles and reaction temperatures used in the PCR assay were optimized to provide a linear relationship between the amount of input template and the amount of PCR product.

Western blotting

Total protein was isolated from cultured cells as previously described.[16] After 12% SDS-PAGE separation, the proteins (15 μg/lane) were electrophoretically transferred to a nitrocellulose membrane (Whatman, London, UK). Blocked with TBST (Tris-Buffered Saline Tween-20; 0.14 mol/L NaCl, 0.02 mol/L Tris base, pH 7.6, and 0.1% Tween) containing 3% BSA (Bovine serum albumin) for 1 hour at room temperature, the membrane was incubated with primary antibody and horseradish peroxidase-conjugated-secondary antibody (Cell Signaling; Danvers, MA, USA), and immunoreactive protein was detected

Table 1. Primer sequences used for RT-PCR

Genes	Product size (bp)	Reverse and forward primer (5'-3')	Ta (°C)	Cycles
APAF1	672	R: TGCTCGTTGATATTGAGTGG F: TGTGAGAGGAGTGTGTGGGG	60	30
GAPDH	432	R: TCCACCACCCTGTTGCTGTA F: ACCACAGTCCATGCCATCAC	58	28

by enhanced chemiluminescence (ECL) (enhanced chemiluminescence; Amersham, Picataway, UK).

Statistical analysis

All data were expressed as means ± SD, and analyzed by one-way ANOVA or Student's *t* test utilizing the SPSS 10.0 statistic software package (SPSS Inc, Chicago, IL, USA)

Results

Oil red O staining

The differentiation of 3T3-L1 preadipocytes was assayed by Oil red O as indicated by the appearance of a microscopic pattern of multiocular fat droplets. These fat droplets appeared as early as day 2 and 95% of cells were differentiated into mature adipocytes by day 10 (Fig. 1).

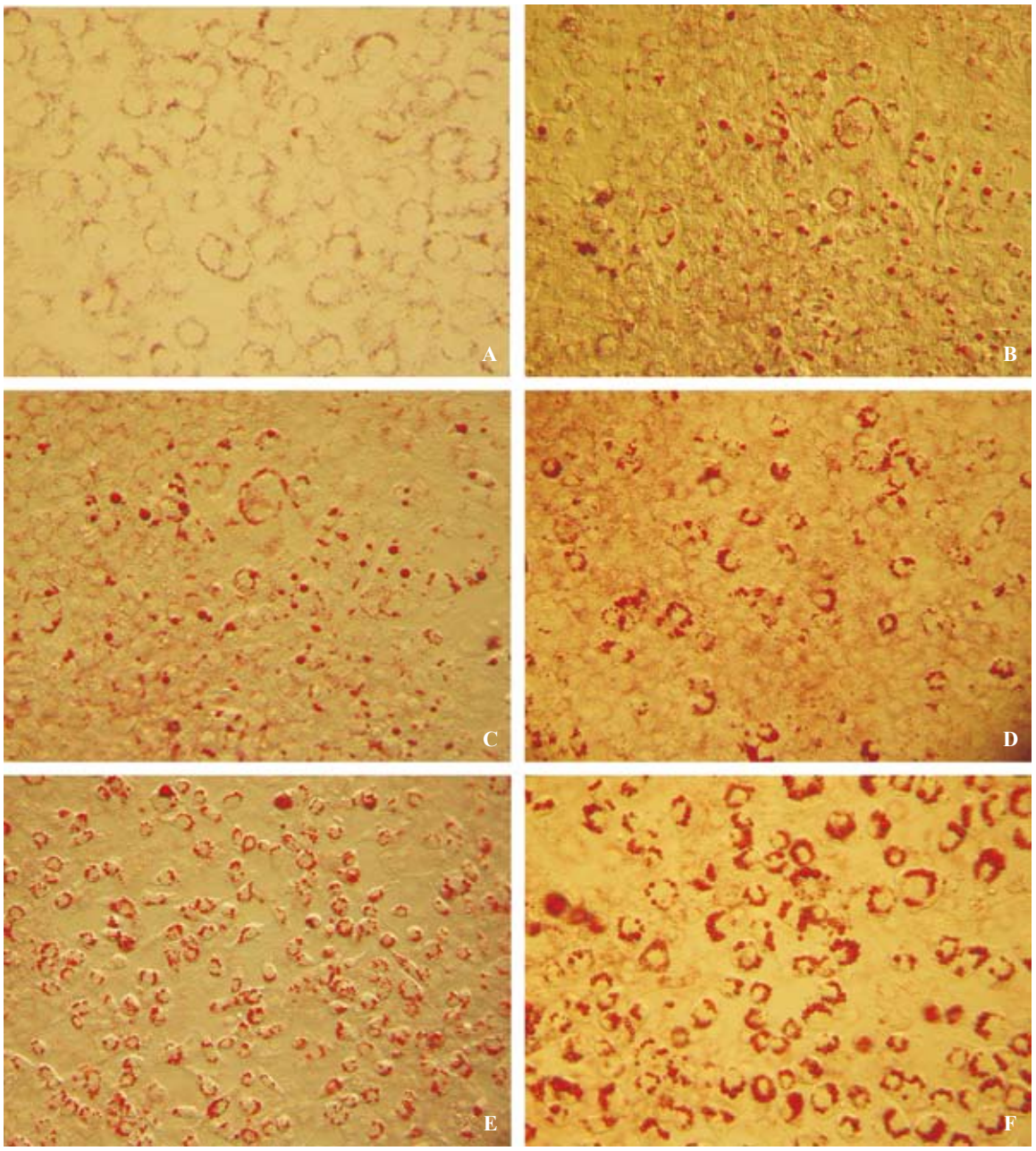

Fig. 1. The differentiation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were induced to differentiate using DMEM plus 15% FBS containing 1 μg/ml insulin, 0.5 mM MIX, and 1 μM DEX. During differentiation, cells were fixed and stained with oil red O to observe the fat droplets. The red fat droplets appeared in cytoplasm as early as day 2, and gradually increased during differentiation. At day 10, about 95% cells were differentiated into mature adipocytes containing a large quantity of red fat droplets. **A:** day 0 of differentiation; **B:** day 2 of differentiation; **C:** day 4 of differentiation; **D:** day 6 of differentiation; **E:** day 8 of differentiation; **F:** day 10 of differentiation. Results are representative of three separate experiments.

Table 2. The change in mRNA level of the APAF1 gene during 3T3-L1 preadipocytes differentiation

Time of differentiation (d)	*n*	mRNA levels of APAF1 (%)
0	6	86.07±7.34*
1	6	70.14±10.72†
2	6	67.89±12.28‡
3	6	63.43±8.13‡
4	6	61.99±6.59‡
5	6	58.67±10.64‡
6	6	59.77±10.04‡
7	6	57.57±12.29‡
8	6	43.74±8.26
9	6	40.06±12.69
10	6	38.87±6.37
F		12.18
P		=0.000<0.01

With the 3T3-L1 preadipocytes differentiated into mature adipocytes, the level of APAF1 gene expression down-regulated gradually and hit to the bottom at day 10. *: vs 1-10 d, $P<0.01$; †: vs 5 d, 7-10 d, $P<0.05$; ‡: vs 8-10 d, $P<0.05$.

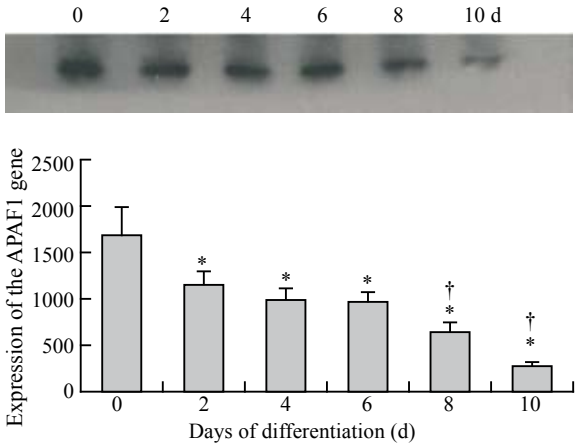

Fig. 2. The change in protein level of APAF1 during 3T3-L1 preadipocytes differentiation. 0-10 d: day 0-10 of differentiation. With the preadipocytes differentiated into mature adipocytes, the protein level of APAF1 down-regulated gradually and hit to the bottom at day 10. *: $P<0.01$ vs day 0, †: $P<0.05$ vs other five groups.

APAF1 gene expression during 3T3-L1 differentiation

As shown in Table 2 and Fig. 2, APAF1 gene mRNA and protein levels were gradually decreased during 3T3-L1 differentiation and hit to the minimum on day 10. Compared to day 0, approximately a 55% decrease in mRNA level was observed on day 10.

Effects of TNF-α on APAF1 gene expression in 3T3-L1 matured adipocytes

With TNF-α treatment (0-24 hours) on matured adipocytes (day 10), APAF1 gene mRNA and protein levels were gradually increased and hit to the maximum at 24 hours (Table 3, Fig. 3). Compared to 0 hour, approximately a 0.52-fold or 1.11-fold increase in mRNA level was respectively observed at 6 and 24 hours.

Table 3. The change in mRNA levels of APAF1 by TNF-α treatment (1 ng/ml) in matured adipocytes

Time of TNF-α treatment (h)	*n*	mRNA levels of APAF1 (%)
0	6	25.58±3.87*
2	6	30.84±4.32*
6	6	38.79±4.28†
12	6	53.54±4.70‡
24	6	54.02±5.77‡
F		46.58
P		=0.000<0.01

With TNF-α treatment, APAF1 gene expression in mature adipocytes was up-regulated and hit to the maximum at 24 h. *: vs 6-12 h, $P<0.01$; †: vs other four groups, $P<0.01$; ‡: vs 0-6 h, $P<0.01$.

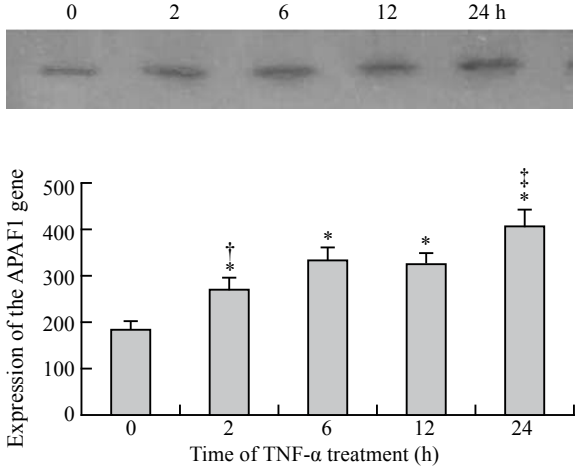

Fig. 3. The change in protein level of APAF1 by TNF-α treatment (1 ng/ml). With TNF-α treatment, the protein content of APAF1 was up-regulated and also hit to the maximum at 24 h. *: $P<0.01$ vs 0 h; †: $P<0.05$ vs other four groups; ‡: $P<0.01$ vs other four groups.

Discussion

Previous studies have suggested that the APAF1 gene plays a key role in cell apoptosis[7-10] and also can mediate differentiation of neurons.[17] Apoptosis and differentiation of cells especially adipocytes have been confirmed to be attributed to the pathophysiology of obesity.[6] The development of obesity involves an increase in number and size of new adipocytes from preadipocytes, therefore the adipocyte plays a critical role in obesity. The number of adipocytes is mediated by apoptosis and proliferation of cells, while the size of adipocytes mainly refers to the preadipocyte differentiation.[18] To consider the above mentioned points and results from our previous studies[3] that APAF1 gene expression was down-regulated in DIO rat adipose tissue, we hypothesized that APAF1 gene

expression may be correlated with obesity and this study was to verify this hypothesis.

Our results showed that APAF1 gene expression was down-regulated with the preadipocyte differentiation and increase of fat droplets. It has been previously reported that the expression of many anti-apoptosis genes, such as B-cell leukemia/lymphoma 2 (Bcl2) and neuronal apoptosis inhibitor protein (NAIP) are up-regulated during the differentiation of 3T3-L1 preadipocytes into matured adipocytes; this up-regulation makes the differentiated 3T3-L1 cells acquire the property of anti-apoptosis;[19,20] while it is well known that the APAF1 gene acts as an apoptotic protease activating gene.[7-10] Based on these findings, we infer that the down-regulation of APAF1 gene expression during the differentiation of 3T3-L1 preadipocytes may reduce cell apoptosis, and this would facilitate the differentiation of 3T3-L1 cells and accumulation of droplets. Additionally, recent study[21] has suggested that APAF1 could down-regulate the expression of the PPARγ (Peroxisome proliferator-activated receptor gamma) gene expression, which acts as a key factor in promoting the differentiation of 3T3-L1 preadipocytes. Therefore, we propose that the down-regulation of APAF1 gene expression during the differentiation of 3T3-L1 preadipocytes may attenuate its effect of inhibited PPARγ's expression and this could enhance the differentiation of PPARγ's effect on 3T3-L1 preadipocytes.

It is well recognized that TNF-α can promote the de-differentiation and stearolysis of matured adipocytes.[22,23] To further study the linkage between the APAF1 gene and the differentiation of 3T3-L1 cells, we applied TNF-α to examine its effect on the APAF1 gene expression in matured adipocytes in this study. Our results showed that TNF-α can increase the expression of APAF1 gene. Together with the above mentioned findings, we suggest that the up-regulation of APAF1 gene expression after TNF-α treatment probably increase matured adipocytes apoptosis and stearolysis and this up-regulation could facilitate the effect of de-differentiation on matured adipocytes by TNF-α treatment. Interestingly, the activated effect of TNF-α on APAF1 gene expression is generally of time-responsive correlation. Further research, however, is needed to elucidate this mechanism of time-dependent trend.

In conclusion, our data demonstrated that APAF1 gene expression was down-regulated during the differentiation of 3T3-L1 preadipocytes and TNF-α treatment in 3T3-L1 matured adipocytes could up-regulate APAF1 gene expression. The APAF1 gene may be partly attributed to the pathophysiology of obesity and a potential target to prevention and cure of obesity.

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