Quantitative Comparison of Adipocytokine Gene Expression during Adipocyte Maturation in Non-obese and Obese Rats

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Adipocytokines secreted from adipocytes have been extensively analyzed due to their role as key factors in various complications of obesity, including arterial sclerosis, liver steatosis, insulin resistance, and diabetes. Several in vivo and in vitro studies have suggested that adipocyte maturation is related to fluctuations in adipocytokine secretion. However, the relationship between adipocyte maturation and adipocytokine levels has not been fully elucidated. Therefore, we sought to clarify the link between adipocytokine gene expression and adipocyte maturation through systematic analysis. We quantified mRNA for six adipocytokine genes: adiponectin, resistin, leptin, plasminogen activator inhibitor 1 (PAI-1), heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), and visfatin, in adipose tissue, in primary cultured adipocytes obtained from an obese Zucker rat, and in the preadipocyte cell line 3T3-L1. Moreover, to elucidate the role of adipocytokines in adipocyte maturation, adipocytokine expression levels were analyzed during maturation. Although fluctuations in adipocytokine gene expression were heterogeneous, gene expression was highly similar during maturation of primary cultured adipocytes from obese and non-obese rats, suggesting that the maturation process is independent from processes that lead to obesity. Moreover, the expression patterns of adiponectin, resistin and leptin mRNA in 3T3-L1 cells were highly similar to those in primary cultured adipocytes, indicating that these adipocytokines could be common markers for primary cultured adipocytes obtained from obese and non-obese rats, and for preadipocyte cell lines.

Key words adipocytokine; adipocyte maturation; Zucker rat; primary cultured cell; mRNA expression

Adipocytokines are known as key mediators of arterial sclerosis and diabetes in the metabolic syndrome.1) “Adipocytokine” is an all-inclusive term used to describe bioactive factors, such as adiponectin and leptin, that are secreted from adipose tissue.2) Adipocytokines can be classified into two types: stimulative and suppressive factors of obesity. Levels of opposing adipocytokines are kept in balance under normal conditions. However, in obesity and some other pathological conditions, stimulative adipocytokine levels are higher than levels of suppressive factors. Therefore, expression and secretion of adipocytokines in vivo, including in adipose tissue, and in cultured cell lines has been widely studied to clarify the relationship between adipocytokines and obesity.3) Fluctuations in the expression and secretion of adipocytokines during adipocyte differentiation are thought to play a key role in the development of metabolic syndrome. However, it is difficult to quantitatively analyze fluctuations in expression and secretion of adipocytokines during adipocyte maturation in vivo, because adipose tissue is a mixture of mature and immature adipocytes and other types of cells, including macrophages.4) Therefore, we analyzed immature adipocytes isolated from the adipose tissue of an obese Zucker rat, which lacks functional leptin receptors, to analyze expression of adipocytokines during adipocyte maturation.

In this study, we quantitated mRNA levels for various adipocytokine genes, including plasminogen activator inhibitor 1 (PAI-1), resistin, visfatin, heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF), adiponectin, and leptin, in adipose tissue from obese Zucker rats, in primary cultured adipocytes isolated from the adipose tissue of a Zucker rat, and in the preadipocyte cell line 3T3-L1. PAI-1,5) resistin6) and visfatin7) are known to advance obesity while HB-EGF,8) adiponectin9) and leptin10) are known to suppress obesity. Moreover, to clarify the relationship between adipocyte maturation and adipocytokine expression, we compared mRNA levels for adipocytokine genes at multiple stages of adipocyte maturation.

MATERIALS AND METHODS

Materials 3,3′,5-Triiodo-L-thyronine (T3), L-thyroxine (T4), 3-isobutyl-1-methylxanthine (IBMX), and insulin (INS) were purchased from SIGMA Aldrich (St. Louis, MO, U.S.A.). D-Biotin was obtained from Nacalai Tesque (Kyoto, Japan). The RNeasy Mini Kit and RNase-Free DNase Set were purchased from QIAGEN (Tokyo, Japan). ISOGEN was obtained from Wako Junyaku Co., Ltd. (Osaka, Japan). TaKaRa EX Taq Hot Start Version, SYBR Premix Ex TaqII (Perfect Real Time) and random primers were purchased from TaKaRa Bio Inc. (Kyoto, Japan). Deoxynucleobonucleotide triphosphate (dNTP) Mixture was obtained from TOYOBO Co., Ltd. (Osaka, Japan). Zucker obese (fa/fa) and non-obese rats (+/+ ) were obtained from SLC Co., Ltd. (Shizuoka, Japan). The 3T3-L1 cell line was purchased from the Japan Health Sciences Foundation (Tokyo, Japan).

Cell Culture and Induction of Adipocyte Maturation in the 3T3-L1 Cell Line Cell culture and adipocyte maturation were performed as described previously.11) Cells were propagated in basal medium (Dulbecco’s modified Eagle’s
medium supplemented with 10% fetal bovine serum (FBS), 10 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES), 0.2% NaHCO₃, 0.2% Na₂HPO₄, 4 mM L-glutamine, and 15% CO₂/air atmosphere at 37 °C until they approached 100% confluence. To induce adipocyte differentiation, cells were incubated in basal medium containing 500 μM DEX, M INS (designated as Day 0). Two days after induction (Day 2), the medium was replaced with growth medium (basal medium supplemented with 100 μM INS and 15 μM D-biotin), and then cells were incubated for 3 d (until Day 5).

**Isolation of Immature Adipocytes from the Adipose Tissue of Zucker Obese (fa/fa) and Non-obese (+/+) Rats, and Induction of Adipocyte Maturation** Isolation of immature adipocytes from adipose tissue and primary culture of adipocytes were performed according to methods previously described by Shima et al.⁵ Five-Week-old male Zucker obese (fa/fa) and non-obese (+/+) rats were sacrificed, and epididymal adipose tissue was collected from four rats of each genotype. Pooled adipose tissue was digested for 5 min to isolate preadipocytes. The primary cultured cells were propagated in basal medium supplemented with 10% FBS, 10 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid (HEPES), 0.2% NaHCO₃, 0.2% Na₂HPO₄, 4 mM L-glutamine, and 15% CO₂/air atmosphere at 37 °C until they approached 100% confluency. To induce adipocyte differentiation, cells were incubated in basal medium containing 500 μM DEX, M INS (designated as Day 0). Two days after induction (Day 2), the medium was replaced with growth medium (basal medium supplemented with 100 μM INS and 15 μM D-biotin), and then cells were incubated for 3 d (until Day 5).

### Transcription

**Isolation of Immature Adipocytes from the Adipose Tissue of Zucker Obese (fa/fa) and Non-obese (+/+) Rats, and Induction of Adipocyte Maturation**

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**Evaluation of Adipocyte Maturation** On days 0, 2, and 5, morphological images were obtained by light microscopy, and cells possessing fat droplets were designated as mature adipocytes. At each time point, total RNA was extracted from adipocytes, and cDNA was synthesized as described above. Adipocyte-fatty acid binding protein (A-FABP), a marker gene for adipocyte maturation, was amplified with the primers listed in Table 1 using Ex Taq Hot Start. PCR products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

### Statistical Analysis

**Statistical Analysis** Statistical significance was determined using Student’s t-test. p values <0.05 were considered to be significant.

### RESULTS AND DISCUSSION

**Quantitative Comparison of Adipocytokine mRNA Expression in Adipose Tissue from Wild-Type and Obese Zucker Rats** We quantitated mRNA levels of the representative adipocytokines adiponectin, resistin, leptin, PAI-1, HB-EGF, and visfatin in epididymal adipose tissue obtained from wild-type and obese Zucker rats. As shown in Fig. 1, mRNA levels of adiponectin, resistin, and visfatin were lower in obese (Zucker) rats than in non-obese (wild-type) rats. Expression of adiponectin, resistin, and visfatin in obese rats was approximately 25%, 15%, and 50% of that in wild-type rats, respectively. Adiponectin and resistin expression levels were similar to those in previous reports. Expression levels of adiponectin and resistin were different in obese and non-obese rats. It is likely that adiponectin and resistin are decreased via feedback inhibition as a result of significant increases in these adipocytokines with the increased progression of adipocyte maturation in obese rats. Visfatin expres-

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**Table 1. Sequences of PCR Primers for Quantification of mRNA**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Accession No.</th>
<th>Forward primer (5’–3’)</th>
<th>Reverse primer (5’–3’)</th>
</tr>
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<tr>
<td>Rat A-FABP</td>
<td>NM_053365</td>
<td>GGACCTGTCGTGATCCCGTCCGTC</td>
<td>CGTTAACCTTGGATAGTCGCACTG</td>
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<tr>
<td>Mouse PPAR-γ</td>
<td>NM_011146</td>
<td>AGTGTGGTTTGCATTTATAGCTGCTATT</td>
<td>TGTCTTTGAGGACTGCTGATAGG</td>
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<tr>
<td>Rat adiponectin</td>
<td>NM_144744</td>
<td>GGAACTTGGTGAGGTGTTGATG</td>
<td>GGTCACCTTGGAGCACAAGAA</td>
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<td>Mouse adiponectin</td>
<td>NM_009605</td>
<td>GCTTCTGGCCAAGCATCTTCA</td>
<td>TACACTGCACTCGACAGTTC</td>
</tr>
<tr>
<td>Rat resistin</td>
<td>NM_144741</td>
<td>GCTTCTGGCCAAGCATCTTCA</td>
<td>TACACTGCACTCGACAGTTC</td>
</tr>
<tr>
<td>Mouse resistin</td>
<td>NM_022984</td>
<td>TCACCTTGTGCTTGTGGTATGAT</td>
<td>TGGCACCTGTGGTGATGAAC</td>
</tr>
<tr>
<td>Rat leptin</td>
<td>NM_013076</td>
<td>TGCTTTTGGTCATCTAAGC</td>
<td>GAATGTCGTATGCTCTAG</td>
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<tr>
<td>Mouse leptin</td>
<td>NM_008493</td>
<td>AAATGGTCTGCTGATAGCAGCTAAT</td>
<td>AAGAGGACGAGCTTGGAGAAC</td>
</tr>
<tr>
<td>Rat PAI-1</td>
<td>NM_012620</td>
<td>TCTTTGATGCTTGGCTGATG</td>
<td>TCCACAGTGAGCTGAGTAG</td>
</tr>
<tr>
<td>Mouse PAI-1</td>
<td>NM_008871</td>
<td>TCTTGCCCTGTGCTTCCCTC</td>
<td>GCATGGCCACGACCCAG</td>
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<tr>
<td>Rat HBG-EGF</td>
<td>NM_012945</td>
<td>ACCTCGAGAAGGGACCATCTG</td>
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<td>Mouse HBG-EGF</td>
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<tr>
<td>Rat visfatin</td>
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<td>TCGACATATCAGGTGTCCTC</td>
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<tr>
<td>Mouse visfatin</td>
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<td>Rat GAPDH</td>
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<td>GAGTGGCGACCTGGCCTGCTACA</td>
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<tr>
<td>Mouse GAPDH</td>
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<td>ATGAGGATCCATGAGGGC</td>
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<td>Mouse β-actin</td>
<td>NM_007393</td>
<td>GACGCGCCCATCACATATTG</td>
<td>CCAAGGATCCCATCACCAC</td>
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*A-FABP, adipocyte-fatty acid binding protein; PPARγ, peroxisome proliferator-activated receptor γ; PAI-1, plasminogen activator inhibitor 1; HB-EGF, heparin-binding EGF-like growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.*
sion has been reported to be induced by leptin. However, in our study, leptin mRNA levels were comparable in obese and non-obese rats, although the Zucker rats used in this study genetically lack functional leptin receptors (Fig. 1). This suggests that visfatin expression is mainly regulated by factors other than leptin. Levels of mRNA for PAI-1 and HB-EGF were 5.8-fold and 4.9-fold higher, respectively, in obese rats than in non-obese rats, in agreement with previously reported results.

Fluctuation of Adipocytokine mRNA Levels during Maturation of Primary Cultured Adipocytes Isolated from Non-obese and Obese Rats

We assessed fluctuations in adipocytokine mRNA levels during maturation of primary cultured adipocytes isolated from non-obese and obese rats. To confirm maturation of primary cultured immature adipocytes, we measured the accumulation of fat droplets in the cytoplasm in comparison with the 3T3-L1 cell line, a representative adipocyte model. As shown in Fig. 2A, fat droplets were observed in 3T3-L1 cells after induction of maturation (Day 5). Similarly, fat droplets were also found in the cytoplasm of adipocytes isolated from adipose tissue of non-obese rats on Days 2 and 5 after induction of maturation (Fig. 2B). Moreover, mRNA for adipocyte-fatty acid binding protein (A-FABP), a marker gene for adipocyte maturation, was increased after induction of maturation (Days 2 and 5), although no A-FABP transcript was detected on Day 0 just after addition of differentiation medium. In primary cultured adipocytes from non-obese rats, A-FABP exhibited a similar expression pattern to that in obese rats, although expression levels were different in non-obese and obese rats. These results confirmed that primary cultured cells isolated from obese and non-obese rats have a similar potential to become mature adipocytes. We next compared adipocytokine mRNA expression during maturation of non-obese and obese rat-derived primary cultured adipocytes. Interestingly, during adipocyte maturation, the expression patterns of adipocytokine genes were nearly identical in non-obese and obese rat-derived cells (Fig. 3). These results strongly suggest that maturation of immature adipocytes derived from non-obese and obese rats involves essentially the same process of fluctuations in adipocytokine production. As shown in Fig. 3, mRNA levels of adiponectin, resistin, and leptin increased throughout the maturation process. On Day 2, adiponectin and resistin mRNA levels were approximately 20-fold higher than on Day 0. This finding suggests that adiponectin and resistin mRNA expression are induced in an early phase of adipocyte maturation. Although little leptin mRNA was detected on Day 2, mRNA levels rapidly increased from Day 2 to Day 5. mRNA for PAI-1 and HB-EGF had decreased by Day 5. In contrast, visfatin mRNA was transiently increased on Day 2. These results indicate that the balance of adipocytokine gene expression changes as adipocytes mature.

Time Profiles of Adipocytokine Expression during 3T3-L1 Maturation

To further characterize the expression patterns of adipocytokines, we analyzed the time profiles of adipocytokine mRNA in 3T3-L1 cells after induction of maturation. Since it was possible that a small fraction of mature adipocytes could be present in the primary culture system (Fig. 2B), we used the established preadipocyte cell line 3T3-L1 to precisely analyze adipocytokine expression throughout the maturation process. mRNA for peroxisome proliferator-activated receptor γ (PPAR-γ), a major marker of adipocyte maturation, was increased in a time-dependent manner after induction of maturation. mRNA for adiponectin, resistin, and visfatin increased until 3—4 d after induction, and reached saturation by Day 5. Leptin mRNA drastically increased on Day 3, but slightly decreased by Day 5. In contrast, mRNA

*Fig. 1. Quantitative Comparison of Adipocytokine mRNA in Adipose Tissue from Non-obese and Obese Rats

RNA was isolated from adipose tissue, and mRNA expression for each gene was quantitated by real-time RT-PCR. Data show relative mRNA expression using GAPDH mRNA as an internal standard. Values represent the means of three samples. Bar represents S.D. *p<0.05 versus non-obese.
Fig. 2. Morphological Images and A-FABP mRNA Expression during Adipocyte Maturation

(A) 3T3-L1 cells and (B) primary cultured adipocytes derived from the epididymal adipose tissue of non-obese rat were treated with insulin, dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX) on Day 0, and morphological images were obtained on the indicated days after induction of maturation. Fat droplets are indicated by white arrows. (C) Agarose gel electrophoresis of A-FABP mRNA amplified by RT-PCR during the maturation of adipocytes derived from non-obese and obese rats.

Fig. 3. Quantitative Comparison of Adipocytokine mRNA during Maturation of Primary Cultured Adipocytes Derived from Non-obese and Obese Rats

Immature adipocytes isolated from pooled adipose tissue from four rats were treated with 20 nM insulin, 0.5 mM dexamethasone, and 500 μM 3-isobutyl-1-methylxanthine to induce differentiation on Day 0. RNA was isolated from adipocytes derived from non-obese (white column) and obese (black column) rats on the indicated days, and mRNA expression of each gene was quantified by real-time RT-PCR. Data show relative mRNA expression using GAPDH mRNA as an internal standard.
levels of PAI-1 were transiently decreased on Day 3. Expression of HB-EGF mRNA was observed only in immature 3T3-L1 cells. Since mRNA for adiponectin, resistin and leptin increased during adipocyte maturation in both the primary culture system and in 3T3-L1 cells (Figs. 3, 4), it is thought that these genes could be used as adipocyte maturation markers. In previous reports, overexpression of adiponectin was shown to induce adipocyte maturation. However, studies of the effects of resistin and leptin on adipocyte maturation have yielded conflicting results. Further examination is needed to clarify the roles of these adipocytokines in the adipocyte maturation process. Although mRNA for adiponectin and resistin increased with adipocyte maturation (Figs. 3, 4), the expression levels of these two genes were significantly lower in obese rats than in non-obese rats (Fig. 1). These results suggest that factors regulating expression of adiponectin and resistin during adipocyte maturation are not essential for obesity. In contrast, the in vitro expression patterns of PAI-1 and HB-EGF in primary cultured cells (Fig. 3) corresponded to the results of in vivo analysis (Fig. 1), i.e., mRNA levels of these two genes were higher both in mature adipocytes and in the obese state than in the immature and non-obese states. Therefore, expression of PAI-1 and HB-EGF may be related to the progression of obesity. In this study, PAI-1, HB-EGF, and visfatin showed differential expression patterns in primary cultured adipocytes and 3T3-L1 cells. Differences in cell types and species differences might be the cause of this discrepancy. To determine the influence of phylogenetic differences, we measured PAI-1, HB-EGF, and visfatin mRNA in primary cultured adipocytes from Wistar rats. We found that during adipocyte maturation, the expression patterns of PAI-1, HB-EGF, and visfatin were very similar in Zucker and Wistar rats (data not shown). Namely, primary cultured adipocytes obtained from both rat strains showed similar patterns of PAI-1, HB-EGF, and visfatin expression. However, since 3T3-L1 cells are derived from mice, differences between animal species might be a possible explanation for the discrepancy. Additionally, in this study, the concentrations of differentiation-inducing agents used in 3T3-L1 cells were much higher than in primary cultured cells. The concentrations of these agents, especially dexamethasone and insulin, have been shown to influence adipocytokine expression. These data suggest that differential induction conditions may have affected the expression of PAI-1, HB-EGF and visfatin. The expression of some adipocytokines can vary depending on the cell type and the concentration of differentiation-inducing agents, but the expression patterns of adiponectin, resistin, and leptin were consistent during adipocyte maturation in this study.

In conclusion, the expression patterns of adiponectin, resistin, leptin, PAI-1, HB-EGF and visfatin during adipocyte maturation were nearly identical in primary cultured adipocytes derived from obese and non-obese rats, although the fluctuations in expression of each adipocytokine gene were diverse. These results suggest that the adipocyte maturation process is distinct from the progression of obesity. Moreover, gene expression patterns for adiponectin, resistin and leptin in 3T3-L1 cells were highly similar to those in primary cultured adipocytes isolated from obese and non-obese rats. Our results indicate that these adipocytokines could be used as common maturation markers for primary cultured adipocytes obtained from obese and non-obese rats, and for preadipocyte cell lines.

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