ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts

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Adipogenesis is a complex process controlled by the interplay of intracellular factors and signals from the environment. During this differentiation, a large number of genes have to be regulated in a selective, coordinated manner, and dramatic changes occur in both cell morphology and gene expression (17, 20). Peroxisome proliferation-activated receptor-γ (PPARγ) and CCAAT/enhancer binding protein-α (C/EBPα) are prominent adipogenic transcription factors, and they are induced in the early stage of adipose differentiation (5, 19, 24). PPARγ and C/EBPα alone or in cooperation with each other induce the transcription of many adipocyte-specific genes encoding proteins and enzymes involved in creating and maintaining the adipocyte phenotype, such as lipogenesis, lipolysis, glucose metabolism, and endocrine functions (9).

ADRP in murine fibroblast cell lines (3T3-L1, NIH-3T3, and Swiss-3T3) by GFP-ADRP fusion protein overexpression. These may provide the direct evidence about the roles of ADRP during adipose differentiation.

RESEARCH DESIGN AND METHODS

Plasmids. A plasmid containing the mouse ADRP was first identified by Jiang et al. (11, 12); they demonstrated that ADRP mRNA is expressed most strongly in adipose tissue and is induced very early during adipose differentiation in murine 1246 cells. In 3T3-L1 cells, ADRP protein levels increased by day 1 of differentiation and then decreased by day 4 (3). ADRP protein localizes at the surface of lipid droplets in cultured 3T3-L1 preadipocytes and early differentiated adipocytes (3). Although these findings suggest that ADRP may play a certain role in the early stage of adipose differentiation, little is known about its function in this stage.

In the present study, we examined the function of ADRP in murine fibroblast cell lines (3T3-L1, NIH-3T3, and Swiss-3T3) by GFP-ADRP fusion protein overexpression. These may provide the direct evidence about the roles of ADRP during adipose differentiation.
ADRP and Lipid Accumulation

(M93275), the open reading frame of mouse ADRP was amplified from C57BL/6 mouse adipose tissue cDNA. Primers for ADRP were 5’-CCAAGCTTGTTAGGCGTCTCTTTTCTTCC-3' and 5’-TGCTCTAGACTGTTGACAAGGAGGGTTA-3', including Hind III and Xba I restriction sites, respectively. The ADRP gene was restricted by Hind III and Xba I and then inserted in the multicloning site of pBluescript SK (TOYOBO, Osaka, Japan).

Construction of green fluorescent protein-ADRP fusion protein expression adenovirus vector. Recombinant adenovirus vector derived from the human type 5 adenovirus was used for this study (Takara, Osaka, Japan). ADRP cDNA was restricted by Hind III and Sac II and inserted in the multicloning site of pEGFP-C2 (Clontech, Palo Alto, CA). pEGFP-ADRP was linearized with Nhe I and Xba I, blunted, and then inserted in the Swa I site of the recombinant cosmids vector pAXCAwt (Takara). The recombinant green fluorescent protein (GFP)-ADRP adenovirus (Ad.GFP-ADRP) was obtained as described in a previous report (18).

Cell culture and adenovirus treatment. NIH-3T3 cells (American Type Culture Collection, Manassas, VA) and Swiss-3T3 cells (Health Science Research Resources Bank, Osaka, Japan) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% calf serum. The cells were injected by adenovirus vector with a multiplicity of infection of ~30 plaque-forming units/cell. For most of the assays, recombinant adenovirus expressing β-galactosidase (Ad.LacZ) was used as a control. 3T3-L1 preadipocytes (kindly provided by Dr. J. M. Olefsky, California University, San Diego, CA) were grown in DMEM containing 10% FCS. Differentiation of 3T3-L1 cells into adipocytes was accomplished by incubating confluent monolayers of cells in DMEM with 10% FCS and 1μM dexamethasone (Sigma), 0.5 mM isobutyryl methylxanthine (IBMX; Sigma), and 10μg/ml insulin (Sigma) for 72 h. After 72 h, the medium was withdrawn and changed to DMEM with 10% FCS and 5μg/ml insulin. The medium was changed every 2 days.

Immunofluorescence microscopy. For determination of GFP-ADRP fluorescence, cells were cultured on 35-mm coverslip-bottomed dishes (Magenta, Ashland, MA) and infected adenovirus vector as described above. Fluorescence imaging of GFP-ADRP was assessed by confocal laser scanning microscopy (Leica TCS-SP system; Leica Microsystems, Heidelberg, Germany). The cells were imaged for GFP by excitation with the 488-nm line from an argon laser, and the emission was viewed through a 496- to 505-nm band-pass filter. To correlate the localization of GFP-ADRP with intracellular structure, cells were viewed with phase-contrast images and fluorescence images.

Oil red O staining. Cells were cultured in 10-cm dishes or 35-mm coverslip-bottomed dishes with adenovirus vector as described above. After 14 days, the cells cultured in 10-cm dishes were washed three times with PBS and then fixed by soaking 10% formalin. After being washed two times with PBS, cells were stained for 30 min at 37°C in freshly diluted Oil red O (Chroma, Mueuster, Germany) solution (six parts Oil red O stock and four parts H2O; Oil red O stock solution is 0.5% Oil red O in isopropanol). The stain was then removed, and the cells were washed two times with water. Nuclei were then stained with hematoxylin (Nichirei, Tokyo, Japan), and the stained cells were examined under a light microscope (Nikon, Tokyo, Japan). As for dual staining with Oil red O and GFP-ADRP, 5 days after adenovirus infection, the cells cultured in 35-mm coverslip-bottomed dishes were washed three times with PBS and then fixed by soaking in 4% paraformaldehyde for 10 min. After being washed two times with PBS, cells were stained for 2 min at room temperature in freshly diluted Oil red O solution. Fluorescence imaging of GFP-ADRP and Oil red O was assessed by confocal laser scanning microscopy. Imaging of Oil red O was provided by excitation with the 563- and 633-nm line, and the emission was viewed through a 650- to 700-nm band-pass filter.

Lipid analysis. For determination of intracellular triacylglycerol content, cells were cultured on 10-cm dishes. After adenovirus vector infection and incubation in 10% FCS or 10% delipidated calf serum (Sigma, St. Louis, MO) for 7 days, cells were washed with PBS, and total lipids were extracted with 2.1 chloroform-ethanol and centrifuged for 5 min at 12,000 g. The chloroform layer, containing triacylglycerol, was extracted and placed under vacuum to evaporate chloroform and methanol. Triacylglycerol content was measured using an enzymatic method (Mizuho Medy, Saga, Japan) and was expressed relative to total cellular protein content measured by the bicinchoninic acid method (Fierce, Rockford, IL).

Northern blot analysis. A plasmid-encoding mouse PPARγ was kindly provided by Dr. Kazuhiko Umesono (Kyoto University, Kyoto, Japan). cDNA probes for murine lipoprotein lipase (LPL), adipose fatty acid-binding protein (aP2/FABP), acyl-CoA synthase (ACS), and CD36/fatty acid transporter (FAT) were prepared by RT-PCR by use of differentiated 3T3-L1 adipocyte cDNA. cDNA probe for human perilin was prepared by RT-PCR by use of human mesenteric adipose tissues, which were obtained from a resected organ from a 58-year-old female patient who had undergone surgery for ovarian cancer (with informed consent for use of tissues).

Total RNA was extracted using Isogen (Waco, Osaka, Japan) from cultured cells. Total RNA (20 μg) was electrophoresed on a 1.0% agarose gel containing 2.2 M formaldehyde and then was transferred to a Hybond(N) + nylon membrane (Amersham Pharmacia Biotech, Little Chalfont, UK). The membrane was hybridized with each probe, which was labeled with 32P-dCTP (Amersham Pharmacia Biotech) using the multiprime DNA labeling system (Amersham Pharmacia Biotech). The radioactivity corresponding to each band was measured by a bioimage analyzer (Multi Bio Imager STORM; Molecular Dynamics, Tokyo, Japan).

Immunostaining of perilin. 3T3-L1 cells were cultured on 35-mm coverslip-bottomed dishes and infected with Ad.GFP-ADRP. After incubation for 15 days, cells were fixed in 10% formalin for 10 min at room temperature, washed with PBS, permeabilized by 0.2% Triton X-100 (Katayama Chemistry, Osaka, Japan) in PBS for 2 min, and washed with PBS. Anti-perilin polyclonal antibody (Research Diagnostics, Flanders, NJ) was diluted 1:400 in PBS with 1% dried bovine milk and incubated for 1 h at room temperature. Alexa Fluor 594, fluorescein-conjugated secondary antibody (Molecular Probes, Eugene, OR), was diluted 1:200 in PBS with 1% dried bovine milk and incubated for 30 min at room temperature. The cells were imaged by Alexa Fluor 594 by excitation with the 633-nm line from an argon laser, and the emission was viewed through a 650- to 700-nm band-pass filter.

Statistical analysis. Statistical analysis was done by ANOVA followed by Student’s t-test.

RESULTS

Effect of overexpression of ADRP in murine fibroblast cell lines. Two murine fibroblast cell lines, NIH-3T3 cells and Swiss-3T3 cells, which don’t differentiate into adipocytes, were infected with control adenovirus (Ad.LacZ) or Ad.GFP-ADRP with a multiplicity of in-
fection of ~30 plaque-forming units/cell and then incubated. Cells were viewed with a confocal laser-scanning microscope. GFP images and phase-contrast images allowed us to correlate the localization of adenovirus-induced GFP-ADRP protein with an intracellular structure. In NIH-3T3 cells, overexpressed GFP-ADRP protein localized around tiny lipid droplets and in the cytosol at day 2 (Fig. 1, A and D). As shown in Fig. 1, B and C, E, and F, the number and size of lipid droplets apparently increased in GFP-ADRP-overexpressed cells. The fluorescence of GFP-ADRP increased around the lipid droplets and decreased in the cytosol at day 7. In Swiss-3T3 cells, overexpressed GFP-ADRP stimulated lipid accumulation more remarkably than in NIH-3T3 cells (Fig. 2). A lot of tiny lipid droplets surrounded by GFP-ADRP appeared at day 2 (Fig. 2, A and D), and the droplets became larger at day 7 (Fig. 2, B and E) and at day 14 (Fig. 2, C and F). The fluorescence of GFP-ADRP appeared to be “ring-shaped.” Analysis by Oil red O staining confirmed that overexpressing ADRP stimulated lipid accumulation in both NIH-3T3 cells and Swiss-3T3 cells (Fig. 3). Dual-staining images clearly showed that the lipid droplets stained with Oil red O were surrounded by GFP-ADRP (Fig. 4, A–D). In addition, the cells contained increased levels of lipid droplets in parallel with the overexpression level of GFP-ADRP (Fig. 4, I–L, yellow arrow). In contrast, the GFP-undetectable cells (Fig. 4, I–L, blue arrow) had few amounts of lipid droplets at the similar levels as Ad.LacZ-infected control cells (Fig. 4, E–H). Intracellular triacylglycerol content in GFP-ADRP-overexpressed Swiss-3T3 cells was significantly increased by 225% (P < 0.001) compared with that of Ad.LacZ-infected cells when the cells were cultured in 10% normal serum (Fig. 5). Even if the cells were cultured in delipidated serum, the intracellular triacylglycerol content in GFP-ADRP-overexpressed cells was still significantly (P < 0.005) increased compared with that in Ad.LacZ-infected cells to a similar degree as in normal serum (Fig. 5).

**Effect of ADRP overexpression on adipocyte-specific gene expression.** To investigate the mechanism of ADRP-induced lipid accumulation, we analyzed PPARγ, which is an early marker and regulator of adipocyte differentiation, and other adipocyte-specific gene expression in ADRP-overexpressed Swiss-3T3 cells. Swiss-3T3 cells infected with Ad.GFP-ADRP or Ad.LacZ were incubated with induction medium containing insulin, IBMX, and dexamethasone, as described in RESEARCH DESIGN AND METHODS. Figure 6 shows that ADRP overexpression did not in-

![Fig. 1. Localization of green fluorescent protein (GFP)-adipose differentiation-related protein (ADRP) in NIH-3T3 cells. A–C: confocal images of immunofluorescence of NIH-3T3 cells at days 2, 4, and 7 after GFP-ADRP adenovirus (Ad.GFP-ADRP) infection. D–F: phase images of cells corresponding to A, B, and C, respectively. Data are representative of 2 independent experiments.](http://www.ajpendo.org/content/283/11/E777/F1)

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duce PPARγ, LPL, or perilipin mRNA expression at days 4 or 8.

Next, we analyzed the expression of three lipogenic genes (ACS, aP2/FABP, and CD36/FAT). Swiss-3T3 cells infected with Ad.GFP-ADRP were incubated with DMEM with 10% FCS. In Swiss-3T3 cells, ectopic GFP-ADRP expression did not induce either ACS, aP2/FABP, or CD36/FAT mRNA (Fig. 7).

Correlation of ADRP and other adipocyte-specific gene expression during 3T3-L1 cell differentiation. To correlate the temporal pattern of expression of ADRP mRNA and other adipocyte-specific gene expression relative to each other, we analyzed gene expression by Northern blot hybridization during the conversion of 3T3-L1 preadipocytes to adipocytes (Fig. 8). ADRP mRNA was present at low levels in 3T3-L1 cells before the conversion to adipocytes. The expression of other adipocyte-specific genes, such as ACS, aP2/FABP, and CD36/FAT, increased concomitantly with the conversion to adipocytes.

Fig. 2. Localization of GFP-ADRP protein in Swiss-3T3 cells. A–C: confocal images of immunofluorescence of Swiss-3T3 cells at days 2, 7, and 14 after Ad.GFP-ADRP infection. D–F: phase images of cells corresponding to A, B, and C, respectively. Data are representative of 2 independent experiments.

Fig. 3. Oil red O staining. Confluent cultured NIH-3T3 cells and Swiss-3T3 cells were infected with Ad.GFP-ADRP or recombinant adenovirus expressing β-galactosidase (Ad.LacZ). At day 14 after infection, the cells were fixed and stained with Oil red O as described in RESEARCH DESIGN AND METHODS. Data are representative of 2 independent experiments.
Fig. 4. Dual staining of GFP-ADRP and Oil red O. A–D: confluent cultured Swiss-3T3 cells were infected with Ad.GFP-ADRP. At day 5 after infection, the cells were fixed and stained with Oil red O as described in RESEARCH DESIGN AND METHODS. A: phase-contrast image. B: confocal image of GFP-ADRP. C: confocal image of Oil red O. D: overlay image of GFP-ADRP and Oil red O. E–L: confluent cultured Swiss-3T3 cells were infected with Ad.GFP-ADRP or LacZ. At day 5 after infection, the cells were fixed and stained with Oil red O as described in RESEARCH DESIGN AND METHODS. E and I: phase-contrast images of Ad.LacZ- or Ad.GFP-ADRP-infected cells, respectively. F and J: confocal images of GFP-ADRP. G and K: confocal images of Oil red O. H and L: overlaid images of GFP-ADRP and Oil red O. L: cells that highly expressed GFP-ADRP (yellow arrows) and those with undetectable GFP (blue arrows).
the initiation of differentiation and increased at day 3 of differentiation. The induction of PPARγ was activated at day 2 before that of ADRP. Expressions of other adipocyte-specific genes, LPL and aP2, were induced at day 2, and its induction was activated at day 3.

GFP-ADRP localizes small lipid droplets in differentiating 3T3-L1 cells. In 3T3-L1 cells, overexpressed GFP-ADRP localized at the surface of lipid droplets and stimulated lipid accumulation similar to Swiss-3T3 and NIH-3T3 cells. At day 15 after infection, some of the cells had started differentiation and had several larger droplets (Fig. 9). Ad.LacZ-infected cells also had large droplets in the same degree as Ad.GFP-ADRP-infected cells (data not shown). Immunostaining using anti-perilipin antibody showed that the larger droplets were surrounded by perilipin instead of GFP-ADRP (Fig. 9, C and G). No staining was seen using nonimmune serum, confirming that perilipin staining is specific. In contrast, tiny or middle-sized droplets were still surrounded by GFP-ADRP (Fig. 9, B and F).
certain cells, GFP-ADRP and perilipin colocalized (Fig. 9, J-L).

DISCUSSION

ADRP is a lipid droplet-associated protein, and its expression increases in the early stage of adipose differentiation (3, 11). Previous reports demonstrated that ADRP was expressed in a wide range of lipid-accumulated cells and the ADRP was a marker of lipid accumulation (10). Although the localization and expression pattern of ADRP has been well examined, little is known about its function in murine preadipocyte cells or other lipid-accumulated cells. Whether increased ADRP promotes lipid accumulation or if the increased ADRP merely reflects the increased storage of lipid remained to be elucidated. Therefore, we analyzed the function of ADRP in murine fibroblasts by GFP-ADRP fusion protein overexpression. In GFP-ADRP-overexpressed cells, the number and size of lipid droplets apparently increased, and overexpressed GFP-ADRP protein localized around lipid droplets. Analysis by Oil red O staining and measurement of intracellular triacylglycerol content confirmed that overexpressing ADRP stimulated lipid accumulation in both NIH-3T3 cells and Swiss-3T3 cells. Furthermore, dual-staining images clearly showed that the cells contained increased levels of lipid droplets, in parallel with the overexpression level of GFP-ADRP. Taken together, these results suggested that ADRP stimulated lipid accumulation and lipid droplet formation in murine fibroblasts.

To determine the mechanism of ADRP-induced lipid accumulation, the effect of ADRP overexpression on adipocyte-specific gene expression, such as PPARγ, LPL, or perilipin, was examined. ADRP overexpression could not induce adipocyte-specific gene expression, suggesting that ADRP-induced lipid accumulation is not the result of activation of the adipose differentiation program. This notion is consistent with the evidence that ADRP is expressed in a wide range of cells and tissues (3, 10). Next, the expression of three lipogenic genes (ACS, aP2/FABP, and CD36/FAT) was also analyzed in ADRP-overexpressed cells. ADRP overexpression did not induce these lipogenic genes, showing...
that ADRP-induced lipid accumulation was independent of other lipogenic genes, such as ACS, aP2/FABP, and CD36/FAT.

One possible mechanism is that the free fatty acid-transporting function of ADRP may be associated with lipid accumulation. Several investigators have mentioned ADRP as a free fatty acid transporter (1, 6, 22). ADRP specifically enhances uptake of long-chain fatty acids by increasing the initial rate of uptake in COS-7 cells (6). A recent report demonstrated that recombinant ADRP protein bound 12-N-methyl-(7-nitrobenz-2-oxa-1,3-diazol)aminostearate at high affinity, and its binding was completed by natural fatty acid (22). ADRP binding fatty acid with high affinity may contribute to the rapid appearance of fatty acids at the surface of lipid droplets, and subsequently the fatty acids may be used for triacylglycerol synthesis (22).

One report showed that ADRP protein translocated to the cell periphery in murine 1246 cells during the differentiating process (6), suggesting that ADRP function as a protein involved in carrier-mediated fatty acid influx from the extracellular environment into the cells. In contrast, in the present study, overexpressed GFP-ADRP protein localized around tiny lipid droplets and in cytosol at day 2, and then it increased around the lipid droplets, not in the cell periphery, and decreased in the cytosol at day 7. These results suggested that ADRP might transport endogenous free fatty acid from the cytosol to the surface of lipid droplets. This notion was supported by the present results showing that ADRP overexpression induced an increase in intracellular triacylglycerol content even if the cells were cultured in delipidated serum to a similar degree as in nondelipidated serum. Thus, in murine fibroblasts, ADRP may stimulate free fatty acid accumulation by shuttling of long-chain free fatty acids, mainly from the cytosol to the surface of lipid droplets to supply the source of triacylglycerol synthesis.

Similar to ADRP, perilipin localizes at the surface of lipid droplets (2, 8), and perilipin also stimulates lipid accumulation (4, 23). A previous report showed that the transition in the surface protein composition of lipid droplets from ADRP to perilipin occurs from 3 to 5 days after the initiation of differentiation (3). In our observation of 3T3-L1 cell differentiation, overexpressed GFP-ADRP appeared to localize at the surface of only small lipid droplets, whereas in turn endogenous perilipin located around large-sized lipid droplets. In the early stage of lipid accumulation, ADRP may play an important role.

The molecular mechanisms for regulation of ADRP expression have not been well understood. ADRP mRNA expression is stimulated by either indomethacin (25) or long-chain free fatty acids (7), and both of them are found to bind directly to PPARγ and to act as a PPARγ agonist (15, 16, 24), so it is possible to assume that their effect on ADRP expression could be secondary to the prior activation of PPARγ. This notion was in agreement with the present finding that ADRP mRNA expression was induced ≥24 h later than that of PPARγ mRNA. Promoter activity analysis of ADRP would give us further information about the regulation of ADRP induction. At the ADRP-to-perilipin switch, the ADRP protein level was reported to be decreased, whereas the ADRP mRNA level still increased, suggesting that ADRP protein levels would also be regulated by a posttranscriptional mechanism (3).

In conclusion, ADRP overexpression stimulated lipid accumulation and lipid droplet formation without induction of other adipocyte-specific genes or other lipogenic genes in murine fibroblast cells. The detailed molecular mechanisms of ADRP on lipid accumulation remain to be elucidated.

This work was supported in part by Grant-in-Aid for Scientific Research no. 11671126 from the Ministry of Education, Science, and Culture, Japan.

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