Thiazolidinediones and Glucocorticoids Synergistically Induce Differentiation of Human Adipose Tissue Stromal Cells: Biochemical, Cellular, and Molecular Analysis

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While adipocyte differentiation has been studied extensively in murine cultures, the lack of a readily available preadipocyte model has hindered equivalent studies in man. We describe methods for the isolation and culture of primary human stromal cells from surgical adipose tissue specimens. In vitro, the stromal cells rapidly differentiate in response to a combination of adipogenic agents. Among these, glucocorticoids and thiazolidinediones act together to induce the formation of lipid vacuoles within the cells. These morphologic changes accompany the increased expression of 2 characteristic adipocyte proteins, the cytoplasmic enzyme glycerol phosphate dehydrogenase (GPDH) and the secreted cytokine leptin. Likewise, stromal cell differentiation results in elevated mRNA levels for the fatty acid binding protein aP2 and the adipogenic regulatory transcription factors CCAAT/enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPARγ) in addition to leptin. The in vitro differentiated stromal cells exhibit a lipolytic response to β-adrenergic agonists, comparable to that reported with primary human adipocytes. These studies demonstrate the validity of human adipose tissue-derived stromal cells as a reliable in vitro model for investigations of adipocyte metabolism in humans.

A MAJORITY of the studies concerning the mechanisms underlying adipogenesis utilize murine cell systems such as the ob17 and 3T3-L1 cell lines.[1-3] These studies have documented the role of peroxisome proliferator-activated receptor (PPAR) ligands (thiazolidinedione) and glucocorticoids in regulating early events in adipocyte commitment and induction.[2,3] Following differentiation, these adipocyte models express lineage-specific gene markers and respond to lipolytic agonists.[2,3] While many of these observations in the murine system can be extrapolated directly to man, it is likely that significant interspecies differences exist. This may preclude the use of murine-based systems for in vitro examination of human conditions such as obesity and diabetes.

Advances concerning the in vitro analysis of human adipogenesis have been hindered by the lack of readily available and reproducible cellular models. Currently, methods for isolation of primary adipocytes and preadipocyte stromal vascular cells demand immediate access to human surgical specimens.[4-6] This places strict limitations on the experimental capacity of most laboratories, particularly those without direct access to a hospital pathology department.

Here, we describe the isolation of human adipose tissue-derived stromal cells in large quantities from liposuction and abdominoplasty specimens. The method is a modification of previously reported techniques.[4,7,8] Based on a detailed biochemical, cellular, and molecular characterization of the adipogenic response in the human adipose tissue-derived stromal cells, our data support the conclusion that the human cells retain their differentiation responsiveness to glucocorticoids and thiazolidinediones in vitro. Consequently, this model has the potential to accelerate the mechanistic explanation of human adipocyte differentiation in health and disease.

MATERIALS AND METHODS

Type I collagenase was obtained from Worthington Biochemical (Lakewood, NJ). Bovine serum albumin (BSA), dexamethasone, biotin, pantothenate, Krebs-Ringer buffer, isoproterenol, and isobutyl methylxanthine (IBMX) were purchased from Sigma (St Louis, MO). Human recombinant insulin was obtained from Boehringer Mannheim (Indianapolis, IN). The fetal bovine serum was from HyClone (Logan, UT). Dulbecco modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), and Ham's F-10 nutrient broth were obtained from Biologos (Naperville, IL). Thiazolidinedione BRL49653 was a gift from Coelacanth (New Brunswick, NJ). All tissue culture flasks and plates were obtained from Corning (Corning, NY).

Tissue Preparation

Subcutaneous adipose tissue was acquired from elective liposuction or abdominoplasty surgeries. The tissues used were from patients of ages 28 to 46 with an average of 36.8 $\pm$ 5.4. The patients displayed a mean body mass index (BMI; kg/m^2) of 25.5 $\pm$ 7.3, with a range between 19 and 40. Other than obesity (defined as a BMI $>$ 25) in some instances, the patients were in good health; no diabetes or other complications were reported. The abdominoplasty tissues were minced with scissors into pieces with diameters between 1 mm and 5 mm; liposuction tissues were used without further processing. The minced tissue was washed at least 3 times with 2 vol of Krebs-Ringer-Bicarbonate (KRB) to remove contaminating blood. The tissue was then digested with 1 vol of collagenase type I (1 g/L of KRB with 1% BSA) for 60 minutes at 37°C with intermittent shaking. The floating adipocytes were separated from the stromal-vascular fraction by centrifugal force (300 $\times$ *g*) for 5 minutes. The preadipocytes in the stromal-vascular fraction were plated in tissue culture flasks without precoating of extracellular matrices. The initial plating density was approximately 3,500 cells/cm^2. The primary cells were cultured for 4 to 5 days until they reached confluence; these cells were defined as "passage 0." The cells were then harvested by digestion with 0.5 mmol/L EDTA/0.05% trypsin (Biologos), centrifuged at 1,200 rpm for 5 minutes, resuspended in DMEM–Ham's F-10 medium (vol/vol, 1:1) supplemented with 10%

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Submitted April 14, 2000; accepted October 17, 2000.
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0026-0495/01/5004-0007$35.00/0
doi:10.1053/meta.2001.21690

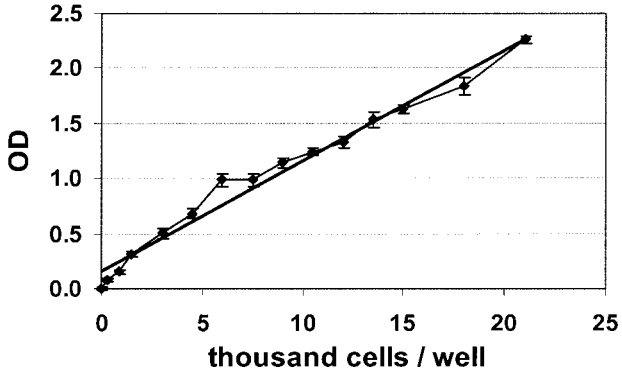

Fig 1. Between 3 × 10^2 and 2.1 × 10^4 human adipose-derived stromal cells were plated into individual wells of a 96-well plate, incubated for less than 16 hours to establish adherence, and assayed according to the cell proliferation reagent WST-1 protocol described in the Methods. Regression analysis of cell number and the OD_{450nm} demonstrated a linear correlation coefficient of >0.98. Representative of n = 2 donors.

fetal bovine serum, 15 mmol/L HEPES (pH 7.4), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (preadipocyte medium), and plated at a density of approximately 10,000 cells/cm^2. Cells were cultured to confluence before a second harvesting and replating procedure as defined above. After reaching confluence a second time, the cells were defined as "passage 2." At this stage, cells were used immediately for assays or cyropreserved in liquid nitrogen in media supplemented with 7% dimethyl sulfoxide (DMSO) prior to subsequent experimentation.

Differentiation of Adipocytes

Unless otherwise noted in the text or figure legends, the following standard conditions were used to induce adipogenesis in the human adipose tissue-derived stromal cells. The stromal cells were trypsinized and plated in multiple-well plates at 30,000 cells/cm^2 for 16 hours in medium containing DMEM–Ham's F-10 medium (vol/vol, 1:1) supplemented with 10% fetal bovine serum, 15 mmol/L HEPES (pH 7.4), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (preadipocyte medium) (day 0). After 24 hours (day 1), the medium was changed to DMEM–Ham's F-10 medium (vol/vol, 1:1) supplemented with 3% fetal bovine serum, 15 mmol/L HEPES (pH 7.4), biotin (33 μmol/L), pantothenate (17 μmol/L), human recombinant insulin (100 nmol/L), dexamethasone (1 μmol/L), IBMX (0.25 mmol/L), and BRL49653 (1 μmol/L) (differentiation medium). After a 3-day induction period (day 4), the cells were fed every 3 days with the same medium without IBMX and BRL49653 supplementation (adipocyte medium) for the remaining 9 to 10 days in the adipocyte differentiation period.

Oil Red O Staining

The Oil Red O staining method is a modification of a published protocol.[1] A stock solution of Oil Red O (0.5 g in 100 mL isopropanol) was prepared and passed through a 0.2-μm filter. Six milliliters of the stock solution was mixed with 4 mL of distilled water, left for 1 hour at room temperature, and filtered through a 0.2-μm filter prior to use. Cells in 96-well plates were rinsed 3 times with PBS, fixed with 10% formalin in PBS for 1 hour, and stained with 30 μL of the Oil Red O working solution per well for 15 minutes at room temperature. The wells were rinsed three times with 50 μL of water. The dye retained by the cells was eluted by incubation with 50 μL isopropanol. The OD_{540} was determined using a Packard Spectrocount plate reader. Blank wells (without cells) were stained with dye and rinsed in the same manner; these values were subtracted from the data points derived from the cell-containing experimental wells to control for any artifactual staining of the plastic alone.

Proliferation Assay

Cell proliferation was determined based on the enzymatic cleavage of the WST-1 tetrazolium salt using reagents from Boehringer Mannheim. Stromal cells from individual donors were cultured in 96-well tissue culture plates at a plating density of 1 × 10^3 cells/well with a final volume of 100 μL/well phenol-red free DMEM supplemented with 5% fetal bovine serum in a humidified incubator. After 24 hours, individual wells were fed with preadipocyte medium alone or supplemented with either 1 μmol/L BRL49653 or 1 μmol/L dexamethasone for the preadipocyte, preadipocyte + BRL, or preadipocyte + dexamethasone studies, respectively. All cells were fed with fresh medium every third day. The adipocyte cultures were fed with differentiation medium for the first 3 days and then converted to adipocyte medium for the remainder of the study. At each time point, 10 μL of cell proliferation reagent WST-1 was added to each. After a 2-hour incubation at 37°C, the plates were shaken thoroughly for 1 minute and the optical density at 450 nm (OD_{450}) was determined using a microtiter plate reader. Final calculations were performed after subtracting out the optical density determined in blank wells containing medium alone. Stromal cells plated at known numbers per well and assayed within 16 hours using the WST-1 reagent served to generate a standard curve for converting the OD_{450} to cell number (Fig 1).

Immunoblot Analysis

Conditioned medium was collected from cells incubated in 24-well plates for 3 to 18 days under control (preadipocyte) and adipogenic conditions and stored at −70°C prior to use. Aliquots (0.5 mL) were precipitated by the addition of 0.1 mL of 100% trichloroacetic acid, incubation for 1 hour at 4°C, and centrifugation. The pellets were resuspended in 1× NuPage sodium dodecyl sulfate (SDS) sample buffer, boiled for 10 minutes, electrophoresed on a NuPage 4% to 12% Bis-Tris acrylamide gel (Novex, San Diego, CA), and transferred to nitrocellulose paper. The blot was blocked for 1 hour at room temperature in 2% BSA in Tris-buffered saline (TBS; 20 mmol/L Tris pH 7.4, 150 mmol/L NaCl) and then incubated with rocking overnight at 4°C in TBS containing 0.1% BSA and a 1:2,000 dilution of a rabbit antileptin polyclonal antibody (Biovendor, Prague, Czech Republic). After the primary antibody incubation, the immunoblot was washed 4 times for 5 minutes at room temperature with TBS, incubated in TBS

Table 1. Number of Stromal Cells Recovered per Gram of Adipose Tissue

BMI (kg/m^2)	Age (yr)	Gender	Cells (×10^4)/g Adipose Tissue
24	43	Female	7.1 ± 2.0
43	50	Male	9.2 ± 3.4
21	39	Female	9.6 ± 2.6

NOTE. Three separate 1-g specimens of adipose tissue were obtained from each donor and processed as described in the Methods. The cells recovered from each gram were incubated in a 10-cm^2 area. After 24 hours of incubation, the cells were recovered by trypsin digestion and counted using a hemacytometer.

Abbreviation: BMI, body mass index.

Table 2. Stromal Cell Proliferation Rates Under Different Culture Conditions

	Days		
	4	7	10
Preadipocytes	3.1 ± 0.6	6.4 ± 2.2	12.4 ± 5.2
Preadipocytes + BRL	2.6 ± 0.3	7.2 ± 2.5	11.6 ± 5.0
Preadipocytes + dexamethasone	1.7 ± 0.3	3.3 ± 1.1	4.7 ± 2.0
Adipocytes	1.9 ± 0.3	2.6 ± 0.5*	2.3 ± 0.7*

NOTE. Cell proliferation was determined on days 0, 4, 7, and 10 after plating, based on the WST-1 reagent. Cells were maintained for 10 days in the presence of preadipocyte medium alone, preadipocyte medium supplemented with either 1 μmol/L BRL49653 or 1 μmol/L dexamethasone, or under adipogenic conditions. Values reported are the mean ± SEM from n = 4 donors with a mean BMI of 23.5 ± 2.4 kg/m^2 normalized relative to day 0, defined as "1." Based on linear regression analysis, the doubling times over the 10-day period are: preadipocytes 43 hours (correlation coefficient, 0.956); preadipocytes + BRL, 44 hours (correlation coefficient, 0.965); preadipocytes + dexamethasone, 127 hours (correlation coefficient, 0.975).

*Significantly different ($P < 0.05$) compared with the preadipocyte value on the same day.

containing 0.1% BSA and a 1:2,000 dilution of a goat anti-rabbit IgG–alkaline phosphatase coupled secondary antibody (BioRad, Richmond, CA) for 1 hour at room temperature. The blot was then washed with TBS 3 times at room temperature and developed with 1-Step NBT/BCIP (Pierce, Rockford IL).

Glycerol Phosphate Dehydrogenase Activity

Adipose tissue-derived stromal cells were cultured in medium containing 3% fetal bovine serum, dexamethasone (1 μmol/L), IBMX (0.25 μmol/L), and human recombinant insulin (100 nmol/L) in the absence or presence of BRL49653 (1 μmol/L). Individual cultures (n= 3 per treatment) were rinsed twice with PBS, harvested into 0.5 mL PBS using a cell scraper, and then frozen at −70°C. The thawed extract was sonicated with two 5-second bursts and centrifuged at 4°C for 30 minutes at 16,000 × *g*. Glycerol phosphate dehydrogenase (GPDH) activity was determined spectrophotometrically by measuring the oxidation of NADH at 340 nm using dihydroxyacetone phosphate as substrate.[9] Protein content of the supernatant was determined using a commercially available reagent (BioRad Protein Assay kit, CA). Data was expressed as mU/mg protein (μmol NADH oxidized)/(min·mg protein).

Lipolysis Assay

Lipolysis was assayed by measuring the glycerol released from the mature adipocytes in respond to the β-adrenergic receptor agonist, isoproterenol. Mature adipocytes plated in 96-well plates were washed with Krebs-Ringer buffer supplemented with 1 g/L glucose and incubated with or without lipolytic reagent (120 μL/well) for 5 hours at 37°C. The conditioned media (100 μL/well) were removed and glycerol determined using the triglyceride reagent A (GPO-Trinder, Sigma) following the manufacturer's instruction.

Polymerase Chain Reaction

Total RNA was isolated from human adipose tissue-derived stromal cells induced with the adipocyte differentiation protocol for 0, 2, 4, or 14 days using TriReagent (Molecular Research Center, Cincinnati, OH).[10] Reverse transcriptase reactions were performed with 2 μg of total RNA using the GeneAmp RNA polymerase chain reaction (PCR) Kit (Perkin Elmer, Branchburg, NJ). The cDNA was divided equally among the following PCRs, which were performed using a 5-minute 94°C hot start, a cycle of 30 seconds at 94°C, 30 seconds at 55°C, and 1 minute at 72°C (the number of cycles is indicated below for each oligonucleotide primer pair) followed by an 8-minute extension at 72°C. The cycle numbers used were determined to be in the linear amplification range of the individual genes using control reverse-transcribed cDNA (data not shown). Sets of forward (F) and reverse (R) oligonucleotide primers were synthesized by Gibco-BRL (Gaithersburg, MD) specific for the following human (h) or murine (m) cDNAs; fragment sizes and number of amplification cycles are indicated in parentheses:

hActin F: AGCCATGTACGTTGCTA (745 bp)
hActin R: AGTCCGCCTAGAAGCA (23 cycles)
hAP2 F: GGCCAGGAATTTGACGAAGTC (251 bp)

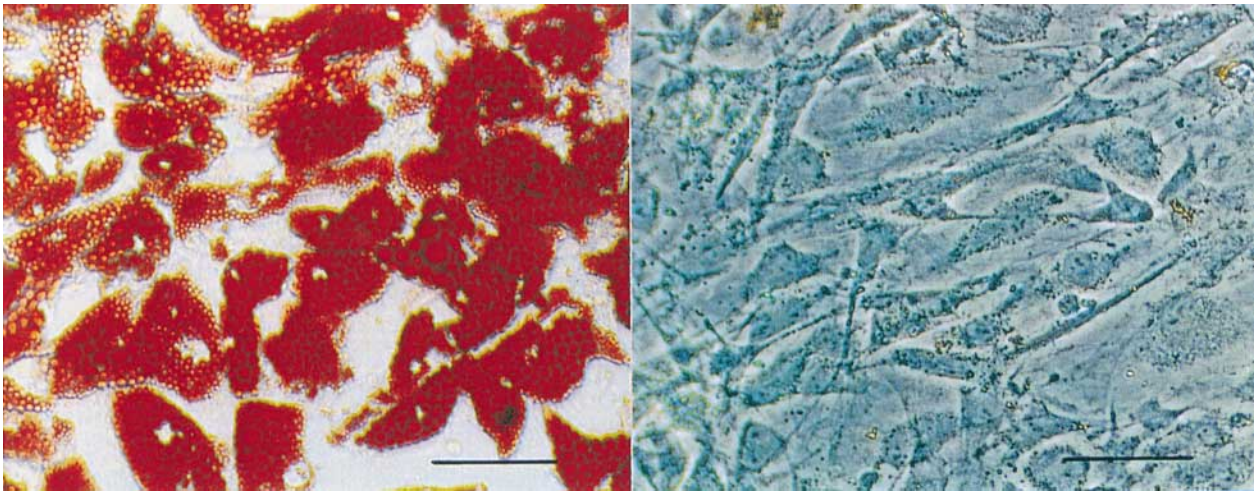

Fig 2. Morphology of cultured human adipose-derived stromal cells under preadipocyte and adipogenic conditions. Phase contrast photomicrographs of the human preadipocytes at passage 2 showed typical fibroblast morphology (right). When differentiated for 14 days, the mature adipocytes had multiple vacuoles and stained positive with Oil Red O (left). The scale bar represents 0.2mm.

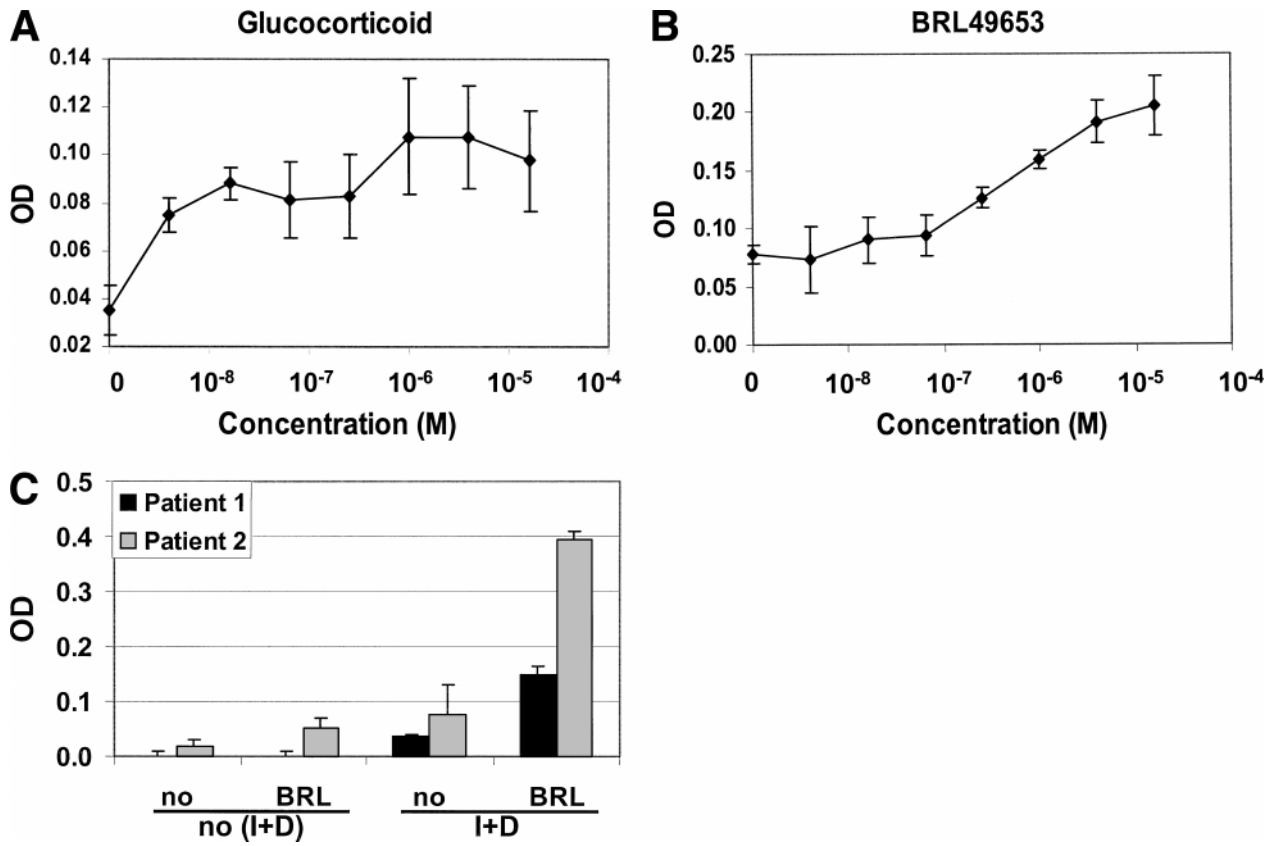

Fig 3. Concentration and time dependence of adipogenesis on thiazolidinedione and glucocorticoid. (A, B) Confluent cultures of human adipose tissue-derived stromal cells were incubated for 3 days in DMEM supplemented with 10% serum, antibiotics, IBMX (0.25 mmol/L), insulin (100 ng/mL), and either (A) increasing concentrations of dexamethasone in the presence of 1 μmol/L BRL49653, or (B) increasing concentrations of BRL49653 in the presence of 1 μmol/L dexamethasone. Cultures were then converted to adipocyte medium (as defined in the Methods). Cells were stained with Oil Red O after 12 days and the amount of retained dye determined by OD_{540}. (C) Confluent cultures of adipose tissue-derived stromal cells were incubated for 3 days in DMEM/ 3% fetal bovine serum supplemented with BRL49653 (1 μmol/L) and/or recombinant human insulin (100 nmol/mL) and dexamethasone (1 μmol/L). After the initial 3-day incubation period, cells were maintained in DMEM supplemented with 3% fetal bovine serum and antibiotics. Cultures were stained with Oil Red O after a total of 14 days in culture and the amount of retained dye determined by OD_{540}.

hAP2 R: ACAGAATGTTGTAGAGTTCAATGCGA (23 cycles)
hC/EBPa F: TGGAGGTTTCCTGCCTCCTTCCC (296 bp)
hC/EBPa R: CCAGCCCCAAGGGAAAGCCAGCC (35 cycles)
mLeptin F: CACCAAAACCCTCATCAAGAC (360 bp)
mLeptin R: AGCCTGCTCAAAGCCACCACC (35 cycles)
hPPARγ2 F: TGGGTGAAACTCTGGGAGATTC (380 bp)
hPPARγ2 R: CATGAGGCTTATTGTAGAGCTG (29 cycles)

Aliquots of the amplified PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photographed. The PCR products were subcloned into the pCR2.1 TA cloning vector (Invitrogen, San Diego, CA) and the sequence confirmed (performed by the Sequencing Core Facility at the Oklahoma Medical Research Foundation, Oklahoma City, OK).

RESULTS

Proliferation Analysis

Primary human subcutaneous stromal cells were isolated from adipose tissue specimens (abdominoplasty or liposuctions) as described in the Methods. One gram of tissue yielded $8.6 \pm 1.4 \times 10^4$ cells (mean $\pm$ SD, n = 3) (Table 1). In the undifferentiated or preadipocyte state, the cells displayed a doubling time of between 45 and 56 hours as determined by hemacytometer cell counts (data not shown). Using a metabolic-based assay to determine the proliferation rate, the control cells (preadipocytes) doubled every 43 hours over the assay period (Table 2). This assay demonstrated a linear relationship between cell number and the OD_{450} over a range of 3,000 to 20,000 cells/well (Fig 1). The proliferation rate of 44 hours in the presence of 1 μmol/L thiazolidinedione BRL49653 was not significantly different from that of the controls; however, the addition of 1 μmol/L dexamethasone increased the doubling time to approximately 127 hours (Table 2). In contrast, under the adipogenic conditions defined in the Methods, containing 1 μmol/L each of dexamethasone and BRL49653 together with insulin (100 nmol/L) and IBMX (0.25 mmol/L), only a single doubling occurred over the 10-day period (Table 2).

Adipogenesis—Neutral Lipid Accumulation

In response to the differentiation protocol outlined in the Methods, the adipose tissue-derived stromal cells accumulated lipid as demonstrated by phase-contrast microscopy (Fig 2). To

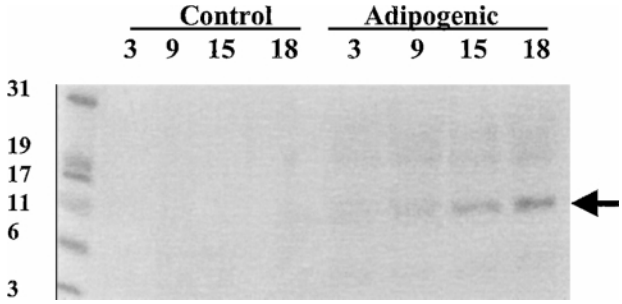

Fig 4. Leptin expression. Conditioned media from human adipose tissue-derived stromal cells cultured under preadipocyte or adipogenic conditions for periods of up to 18 days. Trichloroacetic acid precipitated proteins were examined by western blot analysis with an antileptin antibody. Size markers (in kd) are indicated on the left; arrow indicates the detected leptin protein. Representative n = 2.

determine the responsiveness of the human preadipocyte stromal cells to adipogenic reagents, studies examined the stromal cell's neutral lipid accumulation by Oil Red O staining in response to glucocorticoid and the thiazolidinedione, BRL49653 (Fig 3). In the presence of human recombinant insulin (100 nmol/L), IBMX (0.25 mmol/L), and BRL49653 (1 μmol/L) alone, cells accumulated baseline levels of lipid based on Oil Red O staining. With the addition of increasing concentrations of dexamethasone, the cells increased their staining with Oil Red O and exhibited maximum neutral lipid accumulation at a dexamethasone concentration of 1 μmol/L (Fig 3A). Likewise, in the presence of human recombinant insulin (100 ng/mL), IBMX (0.25 mmol/L), and dexamethasone (1 μmol/L) alone, cells accumulated only a baseline level of lipid. With the added exposure of increasing concentrations of BRL49653, the cells exhibited maximum neutral lipid accumulation at 2.5 μmol/L (Fig 3B). In the absence of IBMX, the actions of glucocorticoid and thiazolidinedione were synergistic (Fig 3C). While the presence of either BRL49653 (1 μmol/L) alone or dexamethasone (1 μmol/L) and insulin (100 nmol/mL) together increased lipid levels above baseline, their combined presence induced lipid accumulation by an additional 3- to 4-fold (Fig 3C). The synergistic actions of BRL49653 and dexamethasone were time-dependent (data not shown).

Adipogenesis–Leptin Expression

The ability to secrete leptin is a unique property distinctive of mature adipocytes. Western blot analysis of conditioned media from adipogenic cultures demonstrated increased expression of secreted leptin protein in a time-dependent manner (Fig 4). Levels increased progressively over an 18-day period. Conditioned media from noninduced preadipocyte controls did not demonstrate increased leptin levels over the same time period (Fig 4).

Adipogenesis–GPDH Activity

During adipocyte differentiation, cells begin to express the cytoplasmic enzyme GPDH. Under preadipocyte conditions, stromal cells expressed GPDH activity of 8 mU/mg protein; the addition of 1 μmol/L BRL49653 did not significantly alter this activity (Table 3). In the presence of insulin, dexamethasone, and IBMX, but in the absence of BRL49653, the level of GPDH activity was not increased significantly relative to the preadipocyte cultured cells (range, 7.8 to 13.5 mU/mg protein); however, the addition of BRL49653 to these reagents increased activity by a mean of 14.4-fold. The fold-induction varied among donors over a range of 4.4 to 27.7, indicating some degree of heterogeneity.

Lipolytic Response to Isoproterenol by In Vitro–Differentiated Mature Adipocytes

The mature adipocytes resulting from this in vitro differentiation protocol exhibited a dose-dependent lipolytic response to isoproterenol (Fig 5). Based on a glycerol release assay, the cells displayed a maximal 4-fold induction of lipolysis in the presence of 100 nmol/L isoproterenol. Preadipocytes failed to display any lipolytic response to isoproterenol in control experiments.

Adipogenesis–mRNA Expression

PCR analysis of total RNA isolated from cells 0 to 14 days following exposure to adipogenic conditions revealed increased expression of adipocyte gene markers. These included the genes for the fatty acid binding protein aP2, leptin, and the transcriptional regulators, CCAAT/enhancer binding protein α (C/EBPα) and PPARγ (Fig 6). Actin levels served as a control for relative equal loading between lanes. The identity of each DNA fragment was verified by subcloning and sequence analysis.

DISCUSSION

Adipose tissue plays a dynamic metabolic role in health and disease. There are many murine-derived in vitro pre-

Table 3. Glycerol Phosphate Dehydrogenase Activity: Induction by BRL49653 Under Adipocyte Culture Conditions

Sample	Minus BRL49653	Plus BRL49653	Fold-Induction by BRL49653
Average of all lots under preadipocyte conditions	8.0 ± 0.8	8.6 ± 0.8	1.1
Lot no. 1 (BMI, 22), adipocyte conditions	7.8 ± 4.4	216.2 ± 71.9	27.7
Lot no. 2 (BMI, 22), adipocyte conditions	13.5 ± 4.4	60.7 ± 12.9	4.4
Lot no. 3 (BMI, 41), adipocyte conditions	8.4 ± 5.8	67.3 ± 19.6	8.0
Lot no. 4 (BMI, 28), adipocyte conditions	9.6 ± 4.6	167.4 ± 53.4	17.4

NOTE. Stromal cells derived from 4 different donors were cultured under preadipocyte conditions or in the presence of dexamethasone, insulin, and IBMX (adipocyte conditions) with or without 1 μmol/L BRL 49653 for 3 days. Adipocyte cultures were then maintained in the presence of medium supplemented with dexamethasone and insulin alone for an additional 11 days. On day 14, protein lysates were prepared and assayed for glycerol phosphate dehydrogenase activity expressed as mU/mg protein. Values are the mean ± SD for all 4 donors under preadipocyte conditions or the mean ± SD of 3 replicate wells for each individual donor under adipocyte conditions.

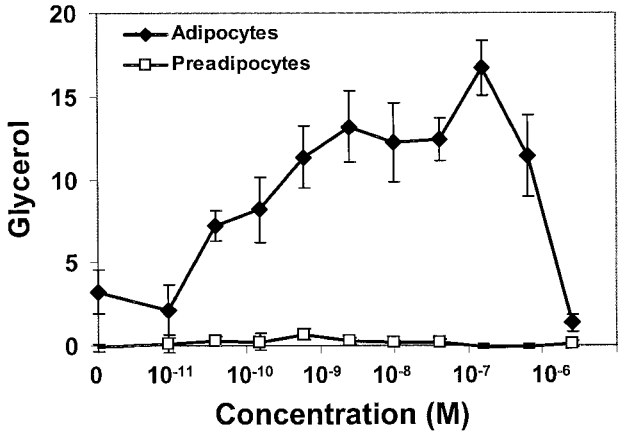

Fig 5. Lipolysis assay. Adipose tissue-derived stromal cells were induced to differentiate according to the protocol defined in the Methods in a 96-well plate format. After a culture period of 14 days, the mature adipocytes were treated with increasing concentrations of isoproterenol. Lipolysis was determined by glycerol release assay and is expressed as nmol glycerol released/h/10^4 cells. Data are the means ± SD of an experiment performed with a single lot of stromal cells and are representative of studies performed with 5 individual donors.

adipocyte models, such as 3T3-L1, 3T3-F442a, and *ob*17, that have yielded a wealth of information concerning the molecular mechanisms regulating adipogenesis.[2,3,11,12] Unlike the murine models, no human preadipocyte cell lines can achieve greater than 50% adipogenesis consistently. Consequently, previous research on human adipose tissue metabolism has focused on primary organoid cultures and primary adipocytes in vitro. Comparison of the adipogenic mechanisms in human and murine systems relies heavily on these models.

Liposuction samples have been an abundant source of viable human adipocytes for other investigators.[5,6] The procedure has not been found to damage the mature adipocytes any more than other excision procedures based on the cellular release of glycerol-3-phosphate dehydrogenase.[5] However, liposuction methods have been reported to destroy up to 6% of the adipocytes in a sample.[6] While intraoperative exposure to lidocaine acutely alters adipocyte function based on glucose uptake and lipolysis, these actions did not persist and the cells regained all function following its removal.[6]

The human preadipocyte stromal cells isolated according to the method described in this manuscript from liposuction and abdominoplasty specimens exhibit adipogenic potential in vitro. This differentiation process is characterized by a time- and dose-dependent expression of lineage-specific secreted protein, enzyme, and mRNA markers. The inductive actions of PPARγ ligands in these cells are comparable to those previously described in primary human adipose tissues.[13-15] Likewise, the differentiation dependence on insulin and glucocorticoids are comparable to previous reports in human and rodent adipose tissue-derived cells.[4,16-20] The PPARγ and glucocorticoid receptor ligands synergistically induce adipogenesis in the current system; however, the mechanism underlying this observation and the contribution of insulin to the differentiation process will require further investigation. The mature adipocytes derived from the current culture methods respond to β-adrenergic receptor agonists in a manner equivalent to human primary adipocytes and adipose tissue organ cultures; these exhibit a similar profile, including a decrease in lipolytic activity at concentrations exceeding 10^{-6} mol/L.[21,22] Consequently, this method represents a reproducible in vitro model for predicting drug effects on human adipose tissue in vivo.

Clearly, many parallels exist between rodent and human adipose tissues. Thiazolidinediones, glucocorticoids, and insulin are adipogenic in both species and induce a common panel of tissue-selective genes. Nevertheless, it remains to be determined if findings in murine systems remain valid in human models. The ready availability of human adipose-derived stromal cells for experimental use will provide a better understanding of adipogenesis and its regulation in humans. These methods have been used to examine the regulation of the PPARγ promoter in human cells.[23] A preliminary profile of expressed genes using microarray technology has also been reported in this human system.[24] It is anticipated that the methods developed in this work will be the basis for future investigations of the adipose tissue

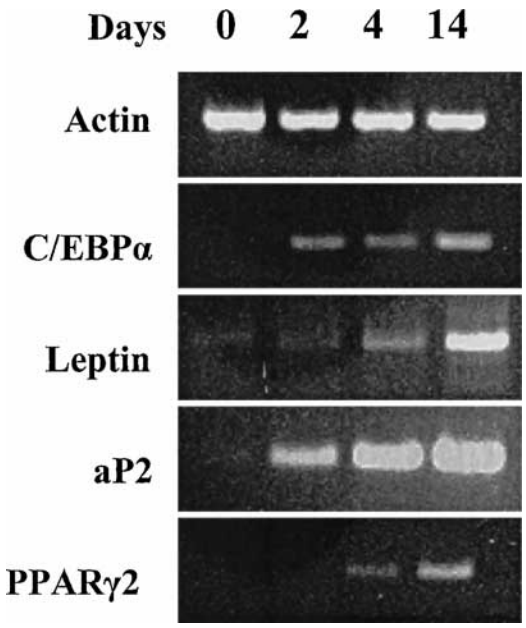

Fig 6. RNA expression. Human adipose tissue-derived stromal cells were plated at 30,000 cells/cm^2 and maintained in preadipocyte medium or induced with differentiation medium for 3 days before conversion to adipocyte medium as defined in the Methods. The cells were maintained for 14 days and harvested for total RNA, which was examined by PCR with primers for the following genes (sizes indicated in parentheses): aP2 (251 bp), actin (745 bp), C/EBPα (296 bp), leptin (360 bp), PPARγ2 (380 bp). PCR products were analyzed after electrophoresis on an agarose gel and detected by ethidium bromide staining. The identity of each DNA fragment was confirmed by sequence analysis.

depot-dependent gene variability in man, an area of increasing attention.[18,25-28]

ACKNOWLEDGMENT

The authors acknowledge the following individuals: Peter Pieraccini for administrative assistance; Sheryl Christofferson (Oklahoma Medical Research Foundation) for sequence analysis of the TA subclones; Dr Joyce Harp (University of North Carolina-Chapel Hill) for critically reviewing the manuscript; Dr Cynthia Buffington and Dr George Cowan (Memphis, TN), Dr Sam Wurster (Cary, NC), Dr Ron Riefkohl (Durham, NC), and Dr Vern Lanier (Durham, NC) for providing adipose tissue specimens for this study.

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