Characterization of differentiated subcutaneous and visceral adipose tissue from children: the influences of TNF-α and IGF-I

Malcolm Grohmann,[1,*] Matthew Sabin,[†] Jeff Holly,[*] Julian Shield,[†] Elizabeth Crowne,[2,†] and Claire Stewart[2,*]

Department of Surgery,* University of Bristol, Bristol Royal Infirmary, Bristol BS2 8HW, United Kingdom; and Department of Pediatric Endocrinology,† Institute of Child Health, Royal Hospital for Children, Bristol BS2 8AE, United Kingdom

Abstract **The relationship between subcutaneous and visceral adipocyte metabolism and development has been extensively studied in adult but not in pediatric tissue. Our aim was to isolate, develop, characterize, and compare primary cell cultures of subcutaneous and visceral preadipocytes from 16 normal prepubertal children (10 male and 6 female). Subculture techniques were developed to increase cell number and allow differentiation using a chemically defined serum-free medium. Removal of insulin from the differentiation medium prevented adipogenesis in both subcutaneous and visceral preadipocytes, whereas coincubation with rosiglitazone markedly enhanced glycerol-3-phosphate dehydrogenase activity, peroxisome proliferator-activated receptor γ expression, and triglyceride accumulation in cells from both fat depots. Adiponectin secretion increased with differentiation from undetectable levels at day 0. Histological analyses demonstrated significant differences in lipid droplet number and size, with subcutaneous cells having fewer but larger vesicles compared with visceral cells. Downregulation and reorganization of the cytoskeleton appeared comparable. We further demonstrate regional differences in adipogenesis manipulation. Tumor necrosis factor-α was more effective at inhibiting differentiation in subcutaneous cells, whereas insulin-like growth factor-I stimulated differentiation more effectively in visceral cells. Insulin-like growth factor binding protein-3 enhanced differentiation equally. These observations may have important physiological and pharmacological implications for the development of obesity in later life.**—Grohmann, M., M. Sabin, J. Holly, J. Shield, E. Crowne, and C. Stewart. **Characterization of differentiated subcutaneous and visceral adipose tissue from children: the influences of TNF-α and IGF-I.** ***J. Lipid Res.*** **2005.** 46: **93–103.**

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Obesity in both adults and children is increasing exponentially throughout the world (1). Childhood obesity is known to track into adulthood (2) and has long-term adverse effects on morbidity, independent of adult weight (3). It is already apparent that the prevalence of type 2 diabetes is increasing in obese adolescents in the United States and United Kingdom (4, 5), but not all obese individuals develop insulin resistance (6). The role of adipose tissue is not simply to store excess energy but to secrete and respond to a variety of metabolites, hormones, and cytokines, which may play important roles in the development of obesity-related pathologies (7). Visceral adiposity is particularly associated with metabolic complications and is an important predictor of increased morbidity and mortality from ischemic heart disease, cancer, and diabetes in adult life (8, 9). Therefore, understanding the mechanisms of site-specific adipose tissue accumulation and those underlying the development of insulin resistance are essential to focus future treatment strategies.

In vitro models of adipocyte development are invaluable tools for studying the pathogenesis of obesity, with the 3T3-L1 mouse cell line (10) the most frequently used. There have been recent advances in both animal (11–13) and human primary cell cultures (14, 15). Studies investigating adipocytes from both subcutaneous and visceral sites in adults identified significant differences in metabolism as well as adipocyte acquisition and loss (16). There have been no studies, however, examining these mechanisms in samples derived from children.

The aims of the present study were as follows: *1*) to develop methods to isolate and culture both subcutaneous

Abbreviations: BMI, body mass index; GPDH, glycerol-3-phosphate dehydrogenase; IGF-I, insulin-like growth factor-I; IGFBP-3, insulin-like growth factor binding protein-3; ORO, Oil Red O; PPARγ, peroxisome proliferator-activated receptor γ; TNF-α, tumor necrosis factor-α.

[1] To whom correspondence should be addressed.
e-mail: m.j.grohmann@bristol.ac.uk
[2] E. Crowne and C. Stewart contributed equally to this work.

and visceral preadipocytes derived from very small biopsies (<0.5 cm^3) of children's adipose tissue; *2*) to differentiate the cells into mature adipocytes using defined serum-free conditions; and *3*) to provide a useful model to study factors that regulate adipogenesis. Here, we present the methodology for the above studies and report site-specific differences in basal and growth factor/cytokine-manipulated growth, morphology, and differentiation.

MATERIALS AND METHODS

Patient data

Paired anterior abdominal wall (subcutaneous) and perinephric (visceral) adipose tissue samples were obtained from 16 prepubertal Caucasian children at the onset of routine elective abdominal surgery at the Royal Hospital for Children undergoing either pyeloplasty or nephrectomy operations (10 male/6 female). Median (range) age was 4.4 (0.5–9) years, and median (range) body mass index (BMI) standard deviation score (SDS) was 0.33 (−2.22–1.85). All had normal blood pressure. Fasting insulin levels were median (range) 1.5 (1–4) mU/l. All displayed normal systemic insulin sensitivity using the Quantitative Insulin Sensitivity Check Index (17), median (range) 0.49 (0.39–0.56). No patients had sepsis, malignancy, or endocrine conditions. This study was approved by the United Bristol Healthcare Trust Ethics Committee, and written informed consent was obtained from all parents.

Reagents

Fetal bovine serum, Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/Ham's F12), HBSS, and trypsin-EDTA were obtained from Gibco Invitrogen (Paisley, UK). Streptomycin and penicillin were obtained from the local hospital pharmacy. Human recombinant insulin was obtained from Novo Nordisk (Bagsverd, Denmark). Insulin-like growth factor-I (IGF-I) and LongR^3 IGF-I [an analog of IGF-I with greatly reduced affinity for the insulin-like growth factor binding proteins (IGFBPs)] were purchased from Gropep (Adelaide, Australia). Recombinant human tumor necrosis factor-α (TNF-α) was obtained from Bachem (St. Helens, UK), and isopropanol was from Fisher Scientific (Leicestershire, UK). Formal saline was purchased from BDH Laboratory Supplies (Poole, UK). Phosphate-buffered saline was obtained from Oxoid (Basingstoke, UK), peroxisome proliferator-activated receptor γ (PPARγ) antibody (sc-7273) was from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-mouse Alexa 488 (A11001) was from Molecular Probes (Eugene, OR). Adiponectin ELISA was kindly donated by Metachem Diagnostics, Inc. (Northampton, UK), and rosiglitazone (BRL 49653-C) was donated by GlaxoSmithKline. All other chemicals and antibodies, unless stated otherwise, were purchased from Sigma (Poole, UK). Tissue culture plasticware was obtained from Greiner Bio-one (Kremsmunster, Austria).

Isolation and culture of adipocyte precursor cells

The isolation of subcutaneous and visceral preadipose cells was performed using an adapted method of Hauner et al. (15). Very small tissue samples (0.2–0.5 g) were collected under sterile conditions at the onset of surgery and taken directly to the laboratory. The adipose tissue was washed three times in 10 ml of HBSS, cut into 1 mm^3 pieces, and digested with 10 ml of 1 mg/ml type II collagenase in HBSS for 60 min at 37°C in a shaking water bath (150 cycles/min). Adipocytes were separated from the stromal-vascular cells by centrifugation at 80 *g* for 3 min. The resulting pellet of sedimented preadipocytes (typically less than 1,000 cells) was resuspended in 10 ml of DMEM/Ham's F12 medium (1:1, v/v) supplemented with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were seeded onto a 75 cm^2 0.2% gelatin-coated tissue culture flask, maintained at 37°C in a humidified atmosphere of 5% CO_2, and medium was changed every 72 h. The primary cultures grew exponentially from preadipocyte cell clusters until confluency was attained (7–14 days) and were then passaged to 3 × 182 cm^2 flasks. At confluency, 50% of the cells were frozen and the remaining cells were used for experimentation. This methodology was successfully adapted to yield sufficient cells from very small biopsies for further investigation. All experiments were performed on cells at passages 3–5.

Cell proliferation assay

Subcutaneous and visceral preadipocytes (passage 4) were seeded at a density of 10,000 cells/cm^2 on 24-well plates and cultured in DMEM/Ham's F12 medium (1:1, v/v) supplemented with 20% FBS. Cell proliferation was assessed over time at days 1, 3, 5, 7, and 10 by manual cell counting using trypan blue exclusion. All experiments were performed before confluency and contact inhibition occurred. Cell growth was expressed as a percentage of the number of cells counted at day 1.

Differentiation of human preadipocytes

Subcutaneous and visceral preadipocytes (passages 3–5) were cultured at a density of 18,000 cells/cm^2 on 12-well plates and after 16 h were induced to differentiate using a modified method of Hauner et al. (15). Cells were washed twice in PBS and cultured for 14 days with a chemically defined serum-free medium of DMEM/Ham's F12 (1:1, v/v) supplemented with 15 mmol/l HEPES, 15 mmol/l $NaHCO_3$, 33 μmol/l biotin, 10 μg/ml apotransferrin, 100 nmol/l insulin, 1 μmol/l dexamethasone, 0.2 nmol/l 3,3,5′-triiodothyronine, 17 μmol/l pantothenate, and, for the first 3 days of culture, 25 μmol/l 3-isomethyl-L-methylxanthine and 10 μM rosiglitazone (optimal concentration after a dose response). Differentiation was manipulated with 100 nmol/l insulin, 10 μM rosiglitazone (for the first 3 days), and, in the absence or presence of a continuous exposure to TNF-α (5, 10, and 20 ng/ml), 200 ng/ml IGF-I, 200 ng/ml LongR^3 IGF-I, or 200 ng/ml IGFBP-3.

Characterization of human preadipocyte and adipocyte cultures

Triglyceride levels of differentiated cell lysates were measured using a glycerol phosphate oxidase triglyceride determination kit (Sigma) and expressed in micrograms per milliliter of lysate. Glycerol-3-phosphate dehydrogenase (GPDH) activity was quantified using a modified method of Kozak and Jensen (18). Cells were washed twice in ice-cold PBS and harvested with scraping in 100 μl of lysis buffer containing 0.05 M Tris-Cl, 1 mM EDTA, and 1 mM β-mercaptoethanol, pH 7.4. GPDH activity of the cell extracts was measured spectrophotometrically at 340 nM in a reaction mix containing 100 mmol/l triethanolamine-HCl, pH 7.5, 12.5 mmol/l EDTA, 0.1 mmol/l β-mercaptoethanol, and 0.12 mmol/l NADH. The reactions were initiated by adding 0.4 mmol/l dihydroxyacetone phosphate, and the spectrophotometer was zeroed. Readings were taken every 30 s for 3 min to ensure linearity. Protein concentrations were quantified using a bicinchoninic acid protein assay (Pierce), and the results are expressed as milligrams of protein per milliliter of lysate. Adiponectin levels in lysates and conditioned media from differentiated adipocytes or from mature adipocytes after 24 h in primary culture after the initial isolation were analyzed using a human adiponectin ELISA (Metachem Diagnostics, Inc.). Differentiation was assessed morphologically and confirmed by Oil Red O (ORO) staining as previously described (19). ORO uptake was

quantified using spectrophotometry at 500 nM in 100% isopropanol. Appropriate blanks were included.

Immunocytochemical characterization

Differentiated cells were washed twice in PBS, fixed in 2% paraformaldehyde, and either permeabilized for 10 min in 0.1% Triton X-100/3% BSA in PBS (for F-actin, α-tubulin, and Nile Red staining) or blocked in 1.5% rabbit serum in PBS (for PPARγ staining). Mouse monoclonal antibodies to PPARγ (Santa Cruz Biotechnology) and α-tubulin were used at dilutions of 1:100, followed by either specific biotinylated secondary antibodies and a streptavidin biotinylated horseradish peroxidase (StrepABComplex/HRP) (Dako, Glostrup, Denmark) coupled with 3,3′-diamino benzidine for PPARγ or an anti-mouse Alexa 488 antibody for α-tubulin. Nuclei were counterstained with hematoxylin or 4′,6-diamino-phenylindole accordingly. F-actin cytoskeletal filaments were visualized by staining with Phalloidin-tetramethyl rhodamine isothiocynate (TRITC) (Sigma) at 1:200 in 3% BSA. Intracellular lipid droplet accumulation was visualized using the selective fluorescent stain Nile Red as previously described (20).

Histological image analysis

Cells were imaged using a Nikon TS100 phase contrast microscope (Melville, NY) and a Nikon 990 Coolpix camera. Histological analyses of fixed cells were made using an Olympus BX40 fluorescence microscope (Olympus America, Inc.). Image acquisitions were calibrated with a 1 mm objective micrometer and were controlled and analyzed using Image Pro Plus 4.0 software from Media Cybernetics (Silver Spring, MD). Three individuals blinded to cell origin assessed lipid droplet size and number.

Statistics

The data were subjected to Student's *t*-test, Scheffe multiple comparison analyses, ANOVA, or nonparametric Wilcoxon matched pairs signed ranks using SPSS (version 11.5). Results are expressed as means ± SEM, and $P < 0.05$ was considered significant.

RESULTS

Proliferation capacity of subcutaneous and visceral preadipocytes

Proliferation of paired subcutaneous and visceral preadipocyte cultures was compared by stimulation with 20% FBS over time for 1, 3, 5, 7, and 10 days, and the results are expressed as percentage increase above day 1 (**Fig. 1**). As expected, there was a significant increase in proliferation between the subcutaneous ($P = 0.005$) and visceral cells ($P = 0.009$) with time. Comparison between the two groups by ANOVA revealed a significant difference at day 7 ($P = 0.04$) and day 10 ($P = 0.024$).

Effects of insulin and rosiglitazone with time on differentiation and triglyceride accumulation

Paired subcutaneous and visceral preadipocyte cultures were induced to differentiate in a chemically defined serum-free adipogenic medium. These studies demonstrated that the cells did not differentiate spontaneously (**Fig. 2A, B**). Inclusion of 100 nM insulin in the medium facilitated differentiation (Fig. 2C, D), which was further enhanced by the addition of 10 μM rosiglitazone for the first 3 days (Fig. 2E, F) in both subcutaneous and visceral preadipocytes. ORO and GPDH analyses confirmed these findings

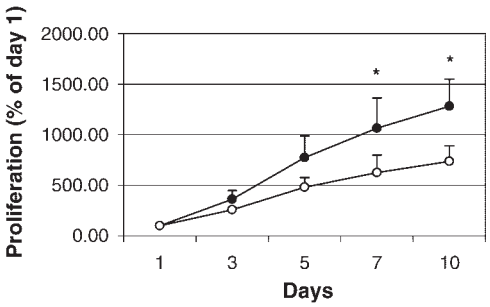

Fig. 1. Proliferation capacity of subcutaneous and visceral preadipocytes. Evaluation of the proliferation rates between paired biopsies of subcutaneous (closed circles) and visceral (open circles) preadipocytes. The data represent percentage increases ± SEM compared with day 1 of triplicate cultures from three individual biopsies. Statistical analysis was performed using three-way ANOVA (* $P < 0.05$).

(data not shown). These morphological studies were confirmed biochemically by assessing triglyceride levels in the differentiated cells (**Table 1**). A stringent Scheffe multiple comparison analysis showed borderline significance between no treatment and insulin ($P = 0.055$) and significant difference not only between control and insulin/rosiglitazone ($P = 0.004$) but also between insulin and insulin/rosiglitazone ($P = 0.033$). Although the data suggest a trend toward increased triglyceride levels in subcutaneous cells, this did not reach statistical significance when analyzed by ANOVA (P = NS).

Triglyceride deposition in subcutaneous and visceral preadipocytes

Additional time-course experiments were undertaken to monitor the rate of triglyceride accumulation during differentiation between the two fat depots. At confluency, the undifferentiated preadipocytes were devoid of lipid droplets, but exposure to serum-free medium containing insulin and rosiglitazone for as little as 24 h resulted in an increase in cytoplasmic lipid accumulation. These lipid-filled vesicles increased in size and number and were easily detected by day 4 of differentiation. Lipid droplets continued to accumulate in a perinuclear manner, and the last 7 days of differentiation resulted in a remarkable increase in both lipid droplet size and number, which coalesced and displaced the nucleus to the periphery. During the differentiation process, it was noted that subtle differences existed in triglyceride deposition, depending on adipose tissue origin. In an attempt to elucidate these differences, we studied 11 paired biopsies at day 14 after differentiation. First, we revealed that the area of lipid-filled cytoplasm in visceral precursor cells was significantly larger compared with subcutaneous cells [2,424 (103) vs. 1,947 (82) μm^2; $P < 0.001$] (**Fig. 3A**). More interesting were the differences in lipid droplet number and size, with visceral fat cells developing a larger number of triglyceride vesicles [107 (5) vs. 42 (3); $P < 0.001$] (Fig. 3B), albeit smaller in volume [77 (7) vs. 451 (85) μm^3; $P < 0.001$] (Fig. 3C), compared with cells of subcutaneous origin.

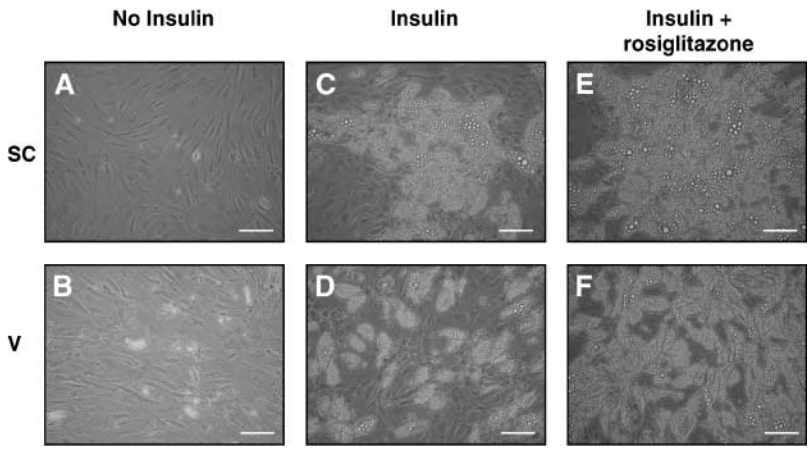

Fig. 2. Insulin and rosiglitazone promote differentiation. Phase contrast micrographs of subcutaneous (SC) and visceral (V) preadipocytes and adipocytes in primary culture after 14 days in adipogenic medium under varying conditions. A, B: Complete absence of insulin. C, D: 100 nM insulin. E, F: 100 nM insulin with 10 μM rosiglitazone added for the first 3 days of differentiation. Representative micrographs are shown. Bars = 100 μm.

GPDH and ORO levels increase with differentiation

To quantify the degree of differentiation between the subcutaneous and visceral precursor cells, we analyzed the cultures for GPDH activity and ORO staining of the accumulated triglyceride, both known to be increased in the adipocyte phenotype. GPDH activity was minimal at day 0 in both cell types; however, after 14 days of differentiation, there was a significant increase vs. day 0 controls in subcutaneous [650 (195) vs. 11 (3) mU/mg/ml; $P = 0.008$] and a borderline significant increase in visceral [781 (363) vs. 14 (3) mU/mg/ml; $P = 0.059$] adipocytes (**Fig. 4A**). Further analysis using a nonparametric Wilcoxon matched pairs signed rank test revealed that GPDH activity at day 14 was significantly different from day 0 controls in both fat cell types ($P = 0.004$). Spectrophotometric analyses of ORO content within the subcutaneous and visceral preadipocytes at day 14 after differentiation vs. day 0 controls confirmed the GPDH data [0.55 (0.26) vs. 0.16 (0.01) optical density; $P = 0.028$; and 0.34 (0.09) vs. 0.21 (0.03) optical density; $P = 0.046$] (Fig. 4B). Although GPDH activity and triglyceride storage both accumulated after 14 days of differentiation, once again, and despite differences in lipid size and number, the degree of differentiation did not differ significantly between the subcutaneous and visceral preadipocytes at passages 3–5. The data were also analyzed for gender; no statistical differences between male and female subjects were found (P = NS).

TABLE 1. Triglyceride net deposition and storage in subcutaneous and visceral preadipocytes

	Triglyceride		
Tissue Type	No Treatment	Insulin	Insulin plus Rosiglitazone
		μg/ml	
Subcutaneous	250 (46)	750 (57)	2,086 (535)
Visceral	280 (55)	423 (86)	1,060 (385)

Subcutaneous and visceral preadipocytes were differentiated for 14 days, and triglyceride deposition and storage were monitored. Treatments to the serum-free media include complete absence of insulin, addition of 100 nM insulin, or addition of 100 nM insulin in combination with 10 μM rosiglitazone. There was borderline significance between no treatment vs. insulin ($P = 0.055$) and significant difference between control vs. insulin/rosiglitazone ($P = 0.004$) and between insulin vs. insulin/rosiglitazone ($P = 0.033$). The data represent means ± SEM of duplicate cultures from three individual biopsies. Statistical analysis was performed on the log-transformed data using three-way ANOVA and a stringent Scheffe multiple comparison test.

Adiponectin secretion increases during differentiation in paired biopsies of subcutaneous and visceral adipose tissue

Adiponectin secretion, known to be increased in differentiated adipocytes (21), was analyzed as a further means of characterizing and validating our cell culture model. Conditioned media accumulated over the last 4 days of differentiation were collected and analyzed by ELISA, and the results obtained were corrected for GPDH activity. Adiponectin levels were undetectable in nondifferentiated preadipocytes; however, after differentiation, adiponectin secretion was detected, and although levels were generally higher from cells of subcutaneous origin [median (range) 38.3 (22.2–116.9) vs. visceral 26.5 (2.1–78.8) ng/mU GPDH], this did not reach significance (P = NS). To consolidate these findings, we analyzed total adiponectin protein levels in children's mature adipose tissue after 24 h in culture medium. There was a propensity for increased adiponectin protein production in mature subcutaneous vs. visceral adipocytes [5,158 (998–13,011) vs. 3,442 (1,108–6,462) μg/mg protein; $P = 0.08$].

PPARγ expression increases and cytoskeletal remodeling occurs during adipogenesis

To further characterize our primary cell culture model during the differentiation process, we used specific antibodies to PPARγ, which is highly specific for adipose tissue

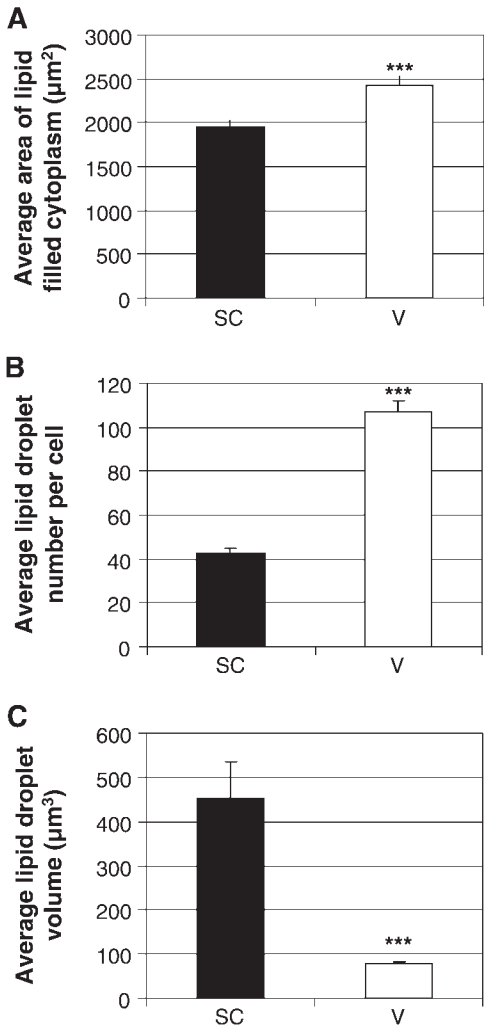

Fig. 3. Net lipid deposition in subcutaneous (SC) and visceral (V) preadipocytes after differentiation. At confluency, cells were subjected to differentiation media for 14 days and the net lipid deposition was monitored by Oil Red O (ORO) staining and analyzed using Image Pro Plus software. Areas of lipid-filled cytoplasm (A), lipid droplet number (B), and lipid droplet volume (C) were compared. All differences were highly significant. The data represent means ± SEM from 11 paired biopsies (n = 66 cells counted for area and lipid droplet number, and n = 660 droplets for lipid droplet volume for each cell type). Statistical analysis was performed using Student's paired *t*-test (*** $P < 0.001$).

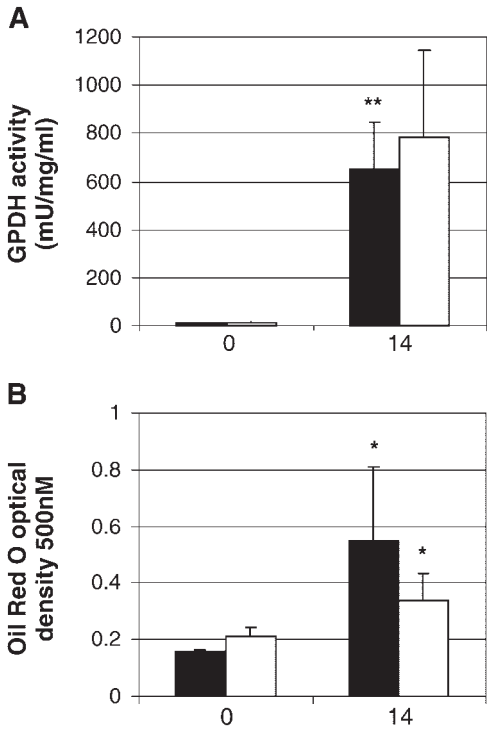

Fig. 4. Glycerol-3-phosphate dehydrogenase (GPDH) and ORO are markers of differentiation. A: GPDH activity was determined from day 0 and day 14 postdifferentiated cell lysates of subcutaneous (closed bars) and visceral (open bars) preadipocytes. Both cell types were comparable at days 0 and 14. When analyzed vs. their respective day 0 controls, a statistical difference was found in the subcutaneous cells ($P = 0.008$) and borderline significance was found in the visceral cells ($P = 0.059$). B: Lipid content of the cells was determined by ORO staining and spectrophotometric analysis. The differences between subcutaneous and visceral cells were not significant at day 0 or 14 ($P = 0.50$), but a statistical difference vs. their respective day 0 controls was revealed ($P = 0.028$ and $P = 0.046$, respectively). The data represent means ± SEM of duplicate cultures from 12 paired biopsies (GPDH) and 6 paired biopsies (ORO). Statistical analysis was performed using Student's paired *t*-test and a nonparametric Wilcoxon matched pairs signed rank test (* $P < 0.05$, ** $P < 0.01$).

and known to be upregulated during adipogenesis (22). Although undetectable in the preadipocytes (data not shown), PPARγ expression increased after 14 days of differentiation and, as expected, was localized in the nuclear region of every cell displaying the adipocyte phenotype (data not shown). There were no obvious visual differences in staining between subcutaneous and visceral adipocytes.

In addition to PPARγ, we also looked at F-actin cytoskeletal reorganization, which has previously been shown to be decreased by as much as 90% upon differentiation in 3T3-L1 adipocytes (23), suggesting that biosynthetic events specific to preadipocyte differentiation may be influenced by alterations in the cytoskeleton or vice versa. To ascertain whether actin remodeling occurred within the two fat depots, both subcutaneous and visceral preadipocytes were differentiated and assessed over time using Phalloidin and Nile Red staining. Before differentiation, the preadipocytes primarily displayed long organized stress fibers of F-actin spanning the entire length of the cell, which extended into lamellipodia- and filopodia-like extensions (**Fig. 5A, B**). After 24 h of differentiation, the actin filaments (in committed cells) appeared to polymerize and coalesce with the plasma membrane (Fig. 5C, D). After an increase in lipid abundance at day 3, the filaments were markedly decreased (Fig. 5E, F), and they were undetectable above the Nile Red staining in fully differentiated cells at days 7–14 (Fig. 5G–J). Comparable remodeling was also observed in the microtubules stained with α-tubulin, appearing as sheet-like morphology in the preadipocytes but colocalizing around the lipid droplet vesicles after adipogenesis (data not shown).

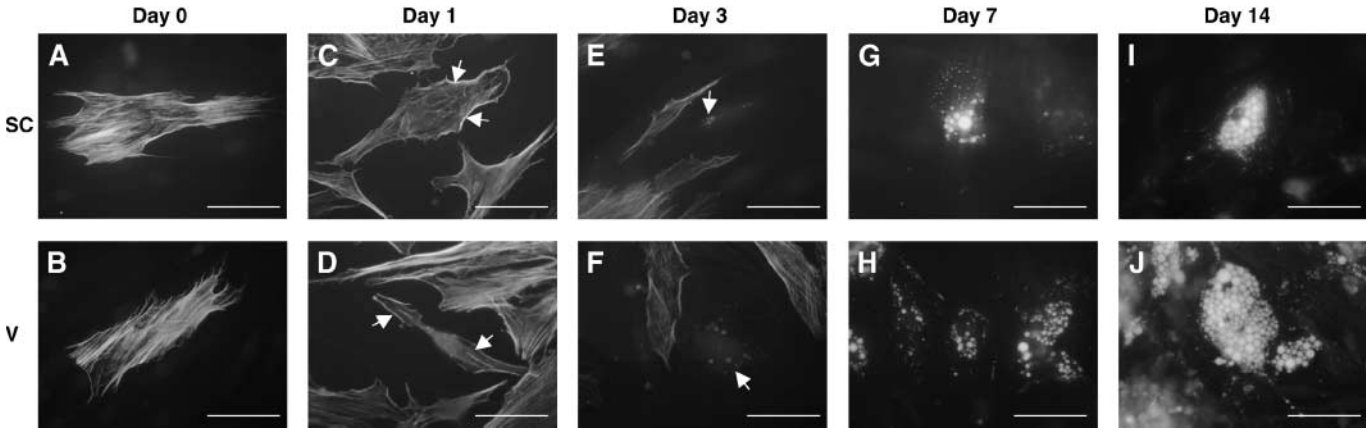

Fig. 5. Cytoskeletal remodeling during the differentiation process: F-actin. Phalloidin and Nile Red immunocytochemistry showing F-actin reorganization and lipid deposition/storage over time: day 0 (A, B), day 1 (C, D), day 3 (E, F), day 7 (G, H), and day 14 (I, J) in differentiating subcutaneous and visceral preadipocytes. Arrows indicate F-actin reorganization at day 1 (C, D) and complete remodeling by day 3 upon the onset of lipid accumulation in committed cells (E, F). Representative micrographs are shown. Bars = 50 μm.

Effect of TNF-α on differentiation

Having developed and characterized our models, we next wished to investigate whether differentiation could be manipulated using various physiologically relevant cytokines and growth factors implicated in the cause of obesity and insulin resistance and whether there were site-specific differences in the capacity of the two depots to respond. TNF-α has been shown to inhibit the differentiation of 3T3-F442A (24) and adult human adipocyte precursor cells (25); therefore, we were particularly interested in whether this cytokine had differential effects in our cultures of subcutaneous and visceral preadipocytes. After 14 days of differentiation, accumulated triglyceride was stained with ORO in subcutaneous and visceral cells. Exposure to a subapoptotic dose (20 ng/ml) of TNF-α blocked lipid accumulation in subcutaneous cells, with visceral cells showing a slightly reduced response toward this cytokine (**Fig. 6A**). These initial observations were substantiated with a dose response to TNF-α (0, 5, 10, and 20 ng/ml). In agreement with the ORO data, GPDH activity was significantly reduced even at the lowest dose tested, with differentiated subcutaneous preadipocytes being more responsive [0 (0), −86.0 (3), −88.6 (2), and −88.6 (2)% change over control vs. 0 (0), −34.8 (12), −41.2 (2), and −46.4 (8)% change over control; $P = 0.04$, $P = 0.001$, and $P = 0.01$ at 5, 10, and 20 ng/ml TNF, respectively] (Fig. 6B). The decrease in triglyceride levels in the presence of TNF mirrored that seen for GPDH in subcutaneous cells [0 (0), −57.3 (7), −57.5 (18), and −59.7 (17)% change over control], but a slightly different pattern emerged in visceral cells (Fig. 6C). The latter were less responsive to TNF at 5 ng/ml but showed increased responsiveness as the doses increased, with similar suppression of triglyceride accumulation being evident at 20 ng/ml [0 (0), −3.3 (15), −25.2 (16), and −48.1 (4)% change over control; $P = 0.02$ at 5 ng/ml TNF] (Fig. 6C). Statistical analysis by three-way ANOVA revealed a significant dose effect ($P < 0.001$), a significant site effect ($P < 0.001$), and a significant interaction between site and dose ($P = 0.02$) for GPDH and only a significant dose effect ($P = 0.007$) for triglyceride measurements.

Effect of IGF-I, LongR^3 IGF-I, and IGFBP-3 on differentiation

The stimulatory role of IGF-I in adipogenesis in vitro has been previously shown in 3T3-L1 cells (26) and in primary adipocyte cultures of pig, rat, and rabbit (12, 27, 28). To begin to define the role of the IGF system in our model, we examined the effects of IGF-I along with LongR^3 IGF-I on the differentiation process. IGF-I (200 ng/ml) markedly enhanced the differentiation of both subcutaneous [33.6 (3)%; $P = 0.009$] and visceral fat cells [170 (54)%; $P = 0.008$] vs. untreated controls (**Fig. 7A**). However, the visceral preadipocytes had an increased response to this growth factor, suggesting that differences may lie either at the receptor level or in variations in binding protein secretion between these two fat depots. To evaluate whether endogenous binding proteins altered the action of IGF-I in our cultures, we used LongR^3 IGF-I (200 ng/ml), which has insignificant affinity toward the IGFBPs and hence can be used to show the effects of IGF-I independent of IGFBP status. Data obtained were equivalent to those derived with IGF-I [4.0 (32) vs. 189 (19)% increase above control; $P = 0.01$] in subcutaneous and visceral cells (Fig. 7B). These findings suggest that endogenous binding proteins have little or no impact on the effects of exogenous IGF-I in these cultures, and although remaining to be substantiated, they suggest that differences lie predominantly at the receptor or postreceptor level. Western ligand blotting analyses of conditioned media confirmed the inference regarding no IGFBP differences, showing undetectable levels of binding protein secretion from both fat cell depots (data not shown). Having determined that little or no IGFBPs were being secreted, we had a very clean model to ascertain the impact of exogenous IGFBPs on adipose cell differentiation. We therefore next wished to investigate the effect of exogenous IGFBP-3 on adipogenesis. IGFBP-3 (200 ng/ml) significantly enhanced the

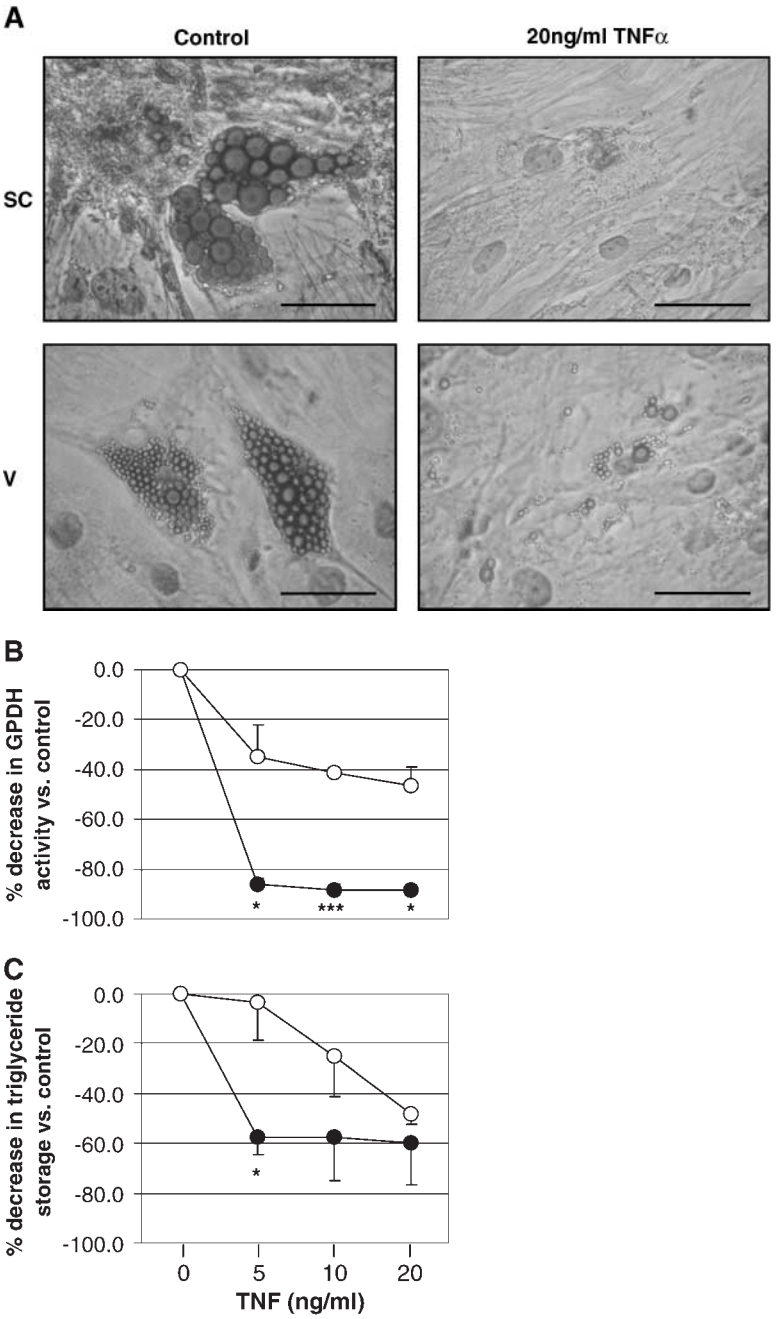

Fig. 6. Tumor necrosis factor-α (TNF-α) inhibits lipid droplet formation, GPDH activity, and triglyceride net deposition. A: Subcutaneous (SC) and visceral (V) preadipocytes were differentiated for 14 days in the absence or presence of 20 ng/ml TNF-α. Representative micrographs are shown after ORO staining. Bars = 50 μm. GPDH activity (B) and triglyceride net deposition (C) were measured in cell lysates from differentiated subcutaneous (closed circles) and visceral (open circles) preadipocytes in the absence or presence of TNF-α at 5, 10, and 20 ng/ml. The data represent means ± SEM of duplicate cultures from five individual biopsies. Statistical analysis was performed using Student's paired *t*-test (* $P < 0.05$, *** $P < 0.001$).

differentiation capacity of both subcutaneous and visceral cell culture models over controls [77.3 (41) vs. 94 (47)%; $P = 0.05$] (Fig. 7C). However, no significant differences were seen between the fat depots. Importantly, although the increase in differentiation was indistinguishable between the two depots, cells of subcutaneous origin were more responsive to IGFBP-3 than to either IGF-I or LongR^3 IGF-I, whereas the converse was true for cells of visceral origin.

DISCUSSION

To our knowledge, this is the first study to develop, comprehensively characterize, differentiate, and manipulate subcutaneous and visceral preadipocytes from prepubertal children, providing a clinically relevant model for the study of adipogenesis and insulin action in fat from children.

This model poses considerable practical problems. Visceral fat is not easily obtained from children, as there are only small quantities present within this age group and there are few indications for open abdominal surgery in nonseptic, nonmalignant conditions in childhood. Therefore, we have used perinephric fat, which although visceral in nature has described limitations (16). It is, however, more available during frequently performed elective operations in children. Biopsy size is, of necessity, very small (0.2–0.5 g) compared with other models (10–20 g) (19, 29). This 20- to 100-fold decrease in biopsy size yields

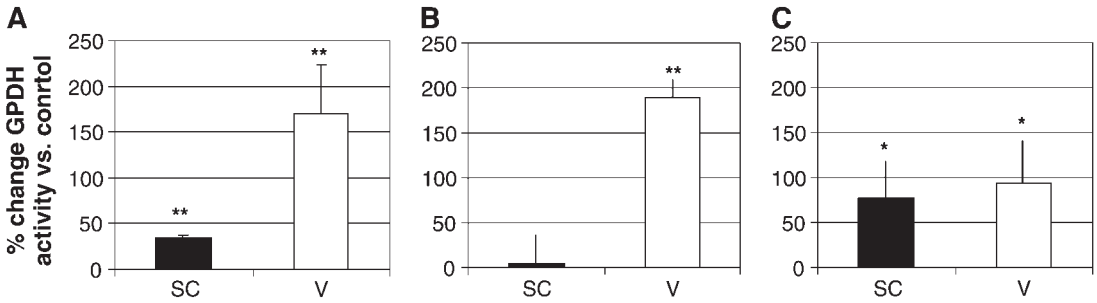

Fig. 7. Effect of insulin-like growth factor-I (IGF-I), LongR^3 IGF-I, and insulin-like growth factor binding protein-3 (IGFBP-3) on differentiation. Subcutaneous (SC) and visceral (V) preadipocytes were differentiated for 14 days in the absence or presence of 200 ng/ml IGF-I (A), 200 ng/ml LongR^3 IGF-I (B), and 200 ng/ml IGFBP-3 (C). Cell lysates were analyzed for GPDH activity and are expressed as percentage change vs. untreated controls. The data represent means ± SEM of duplicate cultures from three individual biopsies. Statistical analysis was performed using Student's paired *t*-test on day 14 lysates vs. untreated controls (* $P < 0.05$, ** $P < 0.01$).

decreased preadipocyte cell numbers after isolation. As inoculation density is reported as a critical parameter for the successful differentiation of cultured fat cells (15, 27), this posed a potential problem. In our model, high seeding densities were not feasible without subculturing, but previous reports have suggested that subculturing leads to a dramatic reduction, related to donor age, in adipose conversion (15). However, we were successful not only in passaging but also in differentiating our cultures after passage, which may have been possible because of the young age of all our subjects (30).

The isolated subcutaneous and visceral preadipocytes were differentiated using a chemically defined serum-free culture medium (14, 15), offering the advantage of studying adipogenic and antiadipogenic agents under controlled conditions without the complications of serum factors that are highly adipogenic. We established not only that insulin was essential for differentiation but that rosiglitazone, which alone was mildly adipogenic, when in combination with insulin markedly enhanced adipogenic potential. Thiazolidinedione treatment has been shown to enhance fat accumulation in adults both in vitro (31) and in vivo (32), through increased insulin-stimulated phosphatidylinositol 3-kinase activity (33), and/or PPARγ activation (34). No differences between the two fat depots were observed in the prodifferentiating effects of rosiglitazone in our cultures. Some studies report significantly greater PPARγ2 expression in subcutaneous compared with omental preadipocytes in response to rosiglitazone, resulting in a greater level of differentiation in the former (31), and significantly greater PPARγ mRNA levels are reported in subcutaneous adipose tissue from subjects with a BMI of <30 kg/m^2 (35). These studies were performed in cells of adult origin and cannot necessarily be extrapolated to our cells.

A time course of differentiation demonstrated similar patterns of lipid accumulation in both pediatric cell models, with tiny lipid inclusions evident within the cytoplasm at 24 h that were visible by days 3–4 of differentiation. In adult studies, lipid droplet formation begins at approximately day 6–9 (36) or even as late as days 10–15 (14). Because no exogenous lipids were present in our differentiation medium, the young age of our study group and the use of rosiglitazone may have contributed to this earlier onset of lipid deposition. There are currently no data concerning thiazolidinedione-regulated gene expression in cultures from children, and models such as ours are required to address this and other reported differences in differentiation of adult cell models.

Histological analyses revealed differences in lipid droplet size and number, with subcutaneous preadipocytes containing fewer vesicles of larger volume. Significant differences in metabolic action have been identified in fat from different sites, and in adults, different rates of triglyceride net storage between the two fat depots might occur as a result of their differences in hormone-sensitive lipase or lipoprotein lipase activity (16). The larger fat droplets in our subcutaneous cells may occur as a consequence of their reported increased sensitivity to the antilipolytic effect of insulin compared with visceral fat cells (37); alternatively, glucocorticoids present in the adipogenic medium may exert preferential activity on subcutaneous adipocyte precursors (38). Further studies are needed to examine these parameters. Further histological examinations demonstrated that at a cytoskeletal level, both fat cell compartments undergo similar structural reorganization during adipogenesis**.** The remodeling of de novo actin polymerization has been shown, using actin-depolymerizing agents such as cytochalasin D, to play an important role in insulin-dependent GLUT4 translocation (39), and the reorganization of F-actin filaments in our cultures has displayed the typical changes seen in 3T3-L1 cells (40).

Biochemical characterization of GPDH activity [a late marker of terminal differentiation (29) reported to be restricted to lipid-containing cells both in human (14) and rodent models (41)] confirmed differentiation of the cells from both sites. In agreement with adult cultures (19), there were no overt differences in GPDH activity between the two sites. These data were also supported using ORO staining techniques, with low levels of ORO staining at day 0 (which could represent background lipid content such as unesterified fatty acids) increasing significantly at day 14. The differences between the two fat regions were not significant.

Adiponectin, an adipose tissue-specific secretory protein with novel insulin-sensitizing and antiatherogenic properties, is inversely related to the degree of visceral adiposity in adults and children (42). Studies to date have focused largely on circulating levels of this adipocytokine or its secretion from mature adipocytes in culture (43) and not from differentiating preadipocytes. We show here that although undetected in preadipocytes, adiponectin secretion increased with differentiation and was more abundant in cells derived from subcutaneous adipose tissue in five out of six individuals. To relate our findings more closely to the in vivo setting, we also investigated total adiponectin levels in mature adipocytes after 24 h in culture after the initial isolation. Comparable results were found with subcutaneous fat cells displaying an increase in adiponectin protein levels when paired against their visceral counterparts. Similar findings have previously been shown by quantitative PCR and Western analysis in samples from nondiabetic adult subjects (44).

Having characterized and differentiated our cultures, we wished to manipulate their adipogenic capacity using TNF-α, which is known to be involved in obesity-related insulin resistance (45) and was previously shown to inhibit the differentiation of adult preadipocyte cultures derived from mammary adipose tissue (25). We demonstrate for the first time that TNF-α blocks the differentiation of both subcutaneous and visceral precursor cells from children, as shown by the retention of their fibroblastic morphology with minimal evidence of lipid accumulation. Interestingly, when cells were exposed to increasing concentrations of TNF-α, the visceral preadipocytes showed a reduced susceptibility toward this cytokine. The effect seen may reflect differences in TNFR1 expression between the two fat depots. Hube et al. (46) have shown a significantly higher expression of TNFR1 in subcutaneous adipose tissue, whereas Xu, Sethi, and Hotamisligil (24) have demonstrated that overexpression of TNFR1 is sufficient to mediate increased inhibition of adipogenesis. Furthermore, TNF-α has been shown to affect the initial stages of differentiation by downregulating adipocyte-specific genes required for adipogenesis, thus inhibiting the expansion of adipose tissue mass by preventing the formation of newly developing fat cells (47, 48). This warrants further investigation in our primary cell culture model, as the ability of TNF-α to inhibit differentiation may have profound physiological implications in disease states as varied as cachexia and insulin resistance.

IGF-I has been shown to enhance the differentiation capacity of adipocytes in primary culture (12, 27). Changes in systemic levels of the growth hormone-IGF axis are well recognized in obesity, with reduced growth hormone but normal or increased circulating IGF-I being reported (49). Growth hormone, nutrition, and the IGFBPs determine the circulating levels of IGF-I and its tissue availability. There are conflicting data regarding IGFBP-3 concentrations in obesity, with levels shown to be either normal or increased (50, 51). We therefore investigated whether regional differences existed in the effects of IGF-I and IGFBP-3 on adipogenesis. We have found that IGF-I upregulated the adipogenic potential of our cells above untreated controls and that this occurred more readily in cells derived from the visceral fat depots. Although not determined in our model, differences may exist in IGF-I receptor abundance or in insulin/IGF-I hybrid receptor distribution, which may lead to differences in overall adipose tissue sensitivity (52). Furthermore, we and others (53) have shown that visceral preadipocytes have a lower proliferation rate compared with subcutaneous cells, and exposure to IGF-I may increase their mitogenic potential during the earlier stages of differentiation, thus leading to an increase in frequency of committed cells toward the differentiation process.

We anticipated that exogenous IGFBP-3 might inhibit differentiation, as previously suggested (54). To our surprise, IGFBP-3 enhanced adipogenesis equally in both cell types, but it is not clear whether this was via an effect on IGF-I availability or via a different mechanism (e.g., mitogen-activated protein kinase), and this warrants further investigation. That IGFBP-3 had a greater effect on subcutaneous adipocyte differentiation compared with IGF-I implies an IGF-independent mode of action.

In conclusion, we have developed novel methodologies to isolate, culture, and differentiate preadipocytes from very small fat tissue biopsies obtainable from children at elective surgery. This method provides a physiologically relevant model to investigate adipogenesis and insulin action in adipose tissue from different anatomical sites in children and may allow us to explore the evolution of regional differences in adipose tissue accumulation and metabolism, which has major implications for understanding the mechanisms leading to central adiposity and insulin resistance in obesity and type 2 diabetes.

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