Published in final edited form as:

Ann Plast Surg. 2008 May ; 60(5): 538–544. doi:10.1097/SAP.0b013e3181723bbe.

Regional Anatomic and Age Effects on Cell Function of Human Adipose-Derived Stem Cells

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Abstract

Adipose tissue has been shown to contain adult mesenchymal stem cells that have therapeutic applications in regenerative medicine. There is evidence that the ability of adipose precursor cells to grow and differentiate varies among fat depots and changes with age. Defining these variations in cell function and molecular mechanisms of adipogenesis will facilitate the development of cell-based therapies. We compared cells harvested from 5 different subcutaneous (SC) adipose depots in 12 female patients classified into 3 age ranges (25–30, 40–45, and 55–60 years old). Capacity for differentiation of isolated adipose-derived stem cells (ASCs) with and without ciglitazone, a strong peroxisome proliferator-activated receptors (PPAR)- γ agonist, was assessed in vitro. ASCs were also characterized by lipolytic function, proliferation, and sensitivity to apoptosis.

Additionally, PPAR- γ -2 protein expression was determined. We observed a difference in the apoptotic susceptibility of ASCs from various SC depots, with the superficial abdominal depot (above Scarpas layer) significantly more resistant to apoptosis when compared with the 4 other depots. We have also demonstrated that a PPAR- γ agonist aids in the induction of differentiation in cells from all depots and ages. Although sensitivity to apoptosis was linked to anatomic depot, differences in cell proliferation were related primarily to age. Stimulated free glycerol release has been shown to be highest in the arm depot. The arm depot has also consistently shown expression of PPAR- γ -2 with and without a PPAR- γ agonist. Younger patients have increased PPAR- γ -2 expression in all depots, whereas the older patients have consistent elevated expression only in the arm and thigh depots. We have shown there is variability in function of ASCs that have been harvested from different SC depots. Additionally, we have shown age-related changes in function. These data will help select patients and cell harvest sites most suitable for tissue engineering therapies.

Keywords

adipose stem cells; adipose tissue; PPAR-gamma; tissue engineering

Adipose-derived stem cells (ASCs) have great potential in the field of tissue engineering. ASCs have been used in addition with various scaffolds and growth factors to engineer adipose tissue. ASCs have most commonly been harvested from the abdominal depot because of its abundance of adipose tissue in most patients. The human body can be segmented into various depots of subcutaneous (SC) adipose tissue based on anatomic location. These depots each vary in their amount of adipose tissue and may produce stem cells with different characteristics.

Prior studies have shown that the anatomic location of adipose tissue has an impact on its metabolic function.¹ This anatomic distribution has also been shown to play a role in metabolic disorders that include glucose intolerance, hyperinsulinemia, dyslipidemia, and hypertension.^{2,3} For example, visceral or omental adipocytes are more resistant to the antilipolytic effects of insulin than SC adipocytes, but are more sensitive to the stimulation of lipolysis by catecholamines.⁴ It has been shown through loss of function studies that peroxisome proliferator-activated receptors (PPAR)- γ is required for adipogenesis both in vivo and in vitro.⁵ Thiazolidinediones (TZDs) act by binding to PPARs, a group of receptor molecules inside the cell nucleus, specifically PPAR- γ . Previous studies have compared PPAR- γ activity, apoptotic susceptibility, and effects of TZDs on adipose differentiation of ASCs isolated from the abdominal SC, mesenteric, and omental tissues.⁶ Tchkonian et al^{6a} found that preadipocytes isolated from the SC depot demonstrate the highest PPAR- γ activity, the least amount of apoptosis, and the greatest effect of TZDs on differentiation. This would imply that the SC tissue produces a stem cell that may be more suited to differentiate into a mature adipocyte than the omental or mesenteric depots. These differences, however, have not been elucidated for the different SC regions. We must recognize variations in SC depots by studying their behavior.

In addition to depot-related variability, we also examined age as a variable and determined its effect on various cell characteristics. By identifying the characteristics of ASCs from various SC depots and age ranges, we may be able to identify a depot that may yield the ideal ASCs for adipose tissue engineering, as well as understand biologic differences of adipose tissue from different regions of the body.

METHODS

Human Subjects

Fat tissue was harvested during elective body contouring procedures in 12 patients who had given informed consent. The body mass index (BMI) of the patients varied from 24 to 28 kg/m². The protocol was approved by the University of Pittsburgh institutional review board for human research. There were a total of 12 patients included in the study. All subjects were female. Patients were classified into 3 age ranges: 25 to 30 (3 patients), 40 to 45 (4 patients), and 55 to 60 (5 patients). The depots included upper arm, medial thigh,

trochanteric, and both superficial and deep abdominal. The anatomic boundary of superficial and deep abdominal fat was Scarpas fascia.

ASC Culture

Fat tissue was placed in sterile conical tubes. A collagenase solution composed of type 2 collagenase (0.1%), with bovine serum albumin (3.5%) in 1× Hanks solution was sterile filtered, then added at a 3:1 ratio of solution to adipose tissue for digestion. This solution was then filtered through double layer gauze, to remove unprocessed debris. The resulting filtered solution was centrifuged at 10,000 rpm for 10 minutes at 20°C. Supernatant was removed and the pellet was resuspended in 10 mL of erythrocyte lysis buffer. Erythrocyte lysis buffer is composed of 154 mM NH₄Cl, 10 mM KHCO₃, <1 mM ethylenediaminetetraacetic acid in water, and sterile filtered. Resuspended pellets were vortexed to lyse red blood cells and debris, and then recentrifuged at 10,000 rpm for 10 minutes at 20°C. The supernatant was then removed and the pellet resuspended in 10 mL of human regular media. Human regular media is composed of DMEM and DMEM F12 (Dulbecco modified eagle medium from Gibco) at a 1:1 ratio, 10% fetal bovine serum, 0.1 mM penicillin, 0.06 mM streptomycin, 0.1 mM dexamethasone, and gentamycin sulfate (10 mg/L). After 6 hours, the ASCs are adherent to the plates and then washed extensively with phosphate-buffered saline (PBS) to remove any contaminants. Plating media was changed 3 times a week. This was continued until the ASCs reached approximately 80% confluence.

Measurement of Proliferation

Approximately 2500 cells from each depot were seeded in triplicate into 24-well plates. After 48 and 96 hours, the plates were then frozen at –80°C for a minimum of 24 hours. The plates were then thawed to room temperature and a CyQUANT cell proliferation assay kit (Cat C35007, Molecular Probes, Eugene, OR) was used to quantify cell number. Fluorescence was measured at 480 nm excitation and 520 nm emission (Tecan SpectraFluor, Tecan, Inc, Research Triangle Park, NC). A standard curve was created and cell number obtained.

Apoptosis Susceptibility

Approximately 30,000 cells from each depot were seeded in triplicate in a 24-well plate. After 24 hours, the media is removed and a solution of 1.0 µM staurosporine (Cat81590, Cayman Chemical Company, Ann Arbor, MI) in plating media is added to each well. A cell death detection enzyme-linked immunosorbent assay-plus kit (Cat 11920685001 Roche, Indianapolis, IN) was analyzed 24 hours after addition of the staurosporine. This enzyme-linked immunosorbent assay is a photometric enzyme immunoassay that is based on a quantitative sandwich enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates. The absorbance is measured at 405 nm and the susceptibility to the apoptotic agent is determined.

Lipolysis Assay

Approximately 20,000 cells from each depot were seeded in triplicate in a 12-well plate. After the cells had reached 100% confluency, they were placed in adipogenic media. The adipogenic media is composed of DMEM/F12 with 33 μ M biotin, 0.5 μ M insulin, 17 μ M D-pantothenic acid, 0.2 nM dexamethasone, 1 μ M ciglitazone, 0.2 nM T3, and 10 mg/L transferrin. For the first 2 days of treatment in adipogenic media, 3-isobutyl-1-methylxanthine (540 μ M) is added to help promote differentiation. ASCs were treated with adipogenic media for 2 weeks. The media was changed 3 times a week for the first week, but left unchanged for the second week. After the 2-week incubation all media is removed and the cells are washed with PBS. A cultured human adipocyte lipolysis assay kit (Cat LIP-1-NC, Zen Bio, Research Triangle Park, NC) was then used to determine free glycerol released after induction of lipolysis. The cells are first treated with a solution containing assay buffer and 1.0 μ M isoproterenol to induce lipolysis. After 8 hours, the absorbance is measured at 540 nm. A standard curve is created and the amount of free glycerol determined.

Lipid Accumulation

Approximately 20,000 cells were placed in triplicate in a 24-well plate and grown to 100% confluence. Plating media was changed 3 times a week. Once confluent, the media was changed to the adipogenic media. One treatment group had ciglitazone and one treatment group did not have ciglitazone added to the media. The cells were kept in this media for a total of 2 weeks with media changes 3 times a week. At the end of the 2 weeks the cells were stained with Oil red O. The cells are first fixed with 10% buffered formalin and then stained. All Oil Red O not contained in the lipid vacuoles was washed away with PBS. For quantification of Oil Red O uptake by the lipids, 1 mL of 2-propanol was added to each well and the absorbance of the sample is measured at 510 nm.

PPAR- γ -2 Protein Isolation

Cells were plated in triplicate in 10 cm petri dishes and grown to 100% confluence in plating media, and cells were either treated with adipogenic media containing ciglitazone, or adipogenic media without ciglitazone.

PPAR- γ -2 protein was then isolated at 7 and 14 days for each group. To isolate the protein, cells were washed with PBS 3 $\times$ to remove any media. Three hundred microliters of a solution containing protease inhibitor and M-PER mammalian protein extraction reagent (Pierce) was then added. The cells were scraped from the dish and collected. The samples were centrifuged at 14,000g for 10 minutes, the supernatant was collected and stored at -80°C .

Western Blot

Isolated protein was quantified using a BCA Protein assay kit (Cat 23225, Pierce, Rockford, IL). Fifteen micrograms of PPAR- γ -2 protein were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis fractionation, blotted onto nitrocellulose membrane, and probed with PPAR- γ -2 primary antibody (Cat MAB3872, Millipore, Billerica, MA).

Immunoreactivity was visualized using an enhanced chemiluminescence kit (Cat RPN2109, ECL, Amersham Corp, Arlington Heights, IL).

Statistical Analysis

Repeated measure analysis of variance was used to evaluate the depot and age range effect on proliferation, apoptosis, lipolysis, and lipid accumulation. We initiated the model with 5 depots (superficial abdominal, deep abdominal, thigh, arm, and trochanteric), 3 age groups (25–30, 40–45, and 55–60). Where the patient was treated as a random effect with unstructured covariance as was the case for lipid accumulation, we considered ciglitazone as a fixed effect. Tukey's adjustment for multiple comparisons was used for post hoc testing on all pair-wise comparison between depots. For each depot, one-way analysis of variance was also used to find the age group effect. The analyses were implemented in SAS using PROC MIXED. All statistical tests were 2-sided and conducted with an α level = 0.05.

RESULTS

Proliferation

There is no statistically significant evidence that depot ($F = 0.88$, $df = 4.9$, $P = 0.514$) or age group ($F = 0.43$, $df = 2.9$, $P = 0.660$) had an effect on proliferation. Though not statistically significant, the superficial abdominal depot showed a trend to proliferate the slowest over the other depots in all age ranges (Fig. 1A). Also, though not statistically significant, there was an age-related difference in proliferation found. The 20-year-old age range proliferated at a higher rate than the other 2 age groups (Fig. 1B).

Apoptosis

There is statistically significant evidence that there are variations among depot ($F = 5.66$, $df = 4.9$, $P = 0.015$) on apoptosis susceptibility. The superficial abdominal depot was statistically significantly less susceptible to an apoptotic stimulus when compared with the deep abdominal, arm, thigh, and trochanteric depot (Fig. 2A). There was however no age ($F = 1.58$, $df = 2.9$, $P = 0.258$) related differences in apoptosis susceptibility (Fig. 2B).

Lipolysis

There is significant evidence that both depot ($F = 21.23$, $df = 4.9$, $P = 0.0001$) and age ($F = 91.19$, $df = 2.9$, $P < 0.0001$) have an effect on lipolysis. We found because of the variable amount of differentiation in each depot, and hence the amount of lipid present that there was much variation in free lipid per patient samples. We did find the arm depot too consistently demonstrate more lipolytic activity than the other depots. To be able to compare the data we normalized all depot samples from a patient to the arm depot of that patient. We then found as before that the arm depot had statistically higher lipolytic activity than the other depots (Fig. 3A). When we stratified by age we found the 20-year-old age group to have statistically more lipolytic activity in all depots when compared with the older age groups (Fig. 3B).

Lipid Accumulation

Ciglitazone (a PPAR- γ agonist) was found to cause a statistically significant increase ($F = 10.79$, $df = 1.20$, $P = 0.004$) in lipid accumulation in all depots when compared with cultures without ciglitazone (Fig. 4A). There was no evidence to detect any depot related differences in lipid accumulation ($F = 1.66$, $df = 4.20$, $P = 0.199$). There was also an age-related statistically significant effect on lipid accumulation noted ($F = 8.83$, $df = 2.20$, $P = 0.002$). We found the 40-year-old age group had statistically more lipid accumulation than both the 50-year-old age group and the 20-year-old age group (Fig. 4B).

PPAR- γ Expression

Protein samples were collected that were differentiated in enriched media with and without ciglitazone. PPAR- γ -2 was found to be highly expressed in all depots of the 20-year-old age group with ciglitazone at both 7 and 14 days, and only showed elevated levels in the arm and thigh depot without the presence of ciglitazone. In the 40-year-old age group PPAR- γ -2 was found to be highly expressed in all depots with ciglitazone at both 7 and 14 days, and the only depots to show elevated levels at 14 days without ciglitazone were the arm and superficial abdominal depot. In the 50-year-old age group all depots showed elevated expression of PPAR- γ -2 at 14 days with ciglitazone. All depots of all age groups strongly expressed PPAR- γ -2 in the presence of ciglitazone. However, the only depot to express PPAR- γ -2 without ciglitazone across all age groups was the arm depot (Fig. 5). In some samples there were multiple bands seen at the level of PPAR- γ -2. This was caused by phosphorylation of the samples and does not change the ability to detect the protein. They are merely artifacts of aging protein samples.

DISCUSSION

The aims of this study were to delineate differences in ASC biology from 5 different SC anatomic locations, and to determine the effect of age on cell function. These differences may help explain the variability in adipose tissue function in various anatomic locations as well as to identify a population of cells that may be suitable for soft-tissue engineering applications. The adherent cell population that is left after our isolation population is a heterogeneous population. Flow cytometry data from our laboratory has shown there to be 4 subpopulations of adipose stem cells within this final adipose stem cell pool. The first is a CD 31⁺, 34⁻ population that we classify as mature endothelial. This group has the endothelial marker of CD 31, but lacks the progenitor marker of CD 34. The second group is 31⁺, 34⁺ that is classified as endothelial stem. This group contains both the progenitor CD 34 and the endothelial CD 31. The next group is 31⁻, 34[±]. This group is classified as the adipose stem cell group. Our final group is CD 146⁺, 90⁺, 31⁻, 34⁻. This group is referred to as the pericyte group. Some investigators feel the origin of the adipose stem cell is from the endothelial tissue within the adipose tissue. CD 146 is a marker for pericytes. In this current study we use all the populations as a mixed group for our work. In future studies from our group we will try and delineate differences in both an in vitro and in vivo setting based on cells in these different sorted groups.

Our data from this current work suggest that there are both depot-dependent phenomena and age-related differences in the various functions studied. When examining cell proliferation rates, younger patients proliferated at a faster rate than the 2 older age groups. Apoptosis susceptibility was demonstrated to be lowest in the superficial abdominal depot and lowest in the younger age groups. This was not surprising as the superficial abdominal depot has been shown to be least susceptible to apoptosis when compared with adipose stem cells from the omental depot.⁶ The lipolysis data demonstrated variability in both age range and depot. The younger age range tended to have the highest activity in each SC depot, although there was variability between samples as seen by the large error bars. When lipolysis was compared between older and younger patients the activity dropped considerably in the older range group in 4 of 5 depots. Only the arm depot maintained high lipolytic activity, regardless of patient age.

In previous studies, abdominal SC preadipocytes demonstrated high adipogenic capacity, as indicated by lipid accumulation and PPAR- γ activity. The addition of TZD (PPAR- γ ligands) resulted in more extensive differentiation of human SC ASCs than omental ASCs.⁷ In our differentiation work with and without the addition of ciglitazone, we found no specific depot-dependent effect. However, the addition of ciglitazone did increase lipid accumulation in all depots when compared with lipid accumulation in samples not containing ciglitazone. When the data is stratified based on age, the 40- to 45-year-old age range has statistically increased adipogenesis when compared with the 25- to 30-year-old group and 55- to 60-year-old age group.

Others have shown that even though TZD such as ciglitazone can cause more extensive differentiation of SC ASCs than omental ASCs, PPAR- γ expression was not different between the depots. We examined this by assessing PPAR- γ -2 protein expression of the various depots at 7 and 14 days, with and without the presence of ciglitazone in the adipogenic media. Without ciglitazone, the only depot to consistently express PPAR- γ -2 at 14 days was the arm depot. In the presence of ciglitazone, however, nearly every depot of all age ranges expressed PPAR- γ -2. This indicates that the effect of ciglitazone is not only grossly evident with the effect of greater differentiation and lipid accumulation, but that this effect is also regulated at the protein expression level. This was expected as Rosen et al¹³ showed that PPAR- γ is required for adipogenesis in vitro as well as in vivo. Therefore, any stimulator of PPAR- γ expression such as ciglitazone should increase adipogenesis. The effect of TZDs such as ciglitazone to greatly increase SC lipid accumulation may further explain its effect as an antidiabetic agent.

Excess visceral adipose tissue in comparison with SC adipose tissue has been shown to produce glucose intolerance.⁸ TZDs have been shown to not only promote larger accumulations of SC fat over visceral adipose tissue, but also significantly increase lipid accumulation across all SC depots as we have demonstrated in this study.^{9,10} This increase in SC fat over visceral fat accumulation may potentially improve glucose tolerance.

The fat specimens evaluated in this study were removed from previously obese patients (BMI >30) undergoing elective body contouring procedures. Preadipocyte capacities for

replication and mitogenic protein release are increased in some massively obese patients compared with lean controls.^{11,12} In our study, the BMI range of the patients was 24 to 28.

In summary, ASCs have many qualities that render these cells well suited for tissue engineering applications. Most have some extensible adipose tissue that can be easily harvested in the operating room or at the bedside. The mesenchymal stem cells extracted from the adipose tissue have reliable cell culture behavior and have been shown to be multipotent.^{6a,13–15} This study may form an increased understanding of the tools needed to successfully engineer adipose tissue. We have shown that PPAR- γ -2 expression is highest in the arm SC tissue. However, in the presence of agonists such as ciglitazone, all SC depots studied express PPAR- γ , which has a direct correlation on adipogenesis. We have also demonstrated that ASCs from younger patients proliferate at a higher rate and are more successful at differentiating into mature adipocytes than are older patients. This may make younger patients more suitable for tissue engineering applications. Finally, as the superficial abdominal depot has been shown to be less susceptible to an apoptotic stimulant, this depot may produce a cell less likely to die of the variable in vivo milieu.

Acknowledgments

The authors acknowledge the excellent technical assistance from Daniel O'Donnell. The authors also acknowledge the NIH RO1CA114246-01A1 (J.P.R.) and the Plastic Surgery Educational Foundation.

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FIGURE 1.

A, Cell proliferation rate by depot at 48 and 96 hours. All age groups combined ($N = 12$ per depot at each time point) ($F = 0.88$, $df = 4.9$, $P = 0.514$). Error bars indicate standard deviation. B, Cell proliferation rate at 96 hours by age range. All depots combined (25–30 $N = 3$, 40–45 $N = 4$, 55–60 $N = 5$) ($F = 0.43$, $df = 2.9$, $P = 0.660$). Error bars indicate standard deviation.

FIGURE 2.

A, Susceptibility to apoptosis by depot. All age groups combined ($N = 12$ for each depot) ($F = 5.66$, $df = 4.9$, $P = 0.015$). Error bars indicate standard deviation. B, Susceptibility to apoptosis by age range. All depots combined (25–30 $N = 3$, 40–45 $N = 4$, 55–60 $N = 5$) ($F = 1.58$, $df = 2.9$, $P = 0.258$). Error bars indicate standard deviation.

FIGURE 3.

A, Lipolytic function by depot. All age groups combined. (N = 12 for each depot) ($F = 21.23$, $df = 4.9$, $P = 0.0001$). Error bars indicate standard deviation. B, Lipolytic function by age and depot (25–30 N = 3, 40–45 N = 4, 55–60 N = 5) ($F = 91.19$, $df = 2.9$, $P < 0.0001$). Error bars indicate standard deviation.

FIGURE 4.

A, Lipid accumulation by depot. All age groups combined (N = 12 per depot with and without ciglitazone) ($F = 1.66$, $df = 4.20$, $P = 0.199$). Error bars indicate standard deviation. B, Lipid accumulation by age. All depots combined. (25–30 N = 3, 40–45 N = 4, 55–60 N = 5) ($F = 8.83$, $df = 2.20$, $P = 0.002$). Error bars indicate standard deviation.

FIGURE 5.
PPAR- γ 2 expression of all depots and ages with and without ciglitazone.