Secretome of Primary Cultures of Human Adipose-derived Stem Cells

MODULATION OF SERPINS BY ADIPOGENESIS

Sanjin Zvonic‡, Michael Lefevre§¶, Gail Kilroy‡, Z. Elizabeth Floyd‡, James P. DeLany**, Indu Kheterpal§, Amy Gravois§, Ryan Dow§, Angie White§, Xiying Wu‡, and Jeffrey M. Gimble‡||

Studies of adipogenic protein induction have led to a new appreciation of the role of adipose tissue as an endocrine organ. Adipocyte-derived "adipokines" such as adiponectin, leptin, and visceral adipose tissue-derived serine protease inhibitor (vaspin) exert hormone-like activities at the systemic level. In this study, we examined the secretome of primary cultures of human subcutaneous adipose-derived stem cells as an in vitro model of adipogenesis. Conditioned media obtained from four individual female donors after culture in uninduced or adipogenic induced conditions were compared by two-dimensional gel electrophoresis and tandem mass spectrometry. Over 80 individual protein features showing ≥2-fold relative differences were examined. Approximately 50% of the identified proteins have been described previously in the secretome of murine 3T3-L1 preadipocytes or in the interstitial fluid derived from human mammary gland adipose tissue. As reported by others, we found that the secretome included proteins such as actin and lactate dehydrogenase that do not display a leader sequence or transmembrane domain and are classified as "cytoplasmic" in origin. Moreover we detected a number of established adipokines such as adiponectin and plasminogen activator inhibitor 1. Of particular interest was the presence of multiple serine protease inhibitors (serpins). In addition to plasminogen activator inhibitor 1, these included pigment epithelium-derived factor (confirmed by Western immunoblot), placental thrombin inhibitor, pregnancy zone protein, and protease C1 inhibitor. These findings, together with the recent identification of vaspin, suggest that the serpin protein family warrants further proteomics investigation with respect to the etiology of obesity and type 2 diabetes. Molecular & Cellular Proteomics 6:18–28, 2007.

From the ‡Stem Cell Laboratory, §Proteomic Core Facility, ¶Lipoprotein Laboratory, and ||Cell Biology Core Facility, Pennington Biomedical Research Center, Louisiana State University System, Baton Rouge, Louisiana 70808 and **Endocrinology and Metabolism Division, Department of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213

Received, June 9, 2006, and in revised form, September 5, 2006
Published, MCP Papers in Press, October 3, 2006, DOI 10.1074/mcp.M600217-MCP200

During the past 25 years, epidemiologists have come to recognize obesity as an international health problem. In the United States alone, the level of obesity has nearly doubled, and it is estimated that 30% of adults are obese (BMI ≥ 30) (1, 2). Scientists and physicians no longer view adipose tissue as just an endocrine target but now appreciate it as an endocrine organ in its own right (3). Mature adipocytes secrete a new class of proteins, known as adipokines. These include adiponectin (4), leptin (5), plasminogen activated inhibitor 1 (PAI-1) (6), resistin (7), visceral adipose tissue-derived serine protease inhibitor (vaspin) (8), and proinflammatory cytokines among others (3). There is growing evidence that adipokines act as endocrine-like factors to modulate physiological functions systemically (3). Moreover adipokines may contribute to the pathogenesis of co-morbidities associated with obesity (3). These discoveries have been the impetus for a more thorough and comprehensive analysis of the secreted proteins derived from adipocytes.

Multiple proteomics tools have been used to characterize the secretome of preadipocyte cell lines, primary adipocytes, and adipose tissue (9–12). One- and two-dimensional gel electrophoresis-tandem mass spectrometry have been applied to murine 3T3-L1 preadipocytes undergoing adipogenesis. With differentiation, the 3T3-L1 secretome changed significantly; multiple classes of proteins were induced, including components of the extracellular matrix, growth regulators, antioxidants, and complement factors (10, 12). Less than 25% of these proteins displayed a classical secretory peptide (12). Two-dimensional liquid chromatography-tandem mass spectroscopy analysis of the rat primary adipocyte secretome has identified 84 proteins that display a secretory signal peptide; this included proteases, protease inhibitors, complement factors, antioxidants, and cytokines (9). An additional 96 proteins were identified that did not meet the criteria for release via the classical endoplasmic reticulum/Golgi secretory pathway (9).

1 The abbreviations used are: BMI, body mass index; ASC, adipose-derived stem cell; 2-D, two-dimensional; DMEM, Dulbecco’s modified Eagle’s medium; HSP, heat shock protein; hu, human; PAI-1, plasminogen activator inhibitor 1; PEDF, pigment epithelium-derived factor; serpin, serine protease inhibitor; vaspin, visceral adipose tissue-derived serine protease inhibitor; FBS, fetal bovine serum; qRT, quantitative real time; SSP, standard spot.
Interstitial fluid from human mammary adipose tissue has been evaluated by a combination of two-dimensional gel electrophoresis-tandem mass spectroscopy and antibody arrays (11). The interstitial fluid contained over 240 individual proteins, many of which were also present in the adipose tissue extracts (11).

Our laboratory has utilized primary cultures of human adipose-derived cells as an in vitro model for the study of adipogenesis (13). In accordance with a consensus reached by investigators attending the Second Annual International Fat Applied Technology Society (October 3–5, 2004), we refer to these cells as human adipose-derived stem cells (huASCs) (14), although we recognize that some prefer the acronym to refer to adipose-derived stromal cells. Our analysis of the huASC proteome using two-dimensional gel electrophoresis-tandem mass spectroscopy and Western immunoblot has identified over 170 individual proteins and determined that multiple members of the heat shock protein (HSP)/chaperone family were induced during differentiation (15). The current work extends this analysis to the huASC secretome. The findings are consistent with previous reports in the literature and suggest that members of the serine protease inhibitor (serpin) family merit further investigation in the context of adipose biology.

EXPERIMENTAL PROCEDURES

Liposuction Aspirate Cell Isolation and Culture—The procedures used are modifications of published methods (14–20). Liposuction aspirates from subcutaneous adipose tissue sites were obtained from female subjects (n = 4) undergoing elective procedures in local plastic surgical offices. Tissues were washed three to four times with phosphate-buffered saline and suspended in an equal volume of PBS supplemented with 1% bovine serum and 0.1% collagenase type I prewarmed to 37 °C. The tissue was placed in a shaking water bath at 37 °C with continuous agitation for 60 min and centrifuged for 5 min at 300 × g at room temperature. The supernatant was removed, and the pelleted stromal vascular fraction was resuspended in Stromal Medium (DMEM/F-12 Ham’s, 10% fetal bovine serum, antibiotic/antimycotic) and plated at a density of 0.156 ml of tissue digest/cm² of surface area in T225 flasks using Stromal Medium for expansion and culture. This initial passage of the primary cell culture is referred to as “Passage 0.” Following the first 48 h of incubation at 37 °C at 5% CO₂, the cultures were washed with PBS and maintained in Stromal Medium until they achieved 80–90% confluence (30,166 ± 3816 cells/cm²). The cells from each donor were passaged by trypsin digestion and seeded at a density of 30,000 cells/cm² (“Passage 1”) on six 48-well plates.

Adipogenic Cell Culture—Four days after seeding, three plates (Uninduced) were maintained in Stromal Medium and fed with this medium every 3rd day. The remaining three plates (Induced) were fed with an Adipogenic Differentiation Medium composed of DMEM/F-12 with 3% FBS, 33 µM biotin, 17 µM pantothenate, 1 µM bovine insulin, 1 µM dexamethasone, 0.25 mM isobutyrylmethoxanine, 5 µM rosiglitazone, and 100 units of penicillin/100 µg of streptomycin/0.25 µg of fungizone. After 3 days, Adipogenic Differentiation Medium was changed to Adipocyte Maintenance Medium, which was identical to the induction medium except for the removal of both isobutyrylmethoxanine and rosiglitazone, and fed every 3rd day. On day 9 following induction, the media were removed from both the Uninduced and induced plates and replaced with Serum Free Medium (DMEM/F-12, 1% antibiotic/antimycotic). After 1 h, the medium was removed and discarded. Fresh Serum Free Medium was added to each well, and the plates were incubated overnight (16 h) after which time all Uninduced and Induced cell conditioned medium for each donor lot was collected, pooled, adjusted to a final concentration of 2 ml PMSF by addition of a 100X stock solution, snap frozen in liquid nitrogen, and stored at −80 °C for future analysis. Two plates of cells from the Uninduced and Induced were harvested for total RNA using TRI Reagent (100 µl/well) according to the manufacturer’s instructions (Molecular Research Center, Cincinnati, OH). One plate of cells under each condition was harvested for total cell protein by addition of 100 µl/well IP buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% Igepal, protease inhibitors, 2 mM PMSF). Protein and RNA samples were stored at −80 °C for future analysis.

Conditioned Medium Concentration—Frozen volumes of conditioned medium (35–40 ml of each) were removed from −80 °C and thawed at 4 °C. As the samples thawed, a fresh aliquot of 100X PMSF (200 mM) was added to each. Fifteen-milliliter aliquots of the solutions were placed in an Amicon Ultra-15 (catalogue number UFC900524) with a 5,000 molecular weight cutoff and centrifuged for 30 min at 4,000 rpm, and the volumes were concentrated between 40- to 200-fold. Protein concentrations of the resulting concentrates were determined (Bio-Rad protein assay) and ranged from 0.19 to 4.78 µg/µl.

Two-dimensional PAGE—Protein samples were solubilized in 8 M urea, 4% CHAPS, 65 mM DTT, 40 mM Tris. Following centrifugation to remove unsolubilized material, 45–70 µg of protein were mixed with rehydration buffer (8 M urea, 4% CHAPS, 1% IPG buffer, 0.3% DTT) and were introduced into the dry IPG strips (typically 18 cm, pH 4–10 non-linear) under conditions of active rehydration (e.g. with a slight voltage applied across the strips). All gels were run in duplicate. Proteins were focused at a maximum of 10,000 V for a total of 90,000 V-h. Upon completion of first dimension electrophoresis, the IPG strips were either directly subjected to second dimension SDS-PAGE or frozen at −80 °C for later analysis. For the second dimension, the IPG strips were equilibrated first with 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT for 15 min followed by a second equilibration with 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 5% iodoacetamide for 15 min. The strips were rinsed with electrophoresis buffer (25 mM Tris, 190 mM glycine, 0.1% SDS) and then embedded in low melting temperature agarose on the top of 25 × 20-cm 12% acrylamide gel. Gels were run at constant voltage until the bromphenol blue dye front reached the bottom of the gel and were stained with SYPRO Ruby. The stained gels were scanned with a Molecular Imager FX with data directly imported into PDQuest. For each gel, the relative abundance of each resolved protein feature was quantified by mathematical fitting of Gaussian curves in two dimensions. Data within each were normalized (expressed as a percentage of total spot abundance), and routine statistical analyses available within the software package were used to identify unique spots, absent spots, or spots up- or down-regulated under specified conditions. Gel spots containing unique spots were excised, and the resulting gel plugs were automatically destained, digested, identified by mass spectrometry, and integrated into a ProteomeList. This Cut List was used by the automated spot cutter to select and excise the protein features in order of least to most abundant from one or more gels. Excised gel plugs were deposited into a 96-well plate and transferred to the MassPrep (Waters/Micromass) station. Proteins within the gel plugs were automatically destained, digested, identified by mass spectrometry, and integrated into a ProteomeList.
reduced, alkylated, dehydrated, rehydrated, and digested with trypsin. The resulting peptides were extracted and deposited into 96-well plates for analysis.

**Q-TOF Analysis**—The peptides from each digested spot were separated and analyzed using a capillary LC system coupled on line with a nanospray Q-TOF mass spectrometer (Waters) as described previously (15). Briefly the peptides were injected onto a 75-μm C18 reverse phase capillary column (Dionex) and eluted using a 50-min gradient from 5 to 50% acetonitrile. The mass spectrometer was operated in a data-dependent acquisition mode in which a full survey MS scan was followed by three MS/MS scans using normalized collision energy. The ProteinLynxGlobalServer 1.1 software (Waters) was used to search Release 49.7 of Swiss-Prot (May 2006) containing 219,361 sequence entries for protein identification using 100 ppm precursor-ion and fragment-ion mass accuracy (21). Potential modifications considered included phosphorylation, oxidation of methionine, cysteines modified with iodoacetamide, one missed cleavage, and digestion with trypsin. Scores above 100 were generally consid-

---

**Fig. 1.** Quantitative real time PCR analysis of representative adipocyte-associated mRNA levels. The relative levels of gene expression for the adipocyte-associated genes aP2, lipoprotein lipase (LPL), and peroxisome proliferator-activated receptor (PPAR) γ2 were normalized relative to cyclophilin B for total RNA isolated from undifferentiated (U1–4) and adipocyte (A1–4) huASCs from four individual donors. Values are the mean ± S.D. for triplicate determinations for each donor sample.

---

**Fig. 2.** Two-dimensional polyacrylamide gel electrophoresis. Two-dimensional PAGE was performed with protein lysates prepared from human ASCs cultured under undifferentiated conditions or maintained under adipocyte differentiated conditions for 9 days following induction. The gels were stained with SYPRO Ruby. The figure displays representative gels from each condition as well as the master composite prepared based on features conserved on replicate gels prepared from protein extracts obtained from the four individual donors.
The database was checked for redundancy and inspected for single proteins listed under multiple names. The molecular weight and pI of identified proteins were evaluated and verified relative to the electrophoretic mobility of the protein feature on the two-dimensional gel. Identified proteins were further analyzed for the presence of predicted secretory signal peptide sequences using SignalP criteria (22).

Criteria Used for Analysis—The proteome of the undifferentiated and differentiated human adipose-derived adult stem cells was defined based on the following guidelines: proteins “induced” or “reduced” during adipogenesis displayed both a 95% significance in comparisons between replicate groups and 2-fold induction or 2-fold reduction with adipocyte differentiation (total of 81 features).

Quantitative Real Time (qRT)-PCR—Total RNA was purified from undifferentiated and adipocyte differentiated (day 9) human ASCs using TRI Reagent (Molecular Research Center) according to the manufacturer’s specifications. Approximately 2 μg of total RNA were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega) with oligo(dT) at 42 °C for 1 h in a 20-μl reaction. Primers for genes of interest were identified using Primer Express software (Applied Biosystems). A complete list of primers used in these studies is listed in Supplemental Table 1. qRT-PCR was performed on diluted cDNA samples with SYBR® Green PCR Master Mix (Applied Biosystems) using the 7900 Real Time PCR system (Applied Biosystems) under universal cycling conditions (95 °C for 10 min, 40 cycles of 95 °C for 15 s, and then 60 °C for 1 min). All results were normalized relative to a cyclophilin B expression control.

Western Immunoblot—Conditioned medium was harvested from confluent, quiescent undifferentiated ASCs and adipocyte differentiated ASCs 9 days after adipogenic initiation. Equal volumes (20 μl) of medium were loaded onto a 15% denaturing acrylamide gel, separated by electrophoresis, transferred to nylon membrane, and probed.
Human Adipose-derived Stem Cell Adipogenic Secretome

sequentially with a primary murine monoclonal antibody recognizing the pigment epithelium-derived factor (Chemicon MAB1059) and a secondary goat anti-mouse Ig antibody coupled to horseradish peroxidase. Signal was detected using an ECL kit (Pierce) according to published procedures (23).

RESULTS

2-D Gel Electrophoresis-Tandem Mass Spectrometry—The huASCs were derived from subcutaneous lipoaspirates obtained from healthy female donors undergoing elective liposuction surgery. The mean age and BMI (±S.D.) of the subjects were 37.0 ± 3.9 years and 25.7 ± 3.8, respectively. Adipogenesis, induced by a 3-day exposure to dexamethasone, insulin, rosiglitazone, and isobutylmethylxanthine, was monitored and confirmed based on the appearance of lipid vacuoles by phase-contrast microscopy (data not shown). In addition, qRT-PCR was used to examine the relative mRNA levels of adipocyte-associated genes in undifferentiated and day 9 adipocyte differentiated huASCs (Fig. 1). The genes encoding adiponectin, a fatty acid-binding protein, and lipoprotein lipase were induced by a mean of 20-fold and 35-fold in four donors, whereas that encoding peroxisome proliferator-activated receptor γ2 was increased in three of the four donors (Fig. 1). Conditioned medium was prepared from undifferentiated and day 9 adipocyte differentiated huASCs from each donor. The concentrated conditioned medium was subjected to two-dimensional gel electrophoresis, and individual protein features were identified by staining with SYPRO Ruby. Representative gels for the undifferentiated and day 9 adipocyte differentiated conditioned medium are presented in Fig. 2. The undifferentiated and adipocyte differentiated conditioned medium gels displayed a mean ± S.D. of 365 ± 72 and 611 ± 64 features, respectively. The data from four individual donor gels were combined to create a “master” map that matched a total of 324 features meeting our selection criteria for protein quantity (>1000 ppm).

The relative signal intensity of individual features was compared between the undifferentiated and adipocyte differentiated huASC gels. A total of 77 features exhibited a ≥2-fold induction, and four features exhibited a ≥2-fold reduction as a consequence of adipocyte differentiation. Representative features meeting these criteria are displayed in Fig. 3; the relative levels of signal intensity in the differentiated and undifferentiated conditioned medium from the four individual donors are presented graphically as is the name of one protein identified from each spot. Individual features were selected for tryptic digestion and tandem mass spectroscopic analysis. Table I presents an abbreviated list of those proteins identified based on a single peptide, the peptide sequence and mass spectrograph are presented in Supplemental Table 3.

qRT-PCR and Western Blot Analysis of Selected Secreted Proteins—Further analyses were performed on a subset of candidate proteins. The mRNA levels of genes encoding proteins within the heat shock protein/chaperone family and the serpin family were examined by quantitative real time PCR (Fig. 5). In all four donors, adipogenesis induced the mRNA levels of the crystallin αB by 17.6 ± 7.3-fold and HSP27 by 3.1 ± 1.5-fold (mean ± S.D.) relative to a cyclophilin B control (Fig. 5). The adipogenic induction was less pronounced for the genes encoding aP2, the fatty acid-binding protein, and lipoprotein lipase encoded by the PAI-1 gene were induced by a mean of 20-fold and 35-fold in four donors, respectively, whereas that encoding peroxisome proliferator-activated receptor γ2 was increased in three of the four donors (Fig. 1).

TABLE I

| Protein                                    | Adipocyte related Ref. |
|--------------------------------------------|------------------------|
| Adiponectin                                | 4, 26                  |
| Albumin                                    | 27–29                  |
| Angiotensinogen                            | 2    |
| Bloodstream-specific protein 2             | 63, 64                  |
| Calreticulin                               | 30                     |
| Calumenin                                  | 12, 65                  |
| Cathepsin D                                | 66                     |
| Cathepsin L                                | 66                     |
| Collagen α 1 (I)                           | 9, 10, 12              |
| Dermatopontin                              | 32                     |
| Follistatin-related protein                 | 67                     |
| Galectin 3                                  | 33, 34                  |
| Glucosidase Iβ                              | 33, 34                  |
| Hexasaminidase                             | 33, 34                  |
| Osteonectin (SPARC)                        | 31                     |
| Pentraxin-related protein (PTX3)           | 31                     |
| Pigment epithelium-derived factor          | 31                     |
| Plasma protease C1 inhibitor               | 31                     |
| Plasminogen activator inhibitor 1          | 31                     |
| Pregnancy zone protein                     | 31                     |
| Prolyl hydroxylase 1α                      | 31                     |
| Proteasome subunit β type 6                | 31                     |
| Protein-disulfide isomerase                | 31                     |
| Retinol-binding protein                    | 31                     |
| Short chain hydroxy acyl-CoA hydroxylase   | 31                     |
| Stromal cell-derived growth factor (IL-25) | 31                     |
| Transthyretin                              | 31                     |
| UMP-CMP kinase                             | 31                     |

All of the proteins were predicted to contain a signal peptide based on the SignalP software algorithm (22). The probability was >0.95 with the exception of proteasome subunit β type 6, short chain hydroxy acyl-CoA hydroxylase, and UMP-CMP kinase where the probabilities were 0.605, 0.513, and 0.933, respectively. SPARC, secreted protein acidic and rich in cysteine; IL-interleukin.
controls (Fig. 5); in contrast, the protein level of PAI-1 increased from non-detectable to detectable levels upon differentiation (Fig. 3, SSP 7302). The relative mRNA levels for the serpin PEDF increased with adipogenesis by 4.5 ± 1.6-fold, whereas HSP47 remained unchanged with induced levels of 1.1 ± 0.3-fold (Fig. 5). Parallel Western immunoblot analysis of conditioned medium from huASCs isolated from two donors demonstrated increased levels of PEDF protein as a function of adipogenic differentiation (Fig. 6). Conditioned medium from primary human adipose tissue incubated in the absence or presence of fetal bovine serum served as positive controls. It should be noted that a signal due to antibody cross-reactivity with serum proteins, most prominent on the longer exposure, is evident above the signal for the PEDF protein itself (Fig. 6).

**DISCUSSION**

**Comparison with Reported Secretomes**—The profile of all proteins identified in the huASC secreome has been compared with selected proteomes of adipocytes and serum reported in the literature (Table II and Supplemental Table 2C). Approximately 49% of the identified proteins (Supplemental Table 2C) were present in the proteome of huASC total cell lysates (15). The rapid transit of proteins through the secretory apparatus of the cell may account for the low abundance and subsequent non-detection of those secreome elements that were not represented within the proteome of the whole cell lysates. The fact that only a subset of all protein features from either the huASC cell lysates or conditioned medium were analyzed by mass spectroscopy may also contribute to any discrepancies in the study outcomes. In total, ~72% of the proteins identified in the huASC secreome had been identified in one or more proteomics analyses of the secretome of human or rodent adipocytes or adipose tissues (Supplemental Table 2C) (9–12, 15, 24). An additional 5% of the huASC secreome proteins had been identified in two or more analyses of human plasma proteins (Supplemental Table 2C) (25). Thus, ~20–25% of the huASC secreome consists of uniquely identified proteins.

**Characteristics of the huASC Secretome**—A total of 101 individual proteins were identified in the conditioned medium from undifferentiated and day 9 adipocyte-differentiated huASCs. A subset of these proteins (29%, 29 of 101) contained a predicted signal peptide (Table I) consistent with secretion via the endoplasmic reticulum/Golgi-dependent pathway (22). In their pioneering studies applying two-dimensional liquid chromatography-tandem mass spectroscopy to the adipocyte secreome, Chen et al. (9) have set the criteria for the identification of an adipokine based exclusively on the presence of a secretory peptide. They have suggested that leakage or cell death may account for any other proteins detected in the conditioned medium (9). Their approach offers technical advantages of improved efficiency and increased sensitivity relative to two-dimensional gel electrophoresis (9). Indeed the high percentage of proteins identified with signal peptides in their study supports the use of this technology for future secreome analyses (9). Even according to these high inclusion criteria, the current study confirmed the presence of multiple adipokines identified during previous analyses of murine adipogenic models including adiponectin (4, 26), angiotensinogen (27–29), cathepsin D (30), pentraxin 3 (31), pregnancy zone protein (32), and retinol-binding protein (33, 34) as well as the protease inhibitors and serpins discussed in the following paragraph. Noteworthy for its absence is leptin, an adipokine secreted by differentiated huASCs (17). Our failure
to detect leptin is not unique because none of the gel electrophoresis/mass spectrometric analyses of adipose tissue or adipocytes included in Table II have reported its detection. Instead it was only detected with antibody-based methods (11, 17). It is possible that structural properties of the leptin protein reduce its detectability by 2-D PAGE and/or mass spectroscopic analysis.

Over 500 identified genes belong to the serpin family, and in the human, these have been categorized into nine clades (35). Both intracellular and extracellular serpins exist, serving multiple functions including coagulation, host defense, and ischaemia protection (35). In the current study, our proteomics approach detected six members of the serpin family derived from five clades in the huASC secretome, the majority of which serve as extracellular protease inhibitors (Table III). Three of these had been identified previously in proteomics analyses of adipocyte secretomes, and we had reported a fourth serpin family member, HSP47, a chaperone protein involved in protein folding, in our proteomics analyses of the huASC cellular protein lysates (15). The current work extends this observation by documenting the expression levels of HSP47 mRNA during adipogenesis. Independent studies, using either proteomics (11) or differential RNA analyses (8), have further identified α₁-protease inhibitor and vaspin, respectively. Visceral white adipose depots uniquely expressed vaspin in mice, and its secretion was increased with obesity (8). Obese mice fed a high sucrose and fat diet improved their insulin sensitivity when treated with vaspin (8). In human subjects, vaspin mRNA has been detected in both visceral and subcutaneous white adipose tissues in a subpopulation of obese but not lean subjects (36). Its levels correlate with BMI and glucose tolerance (36). In addition to the current work, two other studies have associated α₂-macroglobulin with adipocytes (11, 37). In one, the intracellular α₂-macroglobulin was associated with preadipocyte 3T3-L1 cells; however, the protein was not synthesized by the cells themselves and had been derived from the medium (37). The addition of anti-α₂-macroglobulin antibodies increased 3T3-L1 adipocyte numbers, suggesting that the protein played an inhibitory role in adipogenesis (37). In studies of human mammary adipose tissue, α₂-macroglobulin was present both the tissue and interstitial fluid (11).

There has been a substantial body of work on the relationship of PAI-1 with adipogenesis and obesity (6, 38, 39). Proinflammatory cytokines such as tumor necrosis factor α induce expression of PAI-1 mRNA and protein in adipocytes and adipose tissue (39). Due to its role as a regulator of fibrinolysis, PAI-1 has been implicated as a mediator of the cardiovascular co-morbidities associated with obesity (39). Consistent with our current results (Fig. 5), PAI-1 mRNA levels have been found to decrease modestly during the differentiation of human adipocytes in vitro (38) despite the fact that we observed PAI-1 protein levels to increase after differentiation (Fig. 3, SSP 7302) (38). This apparent inconsistency between PAI-1 mRNA and protein levels has precedent in the literature at a global level. A combined proteomics and transcriptomics analysis of osteoblast differentiation models has documented overall low correlation values between mRNA and protein levels in MC3T3-E1 cells as a function of mineralization (40).

| Proteome target                  | Percent similarity (common proteins) | Ref. |
|----------------------------------|-------------------------------------|------|
| Human ASC cell lysates           | 49% (49)                            | 15   |
| Human mammary fat interstitial fluid | 40% (40)                          | 11   |
| Primary rat adipocyte secretome  | 30% (30)                            | 9    |
| Human plasma                     | 21% (21)                            | 25   |
| 3T3-L1 secretome                 | 11% (11)                            | 12   |
| 3T3-L1 secretome                 | 4% (4)                             | 10   |
| 3T3-L1 proteome                  | 13% (13)                           | 37   |

Fig. 5. Quantitative real time PCR levels of representative chaperone and serpin genes. The relative levels of gene expression for the serpins HSP47, PDE5, PAI-1, and protease C1 inhibitor (C1inh) and the chaperones crystallin αB and HSP27 were normalized to cyclophilin B for control (undifferentiated (U)) and adipocyte (differentiated (D)) human ASCs from four individual donors. Values are the mean ± S.D. for triplicate determinations for each donor sample.

Fig. 6. PEDF. For Western immunoblot, conditioned medium was obtained from undifferentiated (day 0 and day 9) and adipocyte differentiated (day 9) human ASCs from two individual donors as well as from freshly isolated human liposuction specimens incubated overnight in the absence (−FBS) or presence (+FBS) of fetal bovine serum. Lanes (left to right) contain conditioned medium from liposuction tissue (−FBS) (lane 1), liposuction tissue (+FBS) (lane 2), undifferentiated day 9 human ASCs (lane 3), empty lane (lane 4), undifferentiated day 0 human ASCs (two donors) (lanes 5 and 7), adipocyte differentiated day 9 human ASCs (two donors) (lanes 6 and 8). A short (upper panel) and long (lower panel) exposure of the same Western immunoblot are displayed. The arrows indicate the location of the BSA and PEDF signals. cond., conditioned; w/o, without.

Comparison with published secretomes of adipocytes, adipose tissue, and plasma

Molecular & Cellular Proteomics 6.1 25


Human Adipose-derived Stem Cell Adipogenic Secretome

### Table III

| Protein name                                      | Serpin identification | Ref.  |
|--------------------------------------------------|-----------------------|-------|
| α1-Antitrypsin inhibitor                         | Serpin A3             | 11    |
| Heat shock protein 47                            | Serpin J6             | Present study: 15 |
| α2-Macroglobulin/pregnancy zone protein          | Serpin F1             | Present study: 9, 11, 37 |
| Pigment epithelium-derived factor                | Serpin B6             | Present study: 9, 10, 12 |
| Placental thrombin inhibitor                     | Serpin E1             | Present study: 6, 9 38 |
| Plasminogen activator inhibitor 1                | Serpin G1             | Present study |
| Protease C1 inhibitor                            | Serpin A1             | 11    |
| α1-Protease inhibitor                            | SerpinA12             | 8     |
| Visceral adipose serine protease inhibitor       |                       |       |

Likewise in proteomics studies of adenocarcinomas, only 20% of the protein and mRNA levels showed a positive correlation (41).

**Pigment Epithelium-derived Factor**—Independent analyses of the adipocyte secretome, including the current study, have detected PEDF consistently (9, 10, 12). In murine 3T3-L1 cells, PEDF protein and mRNA levels were most abundant in the undifferentiated cells; by day 3 of adipogenesis, mRNA levels and protein decreased significantly (10). Our observations were directly opposite those in 3T3-L1 cells; both PEDF protein and mRNA levels were significantly induced during huASC adipogenesis (Figs. 2 and 5). A similar discrepancy between murine and human adipocyte expression profiles has been noted previously for the unrelated secretory products agouti and resistin (7, 42, 43).

In contrast to other serpins, PEDF lacks a serine reactive center and does not inhibit serine protease activity (44). First detected as a secreted protein from retinal epithelial cells, PEDF was identified as a serpin capable of inducing neuronal cell differentiation (44, 45). Further studies demonstrated that PEDF exerted an antiangiogenic action in the retina, countering the effects of vascular endothelial growth factor (46). Levels of PEDF were induced under hyperoxic conditions and reduced under hypoxic conditions (46). Elevated levels of PEDF protect the retina against oxidative damage and inhibit the formation of advanced glycation end products associated with diabetic retinopathy (47, 48). The association of PEDF with both adipocytes and the eye resembles the similar expression pattern we have noted for crystallin, a major component of the lens and a target for diabetes-related glycation (15). PEDF has been found previously in human blood at physiologic levels (100 nM) and to contain several post-translational modifications (49), consistent with our current detection of two unique PEDF-associated spots by 2-D PAGE (Fig. 3, SSP 5301 and SSP 5302). Together these findings indicate that PEDF merits continued evaluation in the context of adipocyte biology.

**Novel Serpins**—Two novel serpins have been associated with huASCs. The C1 inhibitor is a glycosylated serum protein first identified based on its ability to block the activation of the first component of the complement pathway (50). Mutations altering the inhibitory function of the protein are associated with hereditary angioneurotic edema, a rare but life-threatening autosomal dominant disorder that can cause acute airway obstruction due to edematous changes in the soft tissue of the throat (51, 52). Curiously the C1 inhibitor is a cleavage target of the human immunodeficiency virus (type 1) protease and, consistent with this observation, serum from human immunodeficiency virus-infected patients inhibits activation of the classical complement pathway (53, 54). Placental thrombin inhibitor is a prototypic intracellular serpin that was first identified based on its interaction with thrombin (55). The protein is found in monocytes and granulocytes where it is able to form a complex with cathepsin G and inhibit cathepsin G activity (56).

**Conclusions**—We have characterized the secretome of huASCs during adipogenesis using a proteomics approach. This work builds on earlier observations from our laboratory (15) and others (9, 10, 12, 24, 37, 57–62) in profiling the protein changes that accompany adipocyte differentiation. One pattern common among these studies is that adipocyte differentiation modulates the expression levels of the serpin family of protease inhibitors. Therefore, we postulate that further examination of serpin expression profiles will provide mechanistic insights into adipose tissue physiology and pathology.

---

1. Bray, G. A. (2004) The epidemic of obesity and changes in food intake: the fluoride hypothesis. *Physiol. Behav.* **82**, 115–121
2. Bray, G. A. (2004) Medical consequences of obesity. *J. Clin. Endocrinol. Metab.* **89**, 2583–2589

---

* This work was supported by the Pennington Biomedical Research Foundation (to X. W. and J. M. G.) and National Institutes of Health Grant DK072476 (to G. K. and J. M. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be addressed: Stem Cell Laboratory, Pennington Biomedical Research Center, 6400 Perkins Rd., Baton Rouge, LA 70808. Tel.: 225-763-3171; Fax: 225-763-0273; E-mail: gimblejm@pbrc.edu.

---

**REFERENCES**

---

| Protein name                                      | Serpin identification | Ref.  |
|--------------------------------------------------|-----------------------|-------|
| α1-Antitrypsin inhibitor                         | Serpin A3             | 11    |
| Heat shock protein 47                            | Serpin J6             | Present study: 15 |
| α2-Macroglobulin/pregnancy zone protein          | Serpin F1             | Present study: 9, 11, 37 |
| Pigment epithelium-derived factor                | Serpin B6             | Present study: 9, 10, 12 |
| Placental thrombin inhibitor                     | Serpin E1             | Present study: 6, 9 38 |
| Plasminogen activator inhibitor 1                | Serpin G1             | Present study |
| Protease C1 inhibitor                            | Serpin A1             | 11    |
| α1-Protease inhibitor                            | SerpinA12             | 8     |
| Visceral adipose serine protease inhibitor       |                       |       |
Proteomic analysis of adipocyte differentiation: Evidence that alpha2 macroglobulin is involved in the adipose conversion of 3T3 L1 preadipocytes. *Proteomics* 4, 1840–1848

38. Crandall, D. L., Quinet, E. M., Morgan, G. A., Busler, D. E., McHendry-Rinde, B., and Krat, J. G. (1999) Synthesis and secretion of plasminogen activator inhibitor-1 by human preadipocytes. *J. Clin. Endocrinol. Metab.* 84, 3222–3227

39. Dellas, C., and Loskutoff, D. J. (2005) Historical analysis of PAI-1 from its discovery to its potential role in cell motility and disease. *Thromb. Haemostasis* 93, 631–640

40. Conrads, K. A., Yi, M., Simpson, K. A., Lucas, D. A., Camalier, C. E., Yu, L. R., Veenstra, T. D., Stephens, R. M., Conrads, T. P., and Beck, G. R., Jr. (2005) A combined proteome and microarray investigation of inorganic phosphate-induced pre-osteoblast cells. *Mol. Cell. Proteomics* 4, 1284–1296

41. Chen, G., Gharib, T. G., Huang, C. C., Taylor, J. M., Misek, D. E., Kardia, S. L., Giordano, T. J., Iannettoni, M. D., Orringer, M. B., Hanash, S. M., and Beer, D. G. (2002) Discordan protein and mRNA expression in lung adenocarcinomas. *Mol. Cell. Proteomics* 1, 304–313

42. Heilbronn, L. K., Rood, J., Janderova, L., Albu, J. B., Kelley, D. E., Ravussin, E., and Smith, S. R. (2004) Relationship between serum resistin concentration and insulin resistance in nonobese, obese, and obese diabetic subjects. *J. Clin. Endocrinol. Metab.* 89, 1844–1848

43. Smith, S. R., Gawronska-Kozak, B., Janderova, L., Nguyen, T., Murrell, A., Stephens, J. M., and Mynatt, R. L. (2003) Agouti expression in human adipose tissue: functional consequences and increased expression in type 2 diabetes. *Diabetes* 52, 2914–2922

44. Steele, F. R., Chader, G. J., Johnson, L. V., and Tombran-Tink, J. (1993) Pigment epithelium-derived factor: neurotrophic activity and identification as a member of the serine protease inhibitor family. *Proc. Natl. Acad. Sci. U. S. A.* 90, 1526–1530

45. Tombran-Tink, J., Chader, G. G., and Johnson, L. V. (1991) PEDF: a pigment epithelium-derived factor with potent neuronal differentiative activity. *Exp. Eye Res.* 53, 411–414

46. Dawson, D. W., Volpert, O. V., Gillis, P., Crawford, S. E., Xu, H., Benedict, W., and Bouck, N. P. (1999) Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. *Science* 285, 245–248

47. Yamagishi, S., Matsui, T., and Inoue, H. (2005) Inhibition by advanced glycation end products (AGEs) of pigment epithelium-derived factor (PEDF) gene expression in microvascular endothelial cells. *Drugs Exp. Clin. Res.* 31, 227–232

48. Tsao, Y. P., Ho, T. C., Chen, S. L., and Cheng, H. C. (2006) Pigment epithelium-derived factor inhibits oxidative stress-induced cell death by activation of extracellular signal-regulated kinases in cultured retinal pigment epithelial cells. *Life Sci.* 79, 545–550

49. Petersen, S. V., Valnickova, Z., and Enghild, J. J. (2003) Pigment-epithelium-derived factor (PEDF) occurs at a physiologically relevant concentration in human blood: purification and characterization. *Biochem. J.* 374, 199–206

50. Davis, A. E., III, Whitehead, A. S., Harrison, R. A., Dauphinaias, A., Bruns, G. A., Cicardi, M., and Rosen, F. S. (1986) Human inhibitor of the first component of complement, C1: characterization of cDNA clones and localization of the gene to chromosome 11. *Proc. Natl. Acad. Sci. U. S. A.* 83, 3161–3165

51. Roche, O., Blanch, A., Duponchel, C., Fontan, G., Tosi, M., and Lopez-Trascasna, M. (2005) Hereditary angioedema: the mutation spectrum of SERPING1/C1NH in a large Spanish cohort. *Hum. Mutat.* 26, 135–144

52. Bissler, J. J., Aulak, K. S., Donaldson, V. H., Rosen, F. S., Cicardi, M., Harrison, R. A., and Davis, A. E., III (1997) Molecular defects in hereditary angioneurotic edema. *Proc. Assoc. Am. Physicians* 109, 164–173

53. Gerencer, M., and Burek, V. (2004) Identification of HIV-1 protease cleavage sites in human C1-inhibitor. *Virus Res.* 105, 97–100

54. Burek, V., and Gerencer, M. (1998) Inhibition of classical complement activation by sera from HIV-1-positive patients. *Clin. Immunol. Immunopathol.* 81, 114–121

55. Coughlin, P., Sun, J., Cerruti, L., Salem, H. H., and Bird, P. (1993) Cloning and molecular characterization of a human intracellular serine proteinase inhibitor. *Proc. Natl. Acad. Sci. U. S. A.* 90, 9417–9421

56. Scott, F. L., Hirst, C. E., Sun, J., Bird, C. H., Bottomley, S. P., and Bird, P. I. (1999) The intracellular serpin protease inhibitor 6 is expressed in monocytes and granulocytes and is a potent inhibitor of the azurophil granule protease, cathepsin G. *Blood* 93, 2089–2097

57. Bluher, M., Wilson-Fritch, L., Leszky, J., Laustsen, P. G., Corvera, S., and Kahn, C. R. (2004) Role of insulin action and cell size on protein expression patterns in adipocytes. *J. Biol. Chem.* 279, 31902–31909

58. Wilson-Fritch, L., Burkart, A., Bell, G., Mendelsson, K., Leszky, J., Nicoloro, S., Czech, M., and Corvera, S. (2003) Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol. Cell. Biol.* 23, 1085–1094

59. Sanchez, J. C., Converset, V., Nolan, A., Schmid, G., Wang, S., Sennit, M. V., Hochstrasser, D. F., and Cawthorne, M. A. (2003) Effect of rosiglitazone on the differential expression of obesity and insulin resistance associated proteins in lep/lep mice. *Proteomics* 3, 1500–1520

60. Schmid, G. M., Converset, V., Walter, N., Sennit, M. V., Leung, K. Y., Byers, H., Ward, M., Hochstrasser, D. F., Cawthorne, M. A., and Sanchez, J. C. (2004) Effect of high-fat diet on the expression of proteins in muscle, adipose tissues, and liver of C57BL/6 mice. *Proteomics* 4, 2270–2292

61. Aboulaich, N., Vainonen, J. P., Strafrors, P., and Vener, A. V. (2004) Vectorial proteomics reveal targeting, phosphorylation and specific fragmentation of polymerase I and transcript release factor (PTRF) at the surface of caveolae in human adipocytes. *Biochim. J.* 383, 237–248

62. Vainonen, J. P., Aboulaich, N., Turkina, M. V., Strafrors, P., and Vener, A. V. (2004) N-terminal processing and modifications of caveolin-1 in caveolae from human adipocytes. *Biochem. Biophys. Res. Commun.* 320, 480–486

63. Superti-Furga, A., Rocchi, M., Schafer, B. W., and Gitzelmann, R. (1993) Complementary DNA sequence and chromosomal mapping of a human proteoglycan-binding cell-adhesion protein (dermatopontin). *Genomics* 17, 463–467

64. Derfoul, A., Perkins, G. L., Hall, D. J., and Tuan, R. S. (2006) Glucocorticoids promote chondrogenic differentiation of adult human mesenchymal stem cells by enhancing expression of cartilage extracellular matrix genes. *Stem Cells* 24, 1487–1495

65. Yang, R. Y., Hsu, D. K., Yu, L., Chen, H. Y., and Liu, F. T. (2004) Galectin-12 is required for adipogenic signaling and adipocyte differentiation. *J. Biol. Chem.* 279, 29761–29766

66. Feng, J., Romaniouk, A. V., Samal, S. K., and Vijay, I. K. (2004) Processing enzyme glucosidase II: proposed catalytic residues and developmental localization of the mouse mammary gland. *Glycobiology* 14, 909–921

67. Lamande, S. R., and Bateman, J. F. (1999) Procollagen folding and assembly: the role of endoplasmic reticulum enzymes and molecular chaperones. *Semin. Cell Dev. Biol.* 10, 455–464