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Serglycin is a novel adipocytokine highly expressed in epicardial adipose tissue

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ABSTRACT

Much recent work has highlighted the key role of adipose tissue as an endocrine organ that secretes a number of adipocytokines, linking adiposity, especially intra-abdominal visceral fat, and the pathogenesis of cardiovascular and metabolic diseases. However, the role of epicardial adipose tissue (EAT), another important visceral fat depot situated in close proximity to epicardial coronary arteries and myocardium, has been less well studied. In this study, we sought to characterize EAT by comparing gene expression profiles of EAT, omental adipose tissue (OAT), and subcutaneous adipose tissue (SCAT) in patients who underwent elective coronary artery bypass graft surgery for critical coronary artery disease (CAD) and identify molecules involved in inflammation. A total of 15,304 probes were detected in all depots, and 231 probes were differentially expressed. Significantly higher expression of pro-inflammatory genes such as interleukin-1β, -6, and -8, and chemokine receptor 2 was observed in EAT, even when compared with OAT. Among them, serglycin was one of the most abundantly expressed genes in EAT. Serglycin expression was induced during adipocytic differentiation of 3T3L1 cells. Serglycin was secreted from adipocytes, and tumor necrosis factor-α stimulated its expression and secretion in adipocytes. Serglycin was also present in human serum samples. These results suggest that human EAT has strong inflammatory properties in patients with CAD and provide novel evidence that serglycin is an adipocytokine highly expressed in EAT.

1. Introduction

Obesity is associated with an increased risk of morbidity and mortality and is an independent risk factor for coronary artery disease (CAD) [1–3]. Adipose tissue has been widely recognized as not only a simple energy storage organ but also an endocrine organ [4,5]. Adipocytokines are bioactive substances secreted from adipocytes, and increasing evidence has suggested their involvement in the development of metabolic syndrome and cardiovascular diseases [4–6]. Most adipocytokines such as tumor necrosis factor-α (TNFα) and leptin are pro-inflammatory, thereby promoting obesity-related disorders, while a small number of adipocytokines such as adiponectin have protective effects against cardiovascular disorders at various atherogenic stages [4,5].

The link between visceral adiposity and the pathogenesis of cardiovascular and metabolic diseases has been highlighted [7,8]. Visceral adiposity evaluated by the waist-to-hip ratio and computed tomography scanning is related to CAD independently of body mass index [9,10]. Adipocytes in visceral adipose tissue have been suggested to be more endocrinologically active than those in subcutaneous adipose tissue (SCAT) [8,11,12]. Epicardial adipose tissue (EAT), which is situated in close proximity to epicardial coronary arteries and myocardium, also secretes chemokines and inflammatory cytokines [13–15]. Adiponectin expression in EAT has been shown to be lower in patients with CAD than in subjects without CAD [16]. Recent research demonstrates that adiponectin level in EAT is a predictor of cardiovascular prognosis in patients with cardiovascular diseases [17]. Thus, EAT has been suggested to play an important part in the development of cardiovascular diseases. However, adipocytokines secreted from EAT and the differences of expression of adipocytokines among adipose tissues, such as EAT, omental adipose tissue (OAT), and SCAT, have been less well studied.

In this study, we sought to characterize EAT by comparing gene expression profiles of EAT, OAT, and SCAT, and identified serglycin as a novel molecule highly expressed in EAT.

Abbreviations: CAD, coronary artery disease; TNF-α, tumor necrosis factor-α; IL, interleukin; EAT, epicardial adipose tissue; OAT, omental adipose tissue; SCAT, subcutaneous adipose tissue.

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2. Materials and methods

2.1. Tissue collection from CAD subjects

All procedures using biopsy specimens performed in this study were approved by the Ethics Committee of Kyoto Prefectural University of Medicine and informed consent was obtained from all patients. Samples of EAT, OAT, and SCAT were obtained from patients who underwent elective coronary artery bypass graft surgery with the gastroepiploic artery for critical CAD. All of the aspects of animal care and experimentation performed in this study were approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine.

2.2. Microarray analysis

Microarray analysis was performed on a Human Genome U133 Plus2.0 array (Affymetrix, Santa Clara, CA). The arrays were hybridized, washed, scanned according to the standard Affymetrix protocol, and scanned with a Genechip Scanner 3000 7G (Affymetrix, Santa Clara, CA). Data analysis was performed using GeneChip Operating Software (GCOS).

2.3. RNA isolation and real-time PCR

RNeasy Lipid Tissue Mini Kit (QIAGEN) and RNeasy Mini Kit (QIAGEN) were used to isolate total RNAs from human adipose tissues and 3T3-L1 cells according to the manufacturer's instructions, respectively. Total RNA was exposed to RNase-free DNase I (QIAGEN) and reverse-transcribed to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Synthesized cDNA was analyzed by quantitative real-time PCR using the LightCycler (Roche Applied Science) with SYBR Premix Ex Taq (Takara) as previously described [18–20]. GAPDH was used for normalization. Primers used for the PCR were as follows (forward and reverse primers): human serglycin (5′-AATGCAGTCGGCTTGTCCTG-3′ and 5′-GCCTGATCCAGAGTAGTCCT-3′); mouse serglycin (5′-TGATGGAAGGACCCTCAAAA-3′ and 5′-GCTTTCATCTGTTGGCTGGT-3′); mouse peroxisome proliferator-activated receptor γ (PPARγ) (5′-CCCTGGCAAAGCATTTGTAT-3′ and 5′-GAAACTGGCACCCTTGAAAA-3′); and mouse adipocyte fatty acid-binding protein (aP2) (5′-CCGCAGACGACAGGA-3′ and 5′-CTCATGCCCTTTCATAAACT-3′).

2.4. Cell culture and induction of adipocyte differentiation

3T3-L1 cells were maintained as previously described [21]. For differentiation, cells were induced 1 day post-confluence with Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fetal bovine serum (FBS, GIBCO), 1 μg/ml insulin, 1 μM dexamethasone, 500 μM isobutylmethylxanthine, 100 U/ml penicillin, and 100 μg/ml streptomycin for 2 days. Two days after induction, the medium was changed to DMEM supplemented with 10% FBS and 1 μg/ml insulin, and 4 days after induction, the medium was changed to DMEM supplemented with 10% FBS. Days of differentiation were numbered consecutively after the second day of induction (day 0).

2.5. Immunoblot analysis and immunostaining

Cell lysates containing equal amounts of protein were electrophoresed on SDS–polyacrylamide gels and transferred to polyvinylidene difluoride membrane (Millipore) as previously described [22,23]. Blots were performed with primary antibodies against serglycin (D-19 and M-20, Santa Cruz Biotechnology) and horseradish peroxidase-conjugated anti-goat IgG as a secondary antibody. For immunostaining, specimens were fixed in 4% paraformaldehyde and stained with the anti-serglycin antibody and the VECTASTATIN ABC kit (Vector Laboratories).

2.6. Statistical analysis

All experiments were performed at least three times. Data are expressed as means ± SE and were analyzed by unpaired Student's *t*-test for comparisons between two groups or one-way ANOVA with post hoc analysis for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Genetic profiles of EAT

To compare gene expression profiles of EAT, OAT, and SCAT, we performed DNA microarray analysis using samples obtained from patients who underwent elective coronary artery bypass graft surgery for critical CAD. DNA microarrays showed that probes expressed in EAT, OAT, and SCAT in all patients numbered 15,449, 15,359, and 15,427, respectively (Fig. 1A). Among them, 15,304 probes were detected in all three tissues, and 231 probes were differentially expressed. As shown in Fig. 1B, 360 probes had a gene expression ratio of EAT to SCAT (EAT/SCAT) of more than two, and 77 probes had a gene expression ratio of OAT to SCAT (OAT/SCAT) of more than two. Thirty-nine probes were upregulated in both EAT and OAT, 321 probes were selectively upregulated in EAT, and 38 probes were selectively upregulated in OAT (Supplementary Tables 1–3). On the other hand, 158 probes had a gene expression ratio of EAT/SCAT of less than half, 56 probes had a gene expression ratio of OAT/SCAT of less than half, and 35 probes were

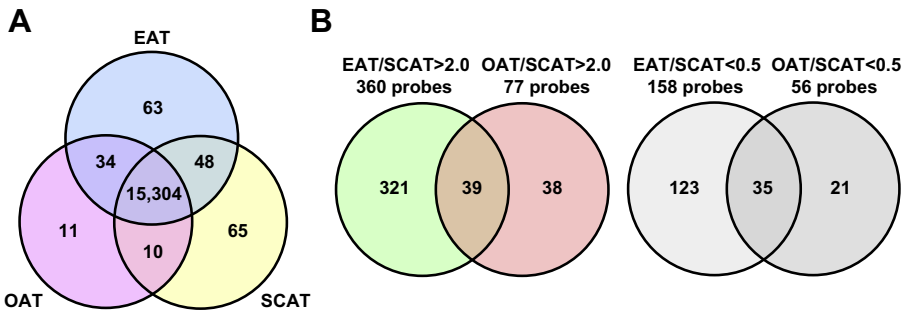

Fig. 1. Gene expression profiles of human adipose tissues. (A) Venn diagram of probes expressed in EAT, OAT, and SCAT in all three patients. (B) Left, Venn diagram of probes that have gene expression ratios of EAT to SCAT (EAT/SCAT) and OAT to SCAT (OAT/SCAT) of more than two. Right, Venn diagram of probes that have gene expression ratios of EAT/SCAT and OAT/SCAT of less than half.

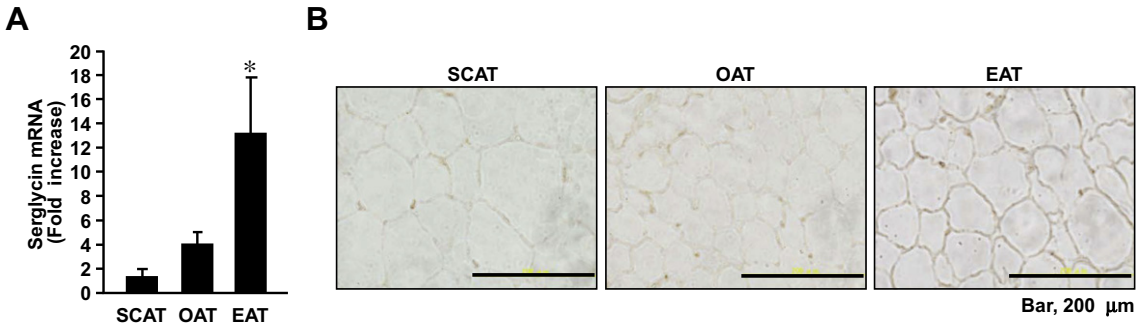

Fig. 2. Serglycin expression in human adipose tissues. (A) Expression of serglycin mRNA was analyzed by quantitative real-time PCR with total RNA isolated from SCAT, OAT, and EAT in patients who underwent elective coronary artery bypass graft surgery for critical coronary artery disease. GAPDH was used as a control for assessing RNA loading. *P<0.05 compared with SCAT. (B) Immunostaining of serglycin in human adipose tissues.

downregulated in both EAT and OAT compared with the level in SCAT.

3.2. Identification of serglycin in human epicardial adipocytes

Among 321 probes selectively upregulated in EAT, 55 genes were involved in inflammatory and immune responses (Supplementary Table 2). Among these 55 genes, we found that 35 genes, including interleukin-1β (IL-1β), IL-6, IL-8, and chemokine receptor 2, had more than 2-fold higher expression in EAT than in OAT. In this screen, we found serglycin to be one of the most abundantly expressed genes in EAT. Quantitative PCR revealed that in patients with CAD, serglycin mRNA expression in EAT was about 13-fold higher than that in SCAT (Fig. 2A). Immunostaining revealed that serglycin was localized to the membrane of adipocytes in EAT (Fig. 2B). Serglycin expression was also observed in adipocytes in SCAT and OAT. Thus, serglycin is expressed in adipocytes, especially those in EAT.

3.3. Expression of serglycin during adipocytic differentiation of 3T3L1 cells

To investigate serglycin expression during adipogenesis, we analyzed mRNA and protein expression using 3T3-L1 cells. As shown in Fig. 3A, upon induction of adipogenesis in 3T3-L1 cells, serglycin mRNA expression was upregulated and peaked at day

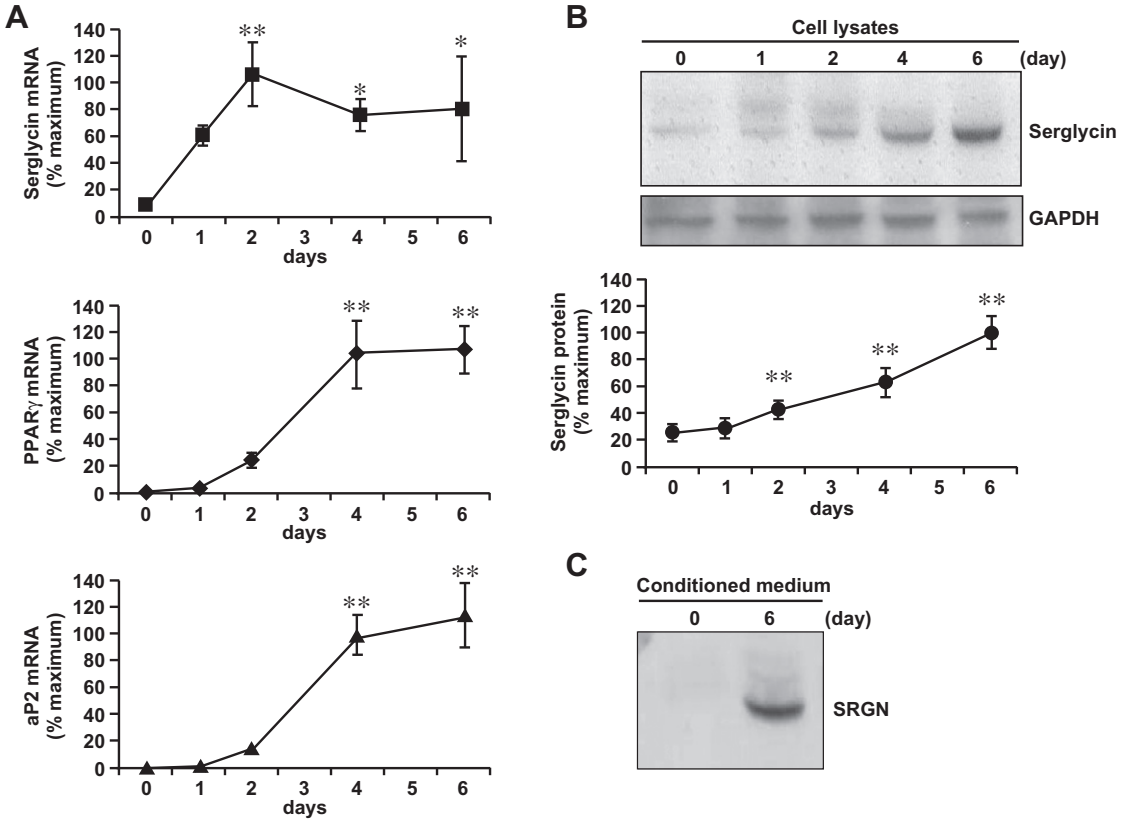

Fig. 3. Serglycin expression during adipogenesis. (A) Expression of serglycin, PPARγ, and aP2 mRNA was analyzed by quantitative real-time PCR with total RNA isolated from 3T3-L1 cells on the indicated days. *P<0.05 compared with day 0, and **P<0.01 compared with day 0. (B) Expression of serglycin protein was analyzed by Western blots using anti-serglycin antibody with cell lysates prepared from 3T3-L1 cells on the indicated days. GAPDH was used as a loading control. **P<0.01 compared with control. (C) Expression of serglycin protein was analyzed by Western blots using the anti-serglycin antibody with conditioned medium from 3T3-L1 cells on the indicated days.

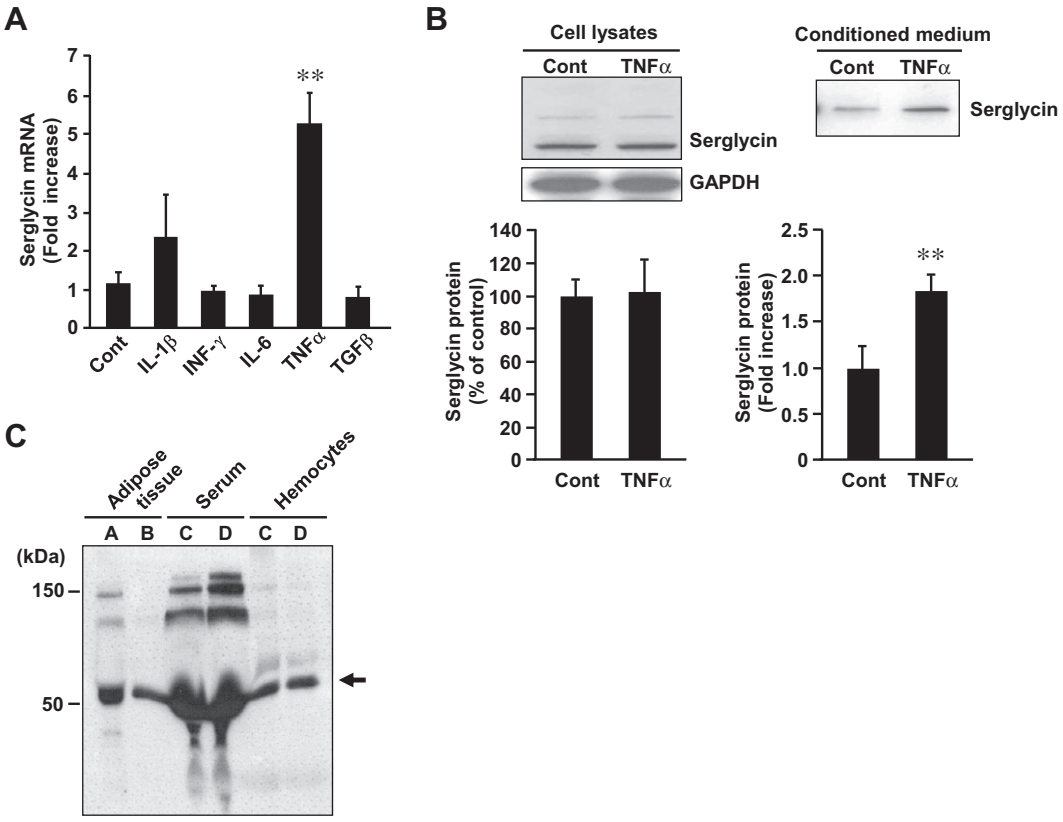

Fig. 4. Secretion of serglycin. (A) Expression of serglycin mRNA was analyzed by quantitative real-time PCR with total RNA isolated from differentiated 3T3-L1 cells. Differentiated 3T3-L1 cells at day 11 were treated with IL-1β (20 ng/ml), interferon-γ (IFN-γ, 10 ng/ml), IL-6 (20 ng/ml), TNFα (50 ng/ml), or transforming growth factor β (TGFβ, 5 ng/ml) for 24 h. GAPDH was used as a loading control. $^{**}P<0.01$ compared with control. (B) Expression of serglycin protein from cell lysates and conditioned medium from 3T3-L1 cells was analyzed by Western blots using anti-serglycin antibody. Differentiated 3T3-L1 cells at day 11 were treated with TNFα (50 ng/ml) for 24 h. GAPDH was used as a loading control. $^{**}P<0.01$ compared with control. (C) Expression of serglycin protein was analyzed by Western blots using the anti-serglycin antibody (D-19) with human subcutaneous adipose tissue, serum, and hemocytes. An arrow indicates serglycin protein. (A)–(D) indicate individuals from which each sample was obtained.

2, which preceded mRNA expression of PPARγ and aP2, which are adipocyte markers [24]. Immunoblotting using lysates from 3T3-L1 cells revealed that serglycin protein expression was barely detectable at day 0 but upregulated during adipogenesis (Fig. 3B). Furthermore, serglycin was detected in conditioned media from differentiated 3T3-L1 cells but not undifferentiated 3T3-L1 cells (Fig. 3C), indicating that serglycin is secreted from adipocytes.

3.4. Induction of expression and secretion of serglycin by TNFα

We then investigated whether serglycin was induced by cytokines involved in inflammation. Among cytokines examined in this study, TNFα induced serglycin mRNA expression in differentiated 3T3-L1 cells (Fig. 4A). Although TNFα did not alter serglycin protein expression in lysates from differentiated 3T3-L1 cells, it increased expression of serglycin protein in conditioned media (Fig. 4B), suggesting that TNFα stimulates the secretion of serglycin from adipocytes. To examine further whether serglycin is present in serum, we performed Western blotting using human serum samples. As shown in Fig. 4C, serglycin was detected in human serum.

4. Discussion

Although EAT has been shown to be a source of both pro-inflammatory and anti-inflammatory mediators, accumulating evidence suggests that local secretion of pro-inflammatory cytokines from EAT is predominant in severe CAD [13–16,25,26]. Using adipose tissues of patients with critical CAD, we detected 55 genes involved in inflammatory and immune responses among 321 probes selectively upregulated in EAT compared with SCAT, suggesting strong pro-inflammatory properties of EAT in patients with CAD, which is in accordance with previous studies [13,14]. Since the pro-inflammatory mediators are supposed to be involved in the atherosclerotic process [27], our data also suggest that inflammation-related genes in EAT contribute to the development of CAD.

Serglycin was originally identified as a hematopoietic cell proteoglycan species, being highly expressed by several hematopoietic cell types, such as mast cells, natural killer cells, cytotoxic T lymphocytes, platelets, and macrophages [28]. Thereafter, serglycin expression was also detected in nonhematopoietic cell types, such as endothelial cells, chondrocytes, and smooth muscle cells, and transformed cell types, such as multiple myeloma cells and nasopharyngeal carcinoma cells [28]. Serglycin has been shown to have a functional impact on processes of inflammatory and immune responses [28,29]. We presented here that serglycin expression in EAT is higher than that in SCAT, and that serglycin is expressed in adipocytes. EAT has been demonstrated to be an abundant source of pro-inflammatory cytokines, such as TNFα and IL-6 [25]. Adipose tissue contains various cell types, such as preadipocytes, mature adipocytes, and the stromal vascular fraction including endothelial cells, blood cells, and macrophages [30]. Substantial inflammatory cell infiltrates, predominantly represented by macrophages, have been observed in EAT obtained during cardiac surgery of subjects with severe CAD [13]. Considering that TNFα is primarily produced by macrophages and monocytes [27], inflammatory cells in EAT are likely the predominant source

of TNFα. In macrophages, serglycin has been shown to regulate secretion of TNFα [31]. We demonstrated that TNFα induces expression and secretion of serglycin in adipocytes. Therefore, serglycin and TNFα probably regulate each other's expression and secretion and mediate paracrine cross-talk between macrophages and adipocytes in EAT, which creates a vicious cycle of inflammatory changes. Furthermore, TNFα exerts potent pro-inflammatory effects in atherosclerosis [27]. TNFα also functions as a determinant of adipocytokine dysregulation in adipocytes of obese subjects [32]. Adipocytokines from EAT have been suggested to promote atheromatous plaque formation in the intima layer by passing into the myocardium via the vasa vasorum [26]. These observations suggest that serglycin and TNFα in EAT likely contribute to the development and progression of CAD through cross-talk between macrophages and adipocytes.

Increasing evidence has suggested that obesity is a chronic low-grade systemic inflammatory state [33,34]. In obesity, adipose tissue is characterized by increased infiltration of macrophages [26,35–37]. We also showed that serglycin is present in human serum. Although it remains unclear what tissue and cell type account for circulating serglycin, it is possible that circulating serglycin, in cooperation with pro-inflammatory proteins secreted from adipose tissue-derived macrophages, might be systemically involved in inflammation in adipose tissues. In addition, serum levels of both TNFα and IL-6 are raised in patients with CAD [38]. Since atherosclerosis has been recognized as a chronic inflammatory disease of the arterial wall [39–41], circulating serglycin and mediators of inflammation might also participate in the mechanisms of systemic vascular insult and atheromatous change.

In conclusion, serglycin is one of the most abundantly expressed proteins in adipocytes of EAT in patients with CAD. Serglycin is secreted from adipocytes, which is induced by TNFα, and present in human serum. These findings provide new evidence that serglycin is an adipocytokine highly expressed in EAT.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.078.

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