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Diminished levels of regulatory T cell subsets (CD8+Foxp3, CD4+Foxp3 and CD4+CD39+Foxp3) but increased Foxp3 expression in adipose tissue from overweight subjects

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ABSTRACT

Objectives: The aim of this study was to identify regulatory T cell (Treg) subsets residing in adipose tissue, demonstrate their immunosuppressive functions, and assess the possible role of Sirt1 in their function in overweight subjects.
Methods: Fat samples were obtained by lipoaspiration from healthy overweight (n = 15) and normoweight (n = 11) subjects. We obtained the stromal vascular fraction and then isolated the mononuclear cells by Ficoll-Hypaque sedimentation. The Treg subsets were analyzed by flow cytometry, the expression of Sirt1 and Foxp3 was detected by western blot, and peroxisome proliferator-activated receptor gamma (PPAR-γ) expression was evaluated by qPCR.
Results: We detected low numbers of Treg cell subsets displaying the phenotypes CD4+CD25−Foxp3+, CD8+CD25−Foxp3+, and CD4+CD39+Foxp3+ associated with increased body mass index in overweight subjects. We found lower levels of mRNA SIRT1 expression in adipocytes from overweight subjects than in those from normoweight subjects. In contrast, increased amounts of the Sirt1 and Foxp3 proteins in adipose tissue mononuclear cells from overweight subjects were observed. The immunosuppressive function of CD4+CD25+ Treg cells is higher in cells from obese subject than in those from normoweight subject.
Conclusions: Low levels of Treg subsets in overweight subjects with a high percentage of inhibition of proliferation could be related to high levels of the Foxp3 protein. Likewise, the low expression of SIRT1 and PPAR-γ mRNA levels and increased concentration of Sirt1 proteins allows adipose tissue mononuclear cells to respond to stimuli dependent on adenosine receptors and sirtuin pathways.

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Introduction

Obesity is considered a low-grade inflammatory disease associated with increased proinflammatory cytokine production, which contributes to the immune and metabolic alterations implicated in the development of insulin resistance [1,2]. Adipose tissue contains a large vascular stromal fraction with macrophages, endothelial cells, T and B lymphocytes, and adipose-derived stem cells [3,4]. The lean adipose tissue has an antiinflammatory phenotype due to infiltrated M2 macrophages, which favors the presence of Th2 cells, and is infiltrated by regulatory T cells (Treg) that help maintain insulin sensitivity and tissue homeostasis [5–8].

Regulatory T cells in adipose tissue are known as Fat-Tregs and possess characteristics distinct from Treg cells found in lymphoid organs [9,10]. The function of Treg cells depends on high Foxp3 expression levels, which are regulated transcriptionally and postranscriptionally [11,12]. Regulatory T cells also exert their suppressive function with the production of adenosine through the participation of the CD73 and CD39 ectonucleotidases and the secretion of interleukin-10 [13]. The numbers of Tregs infiltrated in adipose tissue decreased in studies of obese mice [14]. In contrast, in humans, there has been controversy caused by the failure to observe differences in the numbers of these peripheral cells in children with metabolic syndrome compared to a control group. However, there is an evident reduction in the number of Treg cells in the visceral adipose tissue of obese patients when compared to lean subjects [14,15].

Sirt1 is an nicotinamide adenine dinucleotide-dependent enzyme that deacetylates various histones and transcription factors in addition to influencing gene expression and protein activity. [16]. In adipose tissue, Sirt1 interacts with peroxisome proliferator-activated receptor gamma (PPAR-γ), promoting fat mobilization over storage [17]. Sirt1 is also a negative regulator of Foxp3, causing its deacetylation and polyubiquitination, resulting in proteosomal degradation [18]. Obesity causes the level of Sirt1 expression to decrease, favoring inflammation of the tissue and the abnormal storage of adipocytes [19]. In humans, the mRNA level of Sirt1 has been negatively correlated with the degree of adipose tissue inflammation [19,20]. However, the regulatory role of Sirt1 in adipocytes or adipose tissue mononuclear cells, as well as its relationship with Treg cells, has not been explored in detail. Therefore, the aim of this study was to identify the Treg cell subsets that reside in adipose tissue of normoweight and overweight subjects and to evaluate the possible role of Sirt1 in the function of these Tregs.

Materials and methods

Sample

A group of healthy subjects was divided according to their body mass index (BMI) into overweight (BMI >25, n = 15) and normal weight (BMI <25, n = 11) groups. All subjects were female, and their mean age was 38.7 y (range: 27–58 y). Patients presenting with pregnancy or chronic-degenerative diseases were excluded. The study was approved by the Ethical Committee of Comité de Ética en Investigación y Docencia de la Facultad de Ciencias Químicas, UASLP (CEID2013006).

Adipose tissue sample

Samples of adipose tissue were subjected to digestion with 0.03% collagenase Type I solution (ThermoFisher Scientific, Waltham, MA, USA) in RPMI-1640 (Gibco) and 1.5% bovine serum albumin (Sigma-Aldrich Corporation, St. Louis, MO, USA) for 60 min at 37°C. The stromal vascular fraction from the pellet at the bottom of the tube was recovered, and the adipocyte fraction (at the top of the tube) containing mature adipocytes was used for western blot and quantitative polymerase chain reaction (PCR) assays. Mononuclear cells (adipose tissue mononuclear cells) were isolated from the stromal vascular fraction using the Ficoll-Hypaque method, and cellular viability was determined using trypan blue.

Flow cytometry analysis

The cells were incubated with anti-CD4-PerCP or anti-CD8-APC (eBioscience, San Diego, CA, USA) and anti-CD25-FITC and anti-CD39-FITC (Biolegend, San Diego, CA, USA). For Foxp3 intracellular staining, fixation/permeabilization buffer (eBioscience) was added, and the cells were incubated for 30 min at 4°C in the presence of anti Foxp3-PE (eBioscience). Fluorescence-positive cells were quantified by FACS using a FACSCanto II cytofluorometer (BD Bioscience, San Diego, CA, USA) and BD FACSDiva software (BD Biosciences).

Real time PCR

Total RNA was isolated using the Trizol method (ThermoFisher Scientific). We obtained cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem, San Francisco, CA, USA). cDNA was used for the measurement of SIRT1 mRNA expression by quantitative real time PCR using Taqman Sondes (Applied Biosystem) and PPAR-γ expression using specific primers in a RT thermocycler CFX96 Real Time System (Bio-Rad Laboratories, Hercules, CA, USA). The results obtained were analyzed with the 2-ΔΔCq method. The housekeeping gene 18S was used as an internal control and to normalize the values of the expression of Sirt1 and PPAR-γ (ΔCq).

Western blot

Total protein in the cellular lysate was quantified using the bicinchoninic acid method (BCA Protein Assay Reagent, ThermoFisher Scientific). Each sample was denatured and loaded into a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. The separated proteins were transferred to a nitrocellulose membrane in a Trans-Blot Turbo apparatus (Bio-Rad). The membrane was incubated with anti-Sirt1 or anti-Foxp3 primary antibody (Abcam, San Francisco, CA, USA) followed with goat anti-rabbit HRP (Abcam) or goat anti-mouse HRP (Becton Dickinson, Franklin Lakes, New Jersey, USA) secondary antibody. The membrane was revealed by a chemiluminescence reagent (Pierce ECL Western Blotting Substrate, ThermoScientific Corporation) and imaged in a Chemi Doc system (Molecular Imager, Gel Doc XR+, Bio-Rad). The results were analyzed, and the relation between the mean intensity of β-actin protein (as internal control) and the intensity of Sirt1 or Foxp3 expression was used to normalize the densitometric data obtained.

Isolation of regulatory T cells ($CD4^+CD25^+$)

The CD4+CD25+ Treg cells were freshly isolated from adipose tissue mononuclear cells using the regulatory T cell separation kit (Miltenyi Biotech, Auburn, CA, USA). Then, CD25+ T cells were isolated from the CD4+ T cell population using magnetic microbeads with anti-CD25 antibodies (Miltenyi). We collected CD4+CD25+ Treg cells and CD4+CD25− T cell responders (Tresp).

Proliferation and immunosuppressive assays

Cell proliferation was assessed with the carboxyfluorescein dilution assay. The CD4+CD25− Tresp were stimulated with 5 μg/mL plate-bound anti-CD3 and anti-CD28 (eBioscience). In additional assays, the selective A2A agonist (70 μM CGS21680) or stimulator of sirtuins (300 μM resveratrol) (Sigma-Aldrich) was added 60 min before the anti-CD3/anti-CD28. Then, a classic in vitro Treg and Tresp co-culture system was used to detect the immunosuppressive function of CD4+CD25+ Treg cells. The cells were plated in proportions of 1:1, 1:2, and 1:4 Treg:Tresp for 5 d. The percentage of suppression was evaluated as

Table 1
Anthropometric and biochemical parameters of study subjects

Parameters	Normoweight	Overweight	*P*-value
Number	11	15	
Age (y)	38.1 ± 6.9	42.1 ± 15.9	0.2430
Weight (kg)	58.4 ± 9.6	73.3 ± 15.8	0.0004*
Height (m)	1.60 ± 0.08	1.63 ± 0.09	0.1992
BMI (kg/m^2)	22.9 ± 1.31	27.4 ± 7.2	0.0001*
Glucose (mg/dL)	73.8 ± 31.2	93.4 ± 11.6	0.3409
Creatinine (mg/dL)	0.66 ± 0.06	0.86 ± 0.39	0.2091
Hemoglobin (g/L)	13.08 ± 0.58	14.5 ± 5.41	0.5708
Cholesterol (mg/dL)	220 ± 27	217.8 ± 93.9	0.9551
Triacylglycerols (mg/dL)	159.8 ± 0.2	162.2 ± 156.8	0.9370

Samples of adipose tissue were collected from healthy individuals receiving esthetic plastic surgery. The subjects were divided according to their body mass index (BMI): healthy overweight subjects with BMI >25 (n = 15) and normoweight subjects with BMI <25 (n = 11)

* $P < 0.05$.