Clinica Chimica Acta 438 (2015) 382–387

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Ethnic differences in pre-adipocyte intracellular lipid accumulation and alkaline phosphatase activity

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ARTICLE INFO

Article history:
Received 21 August 2014
Received in revised form 11 September 2014
Accepted 13 September 2014
Available online 1 October 2014

Keywords:
Intracellular lipid accumulation
Histidine
Alkaline phosphatase

ABSTRACT

Alkaline phosphatase (ALP) increases lipid accumulation in human pre-adipocytes. This study was performed to assess whether ethnic differences in the prevalence of obesity in African and European females are related to differences in pre-adipocyte lipid accretion and ALP activity. Pre-adipocytes were isolated from 13 black and 14 white females. Adipogenesis was quantified using the lipid dye, Oil red O, whilst ALP activity was assayed in cell extracts on day zero and 12 days after initiating adipogenesis. Lipid levels (OD units/mg protein) were lower in pre-adipocytes from white than black females on day 0 (0.36 ± 0.05 versus 0.44 ± 0.03, respectively; $p < 0.0005$) and day 12 (1.18 ± 0.14 versus 1.80 ± 0.22, respectively; $p < 0.0005$), as was ALP activity (mU/mg protein) on day zero (36.5 ± 5.8 versus 136.4 ± 10.9, respectively; $p < 0.0005$) and day 12 (127 ± 16 versus 278 ± 27, respectively; $p < 0.0005$). Treatment of pre-adipocytes with histidine, an ALP inhibitor, blocked lipid accumulation. Thus, lipid uptake is higher in pre-adipocytes isolated from black compared to white females which parallels the obesity prevalence rates in these population groups. The reason for higher fat accumulation in pre-adipocytes isolated from black females may be related to higher ALP activity.

1. Introduction

Alkaline phosphatases (ALP; EC 3.1.3.1) are a class of enzymes that catalyse a number of different reactions involving phosphate groups and which include the hydrolysis of phosphate esters from organic molecules of low molecular mass [1], and phosphotransferase [2] and protein phosphatase reactions [3]. There are four alkaline phosphatase isoenzymes, each coded by a different gene and which are termed intestinal, placental and germ cell ALP (collectively known as the tissue specific ALPs) and tissue nonspecific ALP (TNALP). Tissue nonspecific ALP is also known as liver/bone/kidney ALP. The genes encoding the three tissue specific isoenzymes are on the long arm of chromosome 2, whilst the gene for TNALP is located on chromosome 1 [4]. The tissue specific and tissue nonspecific isoenzymes can be differentiated from each other, not only by their gene sequences, but also by their biochemical response to specific inhibitors. Thus, TNALP is inhibited by levamisole, histidine and homoarginine but not by L-phenylalanyl-glycyl-glycine (Phe-Gly-Gly), whilst the reverse is true for the tissue specific forms [5,6]. Despite the importance of ALP in a number of different biological processes including fat accumulation [7,8], tumorigenesis [9], and skeletal mineralization [10], little is known about its mode of action.

Serum levels of the tissue specific ALPs may be elevated during pregnancy (placental ALP) or after food ingestion (intestinal ALP) or due to the presence of different cancer types including lung, ovarian, testicular and colorectal. The principal reasons for high levels of serum TNALP are hepatobiliary disease, liver and bone cancers and diseases involving heightened osteoblastic activity e.g. Paget's disease [11]. Besides being expressed in liver, bone and kidney, TNALP has also been detected in a number of different mammalian tissues, including the mammary gland [12], the adrenal gland [13] and the pancreas [14]. Studies within our laboratory have also shown that TNALP is present within the murine 3T3-L1 pre-adipocyte cell line [7] and human pre-adipocytes [8]. Pre-adipocytes mature into adipocytes by a process termed adipogenesis, the defining characteristic of which is the intra-cellular accumulation of membrane-bound lipid droplets. Tissue nonspecific ALP is located on the membrane of these intra-cellular lipid droplets [7], and intracellular lipid accumulation (ICLA) is inhibited by the treatment of pre-adipocytes with inhibitors of TNALP. Furthermore, the level of ICLA within pre-adipocytes correlates strongly with the cellular level of TNALP activity [8]. These studies suggest that TNALP is an important regulator of lipid storage and that its level of expression may influence

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http://dx.doi.org/10.1016/j.cca.2014.09.022
0009-8981/

the ability of pre-adipocytes to accumulate lipid during the process of adipogenesis.

Studies in adult populations have shown that the prevalence of obesity, particularly in females, is higher in African subjects resident in North America or Africa when compared to European subjects living in the same country [15,16]. The reasons for these ethnic differences in the prevalence of obesity may include cultural and socio-economic influences [15]. It is also possible that physiological factors are involved, most particularly the hypothalamic regulation of appetite and the level of hyperplasia and hypertrophy within adipose tissue. Thus, genome-wide association studies have already demonstrated that polymorphisms within, or lying close to genes controlling appetite regulation and adipocyte function are related to the level of adiposity [17]. It is interesting to note that a polymorphism within the TNALP gene has been associated with waist-to-hip ratio [18], an effect that may be mediated by the ability of TNALP to regulate ICLA during adipogenesis in human pre-adipocytes [8]. There is evidence to suggest that the level of adipogenesis may influence body fat mass and this comes from genetic studies showing that some rare forms of monogenic obesity [19], as well as common polygenic obesity [20], are caused by DNA sequence variants that lie close to, or within genes involved in the regulation of adipogenesis.

Therefore, the hypothesis of the current study is that ethnic differences in the prevalence of obesity may partly be due to ethnic differences in the level of pre-adipocyte adipogenesis. Thus, the principle aim of the present study was to determine whether the adipogenic potential of pre-adipocytes differed between two female population groups with different prevalence levels of obesity (European and African) and whether ethnic differences in pre-adipocytic TNALP activity mirror these differences.

2. Materials and methods

2.1. Reagents

All tissue culture reagents were purchased from Invitrogen (Invitrogen Corporation, Paisley, Scotland) and all laboratory reagents were purchased from Sigma-Aldrich (Sigma-Aldrich, Aston Manor, South Africa), unless otherwise stated.

2.2. Subjects and adipose tissue isolation

Human adipose tissue samples were obtained from the mammary adipose tissue of 14 white and 13 black women. All subjects were undergoing elective surgical mammary gland reduction and all were healthy as assessed by clinical history and physical examination.

Ethical approval for the use of the adipose tissue was obtained from the University of Witwatersrand, Faculty of Health Sciences Human Ethics Committee.

2.3. Isolation of pre-adipocytes from adipose tissue

Adipose tissue was processed immediately after removal, and was transferred to the laboratory in human adipocyte isolation medium consisting of sterile Hanks balanced salt solution supplemented with 25 mmol/l HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin and 3% bovine serum albumin (BSA).

Pre-adipocytes were isolated using a previously published method [8]. After excision of blood vessels, adipose tissue was minced into small pieces, washed once in Hank's balanced salt solution (HBSS), and centrifuged for 5 min at 380 *g*. Tissue was then decanted into isolation medium (0.7 g tissue/ml HBSS supplemented with 25 mmol/l HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 3% BSA, and 0.75 mg/ml collagenase) and digested for 1 h at 37 °C with constant shaking. Suspended cells were then filtered through a 250 μm metal filter and the filtrate spun for 10 min at 380 g. The pellet of stromavascular cells was re-suspended in 100 ml of red cell lysis buffer (0.154 mol/l ammonium chloride, 10 mmol/l potassium bicarbonate, 0.1 mmol/l EDTA and 10% foetal bovine serum) and allowed to settle for 10 min at room temperature followed by 10 min centrifugation at 380 *g*. The cell pellet was resuspended in human pre-adipocyte tissue culture medium (DMEM-Ham's F12 medium containing 15 mmol/l HEPES, 2 mmol/l glutamine, 10% foetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin) and then filtered through a 20 μm nylon mesh and aliquoted into 6-well tissue culture plates. The cells were cultured overnight at 37 °C in humidified atmosphere with 5% CO_2 and the next day washed with DMEM-Ham's F12 medium. The medium was changed every third day until the cells were confluent. Pre-adipocytes were stimulated to differentiate over 12 days in differentiation medium supplemented with 100 nmol/l hydrocortisone, 66 nmol/l insulin (Novo-Nordisk, Copenhagen, Denmark), 0.5 mmol/l 3-isobuty-1-methylxanthine (IBMX) and 1 nmol/l triiodo-1-thyronine.

2.4. Alkaline phosphatase inhibitors

Pre-adipocytes were cultured in the absence or presence of ALP inhibitors, these being added with the differentiation medium and refreshed every third day. The inhibitors included the tissue non-specific ALP inhibitor, histidine (Merck, Darmstadt, Germany) and the tissue specific ALP inhibitor, Phe-Gly-Gly [5,6]. The concentrations of these inhibitors in the differentiation medium were 50 mmol/l and 20 mmol/l respectively. The concentrations chosen were based on a previous study using the murine 3T3-L1 pre-adipocyte cell line [7].

2.5. Extraction of ALP from human pre-adipocytes

Cellular extracts from pre-adipocytes were isolated at baseline and 12 days after initiation of adipogenesis using a previously published method [8]. Briefly, tissue culture medium was removed from the cells and 0.5 ml of an ice cold solution of 10 mmol/l Tris-HCl containing 1% Triton X-100 and 2 mmol/l phenylmethylsulfonyl-fluoride (pH 7.2) was added. The flasks were shaken to detach the cells and the suspension transferred to an Eppendorf tube and centrifuged for 10 min at 15,000 *g*. The supernatant was removed and immediately analysed for alkaline phosphatase activity using an automated colorimetric assay (Roche Diagnostics, Randburg, South Africa). The protein content of the supernatant was analysed using the Bradford method [21]. Alkaline phosphatase activity was calculated as mU of activity per mg protein.

2.6. Measurement of cellular lipid accumulation

Intracellular lipid accumulation, which is the hallmark of adipogenesis was measured on day zero and day 12 using the Oil red O technique [22]. This technique depends on the ability of the lipid droplets in the adipocyte to collect the red stain (the stain only binds to triglycerides and cholesteryl oleate), which is then extracted from the cells using isopropyl alcohol, and the absorbance was measured at 510 nm [22]. Intracellular lipid accumulation was then expressed as OD units per mg of cellular protein.

2.7. Detection of polymorphisms in promoter region of TNALP gene

Genomic DNA was isolated from buffy coat preparations taken from 6 white (BMI, 31.5 ± 4.5; age, 39.5 ± 11.9 years) and 6 black (BMI, 33.0 ± 4.6; age, 49.7 ± 6.3 years) females using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Primers were designed for PCR amplification of the promoter region of the human TNALP gene which provisionally lies within a 3780 bp region 5′ to the major transcription start site [23]. Primer design was accomplished using the Gene Runner software, version 3.05 (Hastings Software, Inc., Hastings, NY, USA). This region was amplified using 7 sets of overlapping PCR primers. The initial denaturation temperature was 95 °C for 11 min whilst the extension temperature was 72 °C for 10 min for all the PCR reactions. The cycling conditions for all reactions