The role of alkaline phosphatase in intracellular lipid accumulation in the human hepatocarcinoma cell line, HepG2

George M Chirambo, Chantal van Niekerk, Nigel J Crowther

Department of Chemical Pathology, National Health Laboratory Service, University of Witwatersrand Medical School, Johannesburg, South Africa
Department of Biochemistry, College Of Medicine, University of Malawi, Blantyre, Malawi

ABSTRACT

Inhibition of tissue non-specific alkaline phosphatase (TNALP) decreases intracellular lipid accumulation in human preadipocytes and the murine preadipocyte cell line, 3T3-L1. Therefore, the current study was performed to determine if TNALP is required for intracellular lipid deposition in the human hepatocyte cell line, HepG2. Intracellular lipid accumulation, TNALP activity and peroxisome proliferator activated receptor (PPAR)γ gene expression were measured in HepG2 and 3T3-L1 cells in the presence and absence of the TNALP inhibitors levamisole and histidine. Sub-cellular TNALP activity was localized using cytochemical analysis. Both PPARγ gene expression and TNALP activity increased during intracellular lipid accumulation in HepG2 and 3T3-L1 cells. Inhibition of TNALP blocked intracellular lipid accumulation but did not alter expression of the PPARγ gene. In HepG2 cells, TNALP co-localized with adipophilin on the lipid droplet membrane. These data suggest a role for TNALP in lipid droplet formation, possibly downstream from PPARγ, within HepG2 and 3T3-L1 cells.

1. Introduction

Alkaline phosphatases (ALPs) are a group of membrane-bound glycoproteins that hydrolyze a broad range of monophosphate esters at alkaline pH optima (Price, 1993). There are four ALP isoenzymes termed tissue non-specific, intestinal, placental and germ cell, each coded by a separate gene (Moss, 1992). Tissue non-specific alkaline phosphatase (TNALP) comprises of three different isoforms called liver, bone and kidney ALP. These enzymes are coded by the same gene but differ in terms of their pattern of glycosylation (Nosjean et al., 1997). Tissue non-specific alkaline phosphatase has a broad tissue distribution however, its precise function and mode of action are not fully understood.

Alkaline phosphatase activity is found in human blood and is a commonly measured analyte in clinical chemistry laboratories. Obstruction of the bile duct is one of the main causes of elevated ALP levels in human serum. Also, increased levels of ALP may be observed in serum of the bile duct is one of the main causes of elevated ALP levels in human serum. Also, increased levels of ALP may be observed in serum of bone or liver pathology. Studies have shown that TNALP is expressed in human preadipocytes (Ali et al., 2006a) and in the murine preadipocyte cell lines, 3T3-L1 (Ali et al., 2005) and 3T3-F442A (Hernández-Mosqueira et al., 2015). The 3T3-L1 cell line has proven to be an excellent model for studying preadipocyte differentiation and maturation (Ntambi and Young-Cheul, 2000). The proliferation and determination of preadipocytes is associated with the specific activation of a number of transcriptional factors and an upward regulation of adipogenic genes (Cornelius et al., 1994). The hallmark of adipocyte maturation is intracellular accumulation of lipid within membrane bound lipid droplets (Gregoire et al., 1998), with the peroxisome proliferator activated receptor (PPAR)γ transcription factor acting as a prime regulator of adipogenesis (Spiegelman, 1998).

A role for TNALP in intracellular lipid accumulation in 3T3-L1 cells and human preadipocytes has been proven using inhibitors of TNALP (Ali et al., 2006a; Ali et al., 2006b; Ali et al., 2005), and studies have shown that this enzyme is localized to the membrane surrounding the lipid droplet (Ali et al., 2006a; Ali et al., 2005). However, the exact role of TNALP in adipogenesis is not yet understood. It is known that TNALP is expressed in hepatocytes (Price, 1993), and that these cells are also able to accumulate intracellular lipid within membrane-bound droplets. These organelles express a number of proteins on their surfaces that are common to both adipocytes and hepatocytes (Okumura, 2011). Excessive acquisition of lipid within hepatocytes is...
observed in alcoholic and non-alcoholic steatohepatitis (NASH) with the latter now being one of the most common liver pathologies observed in developed countries, a probable consequence of the high prevalence of obesity (Press and Sattar, 2008).

The presence of TNALP in preadipocytes and hepatocytes, the ability of both cell types to accumulate lipid within membrane-bound lipid droplets, and the evidence that TNALP is involved in intracellular lipid storage within preadipocytes forms the basis of our hypothesis that TNALP plays a role in lipid accumulation within hepatocytes. This hypothesis was tested by inducing lipid accumulation in the human hepatoma cell line, HepG2 and 3T3-L1 cells in the presence and absence of the TNALP inhibitors, levamisole and histidine. The expression of the adipogenic regulator, PPARγ was also assessed and the sub-cellular localization of TNALP was analysed via cytochemical analysis.

2. Materials and methods

2.1. Culture of HepG2 and 3T3-L1 cells

All tissue culture reagents were obtained from BioWhittaker (Walkersville, MD, USA) and GibCo Invitrogen (Paisley, Scotland) unless otherwise stated. HepG2 and 3T3-L1 cells were obtained from the American Type Culture Collection (ATCC).

HepG2 cells were grown in Earle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/l glutamine, non-essential amino acids, 1 mmol/l sodium pyruvate (maintenance medium) and oleic acid coupled to albumin (Sigma-Aldrich, St Louis, MO, USA) at a final concentration of 400 μmol/l of oleic acid, to induce intracellular lipid accumulation (Brasaemle et al., 1997). The maintenance medium was replaced every third day until the cells were ready for experiments.

The 3T3-L1 cells were maintained in Dulbecco’s modified eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mmol/l glutamine. When the cells reached confluence, 1.70 μmol/l insulin (Novo-Nordisk, Denmark), 0.50 mmol/l 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich, St Louis, MO, USA) and 0.22 mmol/l dexamethasone (Sigma-Aldrich, St Louis, MO, USA) were added to the culture medium to initiate adipogenesis. This transformation medium was removed after 3 days and replaced with similar medium containing insulin only. Following a further 3 days of culture this medium was replaced with the original maintenance medium which was changed every 3 days.

2.2. Use of TNALP inhibitors

In HepG2 cells levamisole was used at a final concentration of 3.0 mmol/l whilst histidine was used at a concentration of 75.0 mmol/l. In 3T3-L1 cells levamisole was used at final concentration of 2.0 mmol/l whilst histidine was used at a concentration of 50.0 mmol/l. The concentrations of the TNALP inhibitors were derived from the literature for the 3T3-L1 cells (Ali et al., 2005) whilst dose response curves were performed for the HepG2 cells based on the concentrations used for the preadipocyte cell line. The final concentrations chosen were those that gave maximal inhibition. The inhibitors were added to the culture medium simultaneous with the induction of lipid droplet formation.

2.3. Measurements of intracellular lipid accumulation, TNALP activity and total protein

Intracellular lipid accumulation was measured at baseline (day 0) and at 4, 7 and 11 days after the induction of intracellular lipid formation in HepG2 and 3T3-L1 cells using the lipid-specific dye Oil Red O (Laughton, 1986). TNALP activity was determined at the same time points as the cellular lipid levels using a previously published method (Ali et al., 2005). The cellular protein content was analysed using the Bradford method (Bradford, 1976).

2.4. PPARγ gene expression in HepG2 and 3T3-L1 cells

Total RNA was isolated from cell cultures on day 0, 4 and 7 post-induction of lipid droplet formation using an RNasy Mini kit (Qiagen, Hilden, Germany). Synthesis of cDNA from the total RNA was then carried out using an oligo dT primer (Inqaba Biotech, Pretoria, South Africa), and Moloney murine leukemia virus reverse transcriptase (Promega, Madison WI, USA). Quantitative real-time PCR was then performed with the TATA-box binding protein (TBP) gene used as an endogenous internal control. Primers were designed using the GeneRunner Software (Hasting Software Inc., Las Vegas, USA) and were synthesized by Inqaba Biotech (Pretoria, South Africa). The TBP primer sequences for the HepG2 cells were: forward, 5′-CAG TGA CCC AGC AGC ATC -3′ and reverse, 5′-GTC AGT CCT GCA TTA AGG C-3′, producing an amplicon of 277 bp. The sequences for the 3T3-L1 TBP primers were: forward, 5′-ACC CTT CAC AAT TGA AAT GCC-3′ and reverse, 5′-ATG ATG ACT GCA GGA AAT GCC-3′, with an amplicon length of 189 bp. The primer sequences of the PPARγ gene for the HepG2 cells were as follows: forward, 5′-GTC TAC AGA CAT GCC A-3′ and reverse, 5′-CAA AGG AGT GGG AGT GGT C-3′, producing an amplicon of 88 bp. The sequences for the 3T3-L1 PPARγ primers were: forward, 5′-CAG GAT CCT GGC TTC GCT -3′ and reverse, 5′-CAC CCA CTA TIG GGT CAC TGC -3′ producing an amplicon of 240 bp.

Real-time quantitative PCR of the PPARγ and TBP cDNA was carried out using a Rotorgene 6000 (Qiagen, Hilden, Germany) thermocycler and a SensiMix SYBR kit (Quantace Ltd., London, UK). The amplification conditions for the PPARγ and TBP gene products from the HepG2 cells comprised of an annealing temperature of 57 °C and an elongation time of 20 s. whilst for the 3T3-L1 cells, the annealing temperature was 60 °C, with the same annealing time as for the HepG2 cells. A 5-point standard curve was set up using cDNA isolated from the respective cell line and ranging in concentration from 14 to 272 ng/μl. The cDNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

2.5. Sub-cellular localization of TNALP

The HepG2 and 3T3-L1 cells were cultured on cover slips coated in gelatin (BDH, Poole, UK), and induced to accumulate lipid using the methods described in Section 2.1. Cells were harvested between days 4–8 of culture, at which point they were fixed using a 3% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) solution and permeabilized with a 0.01% saponin (Sigma-Aldrich, St Louis, MO, USA) solution. The 3T3-L1 cells were incubated with a rabbit anti-perilipin antibody (Cell Signaling Technology Beverly, MA, USA) whilst the HepG2 cells were incubated with a guinea pig anti-adipophilin antibody (Progen Biotechnik, Heidelberg, Germany), overnight at 4 °C. The 3T3-L1 cells were incubated with a goat anti-rabbit immunoglobulin G antibody labeled with Alexa Fluor 594, and the HepG2 cells were incubated with a goat anti-guinea pig immunoglobulin G antibody labeled with Alexa Fluor 488 (both secondary antibodies supplied by Life Technologies, Grand Island, NY, USA). The incubation period was 1 h, in the dark at room temperature. Both cell types were then stained for TNALP activity using a synthetic substrate, ELF97 (Invitrogen Molecular Probes, Leiden, Holland) that gives rise to a fluorescent yellow/green end product that precipitates at the site of TNALP activity. The incubation with ELF97 lasted for 15 min, at room temperature. The cover slips were then mounted onto a microscope slide and visualized at a magnification of 100 using an Olympus 1 × 71 microscope fitted with an Olympus XM10 camera (Olympus, Hamburg, Germany).