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Lipoprotein lipase in chronic lymphocytic leukaemia – Strong biomarker with lack of functional significance

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ABSTRACT

In chronic lymphocytic leukaemia (CLL), lipoprotein lipase (LPL) mRNA overexpression is an established poor prognostic marker, its function, however, is poorly understood. Measuring extracellular LPL enzymatic activity and protein, we found no difference between levels in CLL patients and those of controls, both before and after heparin treatment *in vivo* and *in vitro*. Investigating LPL knock down effects, we determined five potential downstream targets, of which one gene, STXBP3, reportedly is involved in fatty acid metabolism.

While possibly reflecting an epigenetic switch towards an incorrect transcriptional program, LPL overexpression by itself does not appear to significantly influence CLL cell survival.

1. Introduction

Chronic lymphocytic leukaemia (CLL) is the most frequent leukaemia in Western countries. Median age at diagnosis is 64–70 years, survival times differ considerably between patients and range from normal life expectancy (>20 years) to a few years in high-risk groups [1,2]. Prognostic factors include clinical staging systems (Rai and Binet), cytogenetics, serum markers, expression of surface molecules, and genetic markers, particularly the mutational status of immunoglobulin heavy chain variable region genes (IGHV) [1,3–6]. Several surrogate markers for IGHV mutational status have been identified in recent years, ZAP-70 protein and lipoprotein lipase (LPL) mRNA expression being the most reliable ones [7–11]. LPL predicts overall and treatment free survival [12–18]. LPL mRNA expression alone or integrated into scoring systems have established its prognostic value in CLL [19–22].

Given the central role of LPL in lipid metabolism it is attractive to speculate on the impact of its overexpression on the survival of CLL cells. However, data on the functional role of LPL in CLL are very rare and in part conflicting. Our previous data suggest that LPL protein is also found on the cell surface of CLL cells but that mRNA overexpression is mainly associated with an increase of *intracellular* LPL protein [23]. We therefore hypothesized that CLL cells can synthesize LPL protein that in part is secreted and bound to the cell membrane [12]. A correlation of LPL mRNA with protein expression in CLL was also observed by Mansouri and colleagues, who suggested that the majority of protein might be catalytically inactive [24]. On the other hand, Wendtner and colleagues suggested that lipid metabolism is causally related to the survival of CLL cells based on the induction of apoptosis by tetrahydrolipostatin (Orlistat) or erufosine, a synthetic phospholipid analogue with antineoplastic activity, treatment *ex vivo* [25,26]. *In vivo*, decreased high density lipoprotein cholesterol (HDL-C) levels were observed in the plasma of CLL and ALL patients compared to healthy individuals [27]. Nevertheless, so far there are no published data, which prove a distinct and specific functional role of LPL in CLL.

LPL function is best characterized in endothelial cells [28,29]. LPL is produced in parenchymal cells and transported to

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endothelial cells that bind LPL by heparan sulfate–proteoglycan (HSPG) on the luminal cell surface [28]. Intravenous injection of heparin competes for these HSPG binding sites and liberates LPL into circulation, leading to an increase in LPL plasma protein concentration and enzymatic activity [28]. This so called post heparin lipolytic activity (PHLA) is an established diagnostic test for lipid metabolism disorders.

In the present study we assessed LPL protein concentration and enzyme activity in serum and plasma of CLL patients with high or low LPL mRNA expression before and after heparin treatment. These *in vivo* experiments were complemented by *in vitro* treatment of CLL primary cells with heparin and purified LPL protein. LPL knockdown in CLL cells by siRNA was performed to study changes in functional behaviour of CLL cells and gene expression.

2. Patients, materials and methods

2.1. Patients

A total number of 114 fully characterized, untreated chronic lymphocytic leukaemia patients diagnosed at the Division of Haematology at the Medical University of Vienna (Austria) were retrospectively screened for LPL mRNA expression and survival curves were calculated. In this cohort, we confirmed the prognostic power of LPL mRNA expression regarding OS and TFS (Supplementary data and Supplementary Fig. 1). Forty-two patients, for which serum and plasma samples were available, were studied with respect to LPL protein levels in serum and LPL enzymatic activity in plasma. Of these 42 patients, thirteen patients were selected to study LPL release after heparin injection. Seventeen of these patients were selected for LPL knock down experiments. Selection criteria were (1) availability of material needed for knock down and (2) high LPL mRNA expression at sample date, with the goal to maximize mRNA expression reduction through knock down. All patients were untreated. Basic clinical information for the study cohorts is listed in Supplementary Table 1. Three healthy individuals (HIs) and 14 non-CLL patients (lymphoma patients in complete remission) served as controls. All participants gave informed consent according to the Declaration of Helsinki, approval of the institutional Ethics Committee was obtained for this study (Ethics Committee number: 035/2007). Serum, plasma and peripheral blood cells were collected. Peripheral blood mononuclear cells (PBMC) were isolated using standardized Ficoll–Hypaque gradient centrifugation (Seromed, Berlin, Germany).

2.2. Cell culture

Primary CLL PBMC, cells of the cell lines HeLa (cervix carcinoma), Hep3B (hepatocellular carcinoma), CCL228 (colon carcinoma), THP1 (monocytic leukaemia) and RPMI8226 (plasma cells of multiple myeloma) were cultured at 37 °C and 5% CO_2 in GIBCO RPMI 1640 (Life Technologies, Carlsbad, CA, USA) containing 10% foetal calf serum (FCS Gold), 1% penicillin/streptomycin (PS) and 1% L-glutamine (all PAA Laboratories, Linz, Austria).

2.3. Heparin provocation test in vivo

To investigate *in vivo* mobilization of LPL in CLL patients (PHLA), a heparin release study on a cohort of 13 fully characterized CLL patients, was performed. This cohort represents consecutive CLL patients, which consented in writing to participate in this part of the study. Patients received 50 U/kg body weight of standard heparin intravenously (Heparin Immuno, Baxter BioScience, Vienna, Austria). Serum and blood samples were collected before, 10 and 20 min after heparin injection. LPL protein levels in serum, LPL enzymatic activity in plasma, LPL mRNA expression in PBMC and viability of CD3, CD14 and CD19 positive cells were investigated in all samples. For ethical reasons, published values of healthy individuals before and after heparin administration were used as reference.

2.4. Heparin provocation test and LPL treatment in vitro

LPL mobilization *in vitro* was assessed in three (one mutated and two unmutated) CLL samples. Immediately after Ficoll isolation, PBMC were washed twice with phosphate-buffered saline (PBS), counted and 5×10^6 cells were cultured in 5 ml serum-free GIBCO RPMI 1640 containing 1% PS for 2 h at 37 °C and 5% CO_2. Then cells were treated with 20 U standard heparin. After 20 min of incubation, supernatant was harvested and stored at −20 °C for LPL protein and enzymatic activity measurements. Cells were instantly stained and apoptosis was analyzed by flow cytometry. THP1 cells cultured and treated under the same conditions were used as positive control. To evaluate the effect of external LPL on cell viability, cells of the 3 CLL patients were cultured and treated for 24, 48 and 72 h with 10, 100, 1000, and 10,000 ng/ml of purified LPL protein (Sigma, St. Louis, MO, USA). Apoptosis was investigated by flow cytometry.

2.5. LPL protein levels and enzymatic activity

Pre-heparin serum and plasma of 42 CLL, 14 non-CLL patients (lymphoma patients in complete remission) and 3 HIs, as well as pre- and postheparin serum and plasma from 13 CLL patients selected for heparin provocation tests were collected and stored at −20 °C until further analysis. Enzyme-linked immunosorbent assay (ELISA) was performed using the LPL EIA kit (ALPCO diagnostics™, Salem, NH, USA) according to the manufacturer's instructions. Before use, serum was diluted 1:30. Linearity of the assay system was observed from 9 to 500 ng/ml, the coefficient of variation (Cv) was less than 10% (within-run). LPL enzymatic activity assays were carried out at the Department of Molecular Biology and Biochemistry at the University of Graz as described elsewhere [30].

2.6. LPL mRNA expression

RNA and cDNA preparation and real-time PCR were carried out as described previously (LPL TaqMan assay-on-demand: Cat. No. Hs00173425_m12, Life Technologies, Carlsbad, CA, USA) [12].

2.7. Apoptosis and viability

To determine cell phenotype, PBMCs were washed with PBS and re-suspended in 0.3% BSA (bovine serum albumin) and 0.1% Na-azide (both Sigma, St. Louis, MO, USA). Nonspecific binding was blocked by 20% human AB serum (Cat. No. C11-021, PAA Laboratories, Linz, Austria) in PBS/BSA. Cells were stained with mouse anti-human CD3, CD19, CD14 – PE (BD Biosciences, San Diego, CA, USA) using the recommendations of the manufacturer. Cells were incubated for 30 min on ice, washed, re-suspended in 0.2 ml PBS/BSA and analyzed on a Becton Dickinson FACScan. For each analysis 10,000 events were acquired and analyzed using the CellQuestPro software.

For apoptosis detection, the Annexin-V detection kit (Bender MedSystems, Vienna, Austria) was used according to the manual and analyzed on the BD FACScan. The rate of cellular apoptosis was calculated as the percentage of Annexin positive cells.

Viability was detected using the Cell-Titre Blue Assay according to the instructions of the manufacturer (Promega, Madison, WI, USA).

2.8. Transfection of primary CLL cells

Stealth Select 3 RNAi™ set designed to silence human LPL, Stealth RNAi™ scrambled siRNA negative controls for high and low GC-content, and Block-iT™ positive control Fluorescent Oligo for lipid transfection were acquired from Life Technologies (Carlsbad, CA, USA). All three siRNAs targeting LPL were tested and the most effective was used for subsequent experiments. PBMC of 10 CLL patients with high LPL mRNA expression were selected for knock down experiments. Cells were thawed, counted and 10×10^6 cells per well were plated in 6-well cell culture plates using 2 ml phenol red free GIBCO RPMI 1640 (Life Technologies, Carlsbad, CA, USA) containing 10% FBS, 1% L-glutamine, and 1 ng/ml TPA (phorbol-12-myristate-13-acetate, Sigma Aldrich, St. Louis, MO, USA). Cells were then incubated for 1 h at 37 °C. RNAi duplexes and 15 μl Lipofectamine™ 2000 Reagent (Life Technologies, Carlsbad, CA, USA) were diluted in 500 μl Opti-MEM (Life Technologies, Carlsbad, CA, USA) and incubated for 20 min at room temperature according to the manufacturer's instructions. Five hundred microlitres of Stealth™ RNAi-Lipofectamin complexes were added to each well, final siRNA concentration was 60 pmol/ml. Each transfection was performed in duplicate. After 24 h, 1 ml of fresh medium was added to each well. After 48 h, cells were harvested, washed with ice-cold PBS and RNA was prepared using TRIzol. Transfection efficiency and effect on cell viability were monitored by flow cytometry. LPL mRNA expression was measured in all samples (negative control, mock transfection, transfection with scrambled siRNA and transfection with siRNA specific against LPL). The ratio in percent of LPL mRNA expression after transfection with scrambled and LPL specific siRNA, respectively, represents knock down efficiency. In addition, analyses of co-regulated genes were carried out by gene-expression profiling as described below.

2.9. Transfection of cell lines

One day before transfection, cells were seeded in 2 ml culture medium without antibiotics. At 70–80% confluence, cells were transfected. HeLa, Hep3B and CCL228 cells were transfected using Lipofectamine™ 2000 following the cell specific transfection protocols of the manufacturer. RPMI8226 and THP1 cells were transfected using Lipofectamine™ RNAiMAX according to the manufacturer's instructions for THP1 cells. This protocol was modified in one particular aspect: similar to CLL cells, THP1 cells were treated with 1 ng/ml TPA 1 h prior to transfection.

2.10. Microarray analysis

To determine genes up or down regulated after LPL knock down, RNA from paired samples (transfected with scrambled siRNA *vs.* LPL specific siRNA from the same time point) was extracted as described above, purified using the RNeasy Minelute kit (QIAGEN Inc., Valencia, CA, USA). Total RNA (200 ng) was then used for