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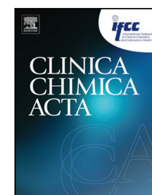

Leukemia cells display lower levels of intracellular cholesterol irrespective of the exogenous cholesterol availability

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ABSTRACT

Background: Different types of cancer cells are previously shown to accumulate intracellular cholesterol. However, the data on intracellular cholesterol levels in leukemia cells provide contradictory evidence. Various previous works indicate either increase, decrease or no difference in total cholesterol levels between leukemia cells and healthy peripheral blood mononuclear cells (PBMCs).
Methods: We studied the intracellular cholesterol levels in acute myeloid leukemia (AML) and acute lymphocytic leukemia (ALL) cells and compared with that in PBMCs from the healthy subjects.
Results: We observed that the PBMCs from AML ($n = 7$) and ALL ($n = 7$) patients displayed significantly lower intracellular levels of total cholesterol in comparison to PBMCs from the healthy subjects ($n = 26$). Consistent with the patient data the ALL (CCRF-CEM and MOLT-3) and AML (KG-1 and THP-1) cell lines also displayed significantly lower intracellular levels of total cholesterol. We confirmed this observation using multiple methodological approaches. Both ALL and AML cell lines also displayed significantly lower levels of free cholesterol and cholesteryl ester contents in comparison to normal hematopoietic cells. We observed that >90% of the total cholesterol in leukemia cells as well as in normal PBMCs was present in the form of cholesteryl esters. It was also observed that the lower levels of cholesterol in leukemia cells are not affected by exogenous cholesterol availability.
Conclusions: Present study provides convincing evidence to prove that the cellular free cholesterol and cholesteryl ester content is significantly reduced in leukemia cells in comparison to normal hematopoietic cells in circulation. Moreover, it was shown that the lower levels of cholesterol in leukemia cells are not affected by exogenous cholesterol availability.

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1. Introduction

Previous works indicate that cholesterol metabolism is deregulated in several types of malignancies including leukemia. In comparison to normal hematopoietic cells, the acute myeloid leukemia (AML) cells display increase in uptake and synthesis of cholesterol. The increased uptake and degradation of low-density lipoprotein cholesterol (LDL-C) is widely reported in AML cells [1–3]. The upregulated expression of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) is also observed in different types of leukemia cells [3]. Moreover, the negative feedback loop of cholesterol regulation is reported to be impaired in the leukemic cells [3]. It has also been noted that various ALL (Acute lymphocytic leukemia) and AML cell lines are sensitive to different types of statins [4,5].

The drug-resistant leukemia cells are shown to possess a fundamentally rewired central metabolism [6]. In comparison to the parental cell lines, the drug-resistant myeloid leukemia cell lines display higher expression of HMGCR [7], whereas, drug-resistant ALL cell lines have increased cholesterol and phospholipid levels [8]. This suggests that cholesterol accumulation in leukemia cells may contribute to drug resistance. It has also been noted that different chemotherapeutic drugs induce increase in intracellular cholesterol levels in AML cells [9]. The up-regulation in the mRNA expression of HMGCR and low-density lipoprotein receptor (LDL-R) is observed in these cells after treatment with various chemotherapeutic drugs [10]. However, detailed analysis showed that *de novo* synthesis is the primary mechanism by which most AML cells increase cholesterol levels during drug exposures. Nevertheless, it was also shown that a subset of AML cells may rely on increased LDL-C accumulation during treatment with particular drugs [10].

Previous studies have shown that solid tumors tissues display abnormally high cellular cholesterol content [11–13]. Experimental evidence also suggests that this elevated cellular cholesterol content increases

Abbreviations: HD, Healthy donors; ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; CE, cholesteryl esters.

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proliferation, migration, and invasion of cancer cells [14,15]. However, existing data on intracellular cholesterol levels in leukemia cells provide contradictory evidence. A relatively recent study indicated that primary myeloid leukemia cells [16] display significant increase in intracellular cholesterol deposits. Another study indicated that total cholesterol levels in primary acute lymphocytic leukemia cells are not significantly different than in PBMCs from healthy donors [17]. On the other hand, some earlier works reported decrease, in intracellular cholesterol levels of leukemia cells in comparison to peripheral blood mononuclear cells (PBMCs) from healthy donor [18–20].

2. Materials and methods

2.1. Cell culture and treatments

The AML (KG-1 and THP-1) and ALL (CCRF-CEM and MOLT-3) cell lines were from the American Type Culture Collection (ATCC). All cell lines and peripheral blood mononuclear cells (PBMCs) were cultivated in RPMI 1640 (*ATCC*, *30-2001*) supplemented with 10% FBS (ATCC, 30-2021) and penicillin–streptomycin solution (*ATCC*, *30-2300*). PBMCs were stimulated with 10 μg/ml phytohaemagglutinin (PHA) (*HiMedia*, *TC209*) for 48 h where stated. Lipoprotein deficient serum (LPDS) was purchased from Merck (*LP4*) and used according to manufacturer's guidelines. For different experiments, cells were cultivated in LPDS containing media for 24 h. Cell cultures were maintained in the atmosphere of 5% CO_2 and 37 °C.

2.2. Human sample collection, study protocol and ethics

For the present study patients that were clinically diagnosed for acute myeloid leukemia (AML) (n = 7) or acute lymphocytic leukemia (ALL) (n = 7) were recruited at the local hospitals. The patients were recruited at diagnosis and had not yet received any anti-neoplastic treatment. In addition to that healthy subjects (n = 26) were included as control population. All the study subjects were above the age of 15 years. We excluded subjects with the following conditions: severe viral/bacterial infection, on anticoagulation therapy, suffering from bleeding disorder (e.g. hemophilia, low platelets, etc.), aplastic anemia, metabolic syndrome, diabetes mellitus, cardiovascular disease or any other cancer. Other exclusion criteria were tobacco smoking and regular use of prescribed medication. Supplementary Tables S1 provides information of age, sex, diagnosis and blast count percentage of ALL and AML patients included in the present study. Supplementary Tables S2 provides information of age and sex of the healthy participants. The study protocol, questionnaires and informed consent forms were approved by the Ethics Committee of School of Biological Sciences, University of the Punjab. Written informed consent was obtained from each study-subject, before sample collection. All the research and experiments were carried out in accordance with Declaration of Helsinki. In order to obtain the basic personal information and medical history, each participant was interviewed and completed a structured questionnaire. The medical history file of each patient was also thoroughly examined. Intravenous blood (3 ml) was collected from all the subjects in vials containing EDTA-anticoagulant agent (BD Biosciences). Blood samples were used for plasma collection and PBMCs isolation. All samples were processed within 30 min of collection. Plasma was promptly separated. PBMCs were isolated from whole blood using Ficoll-based Lymphocyte Separation Medium (*Biowest*, *L0560*). PBMCs were accordingly pelleted or cultivated in appropriate cell culture conditions.

2.3. Determination of plasma lipid levels

Plasma total cholesterol (TC) levels were spectrophotometrically determined using commercially available kit (Analyticon Biotechnologies AG, 4046). For the estimation of high-density lipoprotein cholesterol (HDL-C), other lipoprotein fractions were precipitated using HDL-C precipitation reagent (Analyticon). HDL-C was then estimated using aforementioned Analyticon kit for the quantitative determination of cholesterol. For estimation of low-density lipoprotein cholesterol (LDL-C) we used recently described method by Martin et al. [21].

2.4. Determination of cellular cholesterol content

Lipids were extracted using a modified Bligh Dyer method, as previously described [22]. Total Cholesterol content in the lipid extracts was spectrophotometrically determined using commercially available kit (*Analyticon Biotechnologies AG*, *4046*) against a calibration-curve generated using known concentrations of cholesterol standard (*SUPELCO*, *47127-U*). For quantification of free cholesterol and cholesteryl esters or both (total cholesterol) Cholesterol/Cholesteryl Ester Quantitation Kit (*Abcam*, *ab65359*) was used according to manufacturer's guidelines. For the histochemical identification of unesterified cholesterol, *Filipin* staining was performed using Cholesterol Assay Kit (Cell-Based) (*Abcam*, *ab133116*) according to the manufacturer's guidelines. Samples were imaged with a fluorescent microscope. The quantification of total cholesterol by all methods gave similar results.

2.5. ELISA

The expression levels of HMGCR in cell lysates were measured by standard ELISA protocol using anti-HMGCR antibody (*Abcam*, *ab98018*).

2.6. Statistical analysis

The results were analyzed by Students' *t*-test (GraphPad Prism Software), where applicable. P-values < 0.05 were considered statistically significant. Correlation analysis was performed to study association between intracellular cholesterol levels and exogenous cholesterol availability and Pearson's correlation coefficient (r) values were calculated.

3. Results

3.1. Acute lymphocytic leukemia cells and acute myeloid leukemia cells display significantly low levels of intracellular cholesterol in comparison to mononuclear cells isolated from peripheral blood of healthy donors

We first compared the cellular cholesterol content in peripheral blood mononuclear cells (PBMCs) of healthy donors versus patients meeting the diagnostic criteria for ALL or AML. We observed that PBMCs from healthy donors display significantly higher cellular levels of cholesterol in comparison to ALL (Fig. 1a) and AML cells (Fig. 1b). To complement this patient data we also studied the cellular cholesterol content in various acute leukemia cell lines. Consistent with the patient data, both, ALL and AML cell lines displayed significantly lower intracellular cholesterol levels in comparison to PHA-stimulated (48 h) PBMCs from the healthy donors (Fig. 1c). We also compared the intracellular cholesterol content in ALL and AML cell lines with that in freshly isolated PBMCs from healthy donors. Again, ALL and AML cell lines displayed significantly lower intracellular cholesterol levels in comparison to freshly isolated PBMCs from the healthy donors (Fig. 1c). To further confirm these observations we used another kit-based (*Abcam*, *ab65359*) approach to study the intracellular cholesterol levels in leukemia cell lines. These analyses also gave similar results i.e. leukemia cells display significantly lower intracellular cholesterol levels (Supplementary Fig. S1).

Next, we sought to determine the levels of free cholesterol and cholesteryl esters in acute leukemia cells. We observed that both ALL and AML cell lines display substantially lower intracellular levels of free cholesterol (Fig. 1d) and cholesteryl esters (Fig. 1e) in comparison to these in PBMCs from healthy donors. Cholesterol labeling with filipin also verified that CCRF-CEM cells display lower cellular levels of cholesterol in comparison to PBMCs from healthy donor (Supplementary

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