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ABCB1, ABCC1, and LRP gene expressions are altered by LDL, HDL, and serum deprivation in a human doxorubicin-resistant uterine sarcoma cell line

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ABSTRACT

Multidrug resistance (MDR) is the major cause of cancer treatment failure. The ATP-binding cassette-B1 (ABCB1) transporter, also known as MDR1 or P-glycoprotein, is thought to promote the efflux of drugs from cells. MDR is also associated with the multidrug resistance-associated protein 1 (ABCC1) and the lung resistance-related protein (LRP), a human major vault protein. Moreover, MDR has a complex relationship with lipids. The ABCB1 has been reported to modulate cellular cholesterol homeostasis. Conversely, cholesterol has been reported to modulate multidrug transporters. However, results reported to date are contradictory and confusing. The aim of this study was to investigate whether LDL, HDL, and serum deprivation could influence ABCB1, ABCC1, and LRP expression in a human doxorubicin-resistant uterine sarcoma cell line.

ABCB1 and ABCC1 expression increased after 24 h of serum deprivation, and expression returned to basal levels after 72 h. LDL, depending on concentration, increased ABCB1, ABCC1, and LRP expression. ABCB1 expression increased at low HDL, and decreased at high HDL concentrations.

We demonstrated that serum deprivation and lipoproteins, particularly LDL, modulated ABCB1 expression and, to a lesser extent, ABCC1 expression. This finding may link the phenomena of drug transport, cholesterol metabolism and cancer.

1. Introduction

The major cause of cancer treatment failure is due to the development of resistance to anticancer agents, or multidrug resistance (MDR). The typical mechanism of MDR is to expel drugs out of the cell via transport proteins; this action maintains the intracellular drug concentration below the drug cytoxicity threshold [1]. Commonly, MDR results from the expression of ATP-dependent efflux pumps [2]. The ATP-binding cassette (ABC) superfamily comprises transporters that actively export substrates across the membrane, coupled to the hydrolysis of ATP [3]. One of these ABC transporters, ABCB1, also known as MDR1 or P-glycoprotein (Pgp), has been intensely studied [1,2,4–6]. ABCB1 is the 170 kDa transmembrane protein product of the human MDR1 gene, localized to chromosome 7q21 [7]. It is expressed in a wide range of cells and tissues, and it actively exports numerous organic compounds from the cytosol, across the cell plasma membrane, and into the extracellular fluid [2,4–6].

Another member of the ABC family of transporters is the multidrug resistance-associated protein 1 (ABCC1), which is also capable of conferring MDR by virtue of energy-dependent drug efflux. ABCC1 is an integral membrane glycophosphoprotein with an apparent molecular weight of 190 kDa [8,9]. It has been linked to the development of clinical MDR in several types of cancer [10].

Another protein related to MDR is the lung resistance-related protein (LRP), with a molecular weight of 110 kDa. LRP was identified as a component of the human major vault protein (MVP), a large-sized ribonucleoprotein complex. These vaults are present in various cell types, and they may mediate MDR by operating as cytoplasmic and/or nuclear membrane-associated drug transporters, perhaps in conjunction with ABC transporters [11].

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There is strong evidence that the lipid composition of biological membranes is closely related to the function of several proteins. Most ABC family members are integral membrane proteins that interact intimately with membrane lipids. Several studies have linked ABCB1 functional activity with cholesterol [1,12,13]. Cholesterol is a major constituent of mammalian plasma membranes. It is well known that the tissue contents of cholesterol and other lipids are strongly affected by exogenous and endogenous factors [14,15]. In normal cells, cholesterol is either synthesized *de novo*, or it is derived from the uptake of circulating low-density lipoprotein (LDL) complexes. The uptake of LDL is mediated by the LDL receptor, located on the cell membrane. Cancer cells possess elevated LDL receptor levels [16,17].

MDR has a complex relationship with lipids. In addition to its role in drug efflux, ABCB1 has been reported to modulate cellular cholesterol homeostasis; in fact, it is involved in several cholesterol-related processes in the cell [18]. Reports have shown that ABCB1 is involved in cholesterol transport through an ATP-dependent mechanism [13]. ABCB1 also appears to be involved in the transport of sterols from the plasma membrane to the endoplasmic reticulum, where cholesterol is esterified by the enzyme, acyl-CoA-cholesterol acetyltransferase [19–22]. Inhibition of this transport can prevent cholesterol esterification and cholesterol biosynthesis [20]. However, other studies did not show an effect of ABCB1 expression on the cellular content of free- and esterified-cholesterol, or on cholesteryl ester uptake from LDL and HDL particles [23]. Nonspecific inhibitors of ABCB1 have also been reported to inhibit cholesterol biosynthesis [20]. Therefore, the effects of ABCB1 on cholesterol homeostasis previously described in drug-selected cells might result from non-ABCB1 pathways [23].

Conversely, cholesterol has been shown to modulate multidrug transporters, particularly ABCB1 (MDR1) [4]. The presence of high ABCB1 content in membrane regions with low fluidity (i.e., rafts) suggested that the membrane lipids that surround ABCB1, particularly cholesterol, may play an important role in regulating ABCB1 activity [5]. In fact, the activity of the transporter has been described to be highly sensitive to the presence of cholesterol [6]. Indeed, membrane fluidization has been shown to impair ABCB1 function [24]. Alterations of the membrane cholesterol concentration by methyl-β-cyclodextrin, which affected lipid-raft membrane structure, also affected the ATPase activity of ABCB1 [7,13]. On the other hand, other studies reported that the effect of membrane cholesterol on the transporter is nearly negligible [6]. Studies with isolated MDR1 protein have shown that cholesterol increased the basal ATPase activity, but did not significantly modulate the drug-stimulated MDR1-ATPase in proteoliposomes [4]. Therefore, the results reported in the literature are contradictory and confusing.

It should be emphasized that most previous experiments were performed with drugs, like methyl-β-cyclodextrin, to change the cholesterol levels in cell membranes. Few reports have used LDL-cholesterol [17,18], and no systematic studies have been reported. Moreover, to the best of our knowledge, there are no descriptions on the influence of LDL-cholesterol on ABCC1 or LRP expression. This influence might be of particular interest, because, as stated, these transporters have been shown to represent important parameters in the kinetics of a wide variety of hydrophobic amphipatic drugs.

The aim of this study was to investigate whether LDL, HDL, and serum deprivation could influence ABCB1, ABCC1, and LRP gene expression.

2. Materials and methods

All subjects provided written, informed consent. The protocol of this study was approved by the Ethics Committee of the Institution.

2.1. Reagents

MacCoy's 5A medium, penicillin and streptomycin were from Sigma (Poole, UK). Fetal bovine serum (FBS) was obtained from Grand Island Biological Company (GIBCO, New York, USA). Tissue culture flasks were from Corning glass works (New York, USA). Trizol and SuperScript® III Platinum® SYBR® Green One-Step qPCR Kit were from Invitrogen (Paisley, UK). Polymerase chain reaction (PCR) primers were purchased from Integrated DNA Technologies (IDT, Iowa, USA).

2.2. Preparation of LDL and HDL

Blood was drawn from 3 healthy donors. LDL and HDL were isolated by sequential ultracentrifugation, after adding KBr for density adjustments, according to the method described by Havel et al. [25]. LDL and HDL were then extensively dialyzed against 150 mM NaCl. All lipoproteins were filtered through a 0.22 μm filter and stored at 4 °C, under nitrogen atmosphere, for no more than 1 week before using in experiments. Protein concentrations were measured with Lowry methodology [26].

2.3. Cell culture

MES-SA/Dx5 cells were obtained from American Type Culture Collection (ATCC CRL-1977, Virginia, USA). These cells were derived from sarcomatous elements of a uterine, mixed Mullerian tumor, and they exhibit MDR [27]. MES-SA/Dx5 were seeded into flasks containing McCoy's 5A medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) to obtain monolayer cultures. Cells were maintained at 37 °C in a humidified incubator with an atmosphere of 5% CO_2. Cells were subcultured after reaching 80–90% confluence (every 5–7 days).

2.4. Effects of LDL, HDL, and starvation on ABCB1, ABCC1, and LRP expression

MES-Dx5 cells were seeded into 6-well plates at a density of 1×10^6 cells/well. Cells were maintained in MacCoy's 5A medium with no FBS for up to 72 h (starvation group). In another set of experiments, medium was changed after 24 h of plating, and different concentrations of LDL or HDL (0.45, 0.65, and 0.85 μM) were added. Cells were incubated for 48 h.

At the end of the experimental periods, total RNA was extracted from cells with Trizol™ reagent. cDNA synthesis and quantitative PCR were performed on a Rotor Gene 3000 (Corbett Research, USA) with the SuperScript® III Platinum® SYBR® Green One-Step qPCR kit. Each reaction consisted of 1 × Sybr® green reaction mix, 1 μl SuperScript™ III RT/Platinum® Taq Mix, 10 μM each of forward and reverse primers, 125 ng RNA, and H_2O to a final volume of 12.5 μL. Primer sequences were: *ABCB1 forward* – CCC ATC ATT GCA ATA GCA GG, *ABCB1 reverse* – GTT CAA ACT TCT GCT CCT GA; *ABCC1 forward* – CTG AAA CCA TCC ATG ACC TCA ATC C, *ABCC1 reverse* – GCC TCC TCG TTC ACG TCC ACC TGG G; and *LRP forward* – GGG TTG TGC CCA TCA CCA CC, *LRP reverse* – GGT CCG CGG ATG AGC CAG TGG. Thermal cycler conditions included an incubation at 50 °C for 3 min, another incubation at 95 °C for 5 min, followed by 40 cycles of: 95 °C for 15 s and 60 °C for 30 s. Following the 40 cycles, the products were heated from 60 °C to 95 °C over 20 min to perform a melting curve analysis. This allowed the specificity of the products to be determined (single melting peak) and confirmed the absence of primer dimers.

To enable the relative quantification of transcripts, standard curves were generated with serial dilutions of MES-Dx5 RNA. The β_2-microglobulin (β_2M) gene was used to standardize the samples

(primer sequences: β_2M *forward* – ACC CCC ACT GAA AAA GAT GA and β_2M *reverse* – ATC TTC AAA CCT CCA TGA TG). The relative expression levels of ABCB1, ABCC1, and LRP transcripts were calculated with the $2^{-\Delta\Delta CT}$ method [28].

2.5. Statistical analysis

Data are shown as means ± SD. Values of $p < 0.05$ were considered significant. All analyses were performed with GraphPad Prism 5 (GradPad software, California, USA).

3. Results

3.1. Effect of serum deprivation

ABCB1 expression increased almost 40-fold after 24 h of serum deprivation. After 48 h, the expression decreased relative to the level at 24 h, but it remained higher than basal levels (10-fold increase). After 72 h of incubation, ABCB1 expression returned nearly to control cell expression levels. ABCC1 expression increased after 24 h, but less intensely than ABCB1 expression (2.5-fold increase compared to controls), and it showed no decrease over time. LRP gene expression did not significantly change over time (Fig. 1).

3.2. Effect of LDL cholesterol

ABCB1 expression increased with LDL concentrations above 0.65 mg/ml, in a concentration-dependent manner (see Fig. 2). At 1.05 mg/ml, ABCB1 expression increased almost 40-fold compared to basal values. ABCC1 and LRP expression increased only with LDL concentrations above 0.85 mg/ml, and the maximum value was observed with 0.85 mg/ml LDL.

3.3. Effect of HDL cholesterol

The effect of HDL on ABCB1 expression depended on the concentration. Expression increased with HDL concentrations up to 0.65 mg/ml. Unexpectedly, with HDL concentrations above 0.85 mg/ml, a strong decrease in ABCB1 expression was observed. ABCC1 expression was not affected by HDL. In contrast, LRP expression decreased at low HDL concentrations, and returned to basal levels with increasing HDL concentrations (Fig. 3).

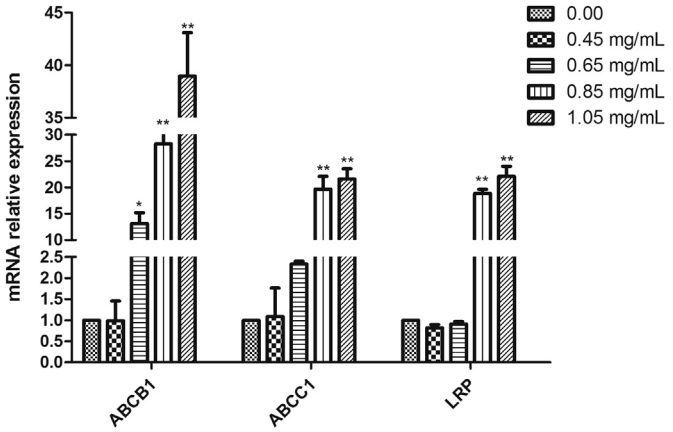

Fig. 2. Relative expression of ABCB1, ABCC1, and LRP in human uterine sarcoma cell line treated with different concentrations of LDL (mg/ml) for 48 h. Means represent results for three different donors, tested in triplicate. *p < 0.05, **p < 0.001.

4. Discussion

The response of tumor cells to drugs can be defined by a number of molecular mechanistic properties that operate at different stages. These properties include the ability of the drug substance to adequately penetrate the cell and the cell's capacity to expel the drug compound [21]. Although little studied, lipids could be important in this regard; but currently, reports are contradictory. Acute cholesterol depletion or saturation inhibited transport activity in different cell lines. In certain cell types, cholesterol saturation enhanced active drug efflux (7,19). Moreover, there is strong evidence that the lipid composition of cell membranes is closely related to the function of ABCB1, the most studied MDR-related protein [29].

ABCB1 is an unusual transporter, because its mode of action includes lipid/drug flippase characteristics, which make it particularly sensitive to the properties of the surrounding lipid bilayer. Various aspects of its function appear to be modulated by the lipid environment in complex ways [30]. ABCB1 is mainly localized within membrane microdomains, termed lipid rafts and caveolae, which are characterized by relative rigidity and reduced fluidity [31,32]. Cells that overexpressed native or recombinant ABCB1 accumulated reduced amounts of both short- and long-chain fluorescent phospholipid derivatives, due to increased outward transport of these analogs. Moreover, lipid transport decreased

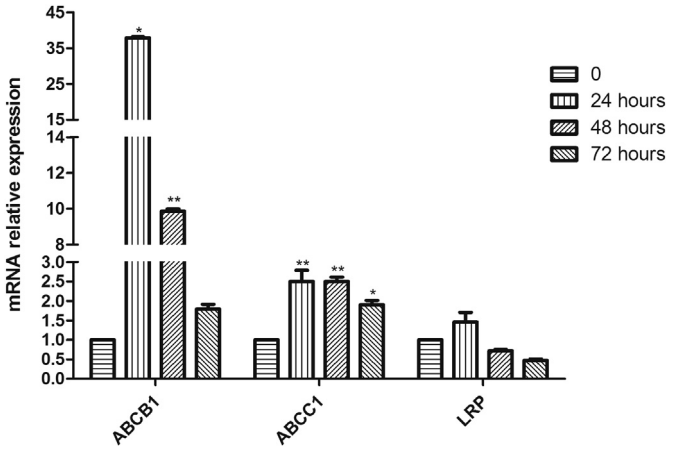

Fig. 1. Relative mRNA expression of ABCB1, ABCC1, and LRP in human uterine sarcoma cell line after serum deprivation for up to 72 h *p < 0.05, **p < 0.001.

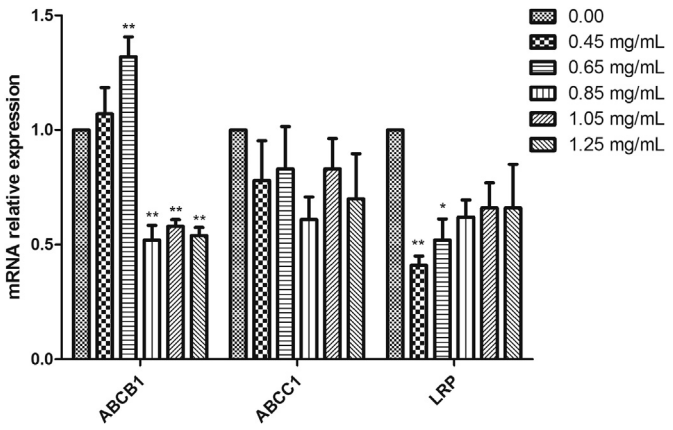

Fig. 3. Relative expression of ABCB1, ABCC1, and LRP in human uterine sarcoma cell line treated with different concentrations of HDL (mg/ml) for 48 h. Means represent results for three different donors tested in triplicate. *p < 0.05, **p < 0.001.

after treatment with ABCB1 modulators [33,34]. Several studies have suggested that membranous cholesterol content may modulate ABCB1. The ATPase activity of ABCB1 appeared to exhibit a strong dependence on the amount of cholesterol incorporated into membrane vesicles [7]. In addition, cholesterol directly and variably modulated the drug-affinity of the isolated ABCB1 protein, but it did not affect the maximum turnover rate [22]. In a NIH-G185 cell line, which overexpressed the human ABCB1 transporter, added cholesterol caused dramatic inhibition of daunorubicin transport, yet had no effect on rhodamine 123 transport [35]. Those results suggested that cholesterol affected the transport characteristics of ABCB1.

To date, transport has not been assessed systematically under different cholesterol conditions (depletion, repletion, and saturation), and no data are available on native human cells [36]. Therefore, we chose to use human LDL, the natural cholesterol carrier, as a source of cholesterol in this study. Moreover, we studied cholesterol depletion by adding either human HDL, as an acceptor of membrane cholesterol, or lipid starvation.

Few studies have reported the effect of LDL on ABCB1 levels. However, one study showed that LDL significantly decreased ABCB1-associated ATPase activity. That study analyzed ABCB1 ATPase activity in a vinblastine-resistant human lymphoblastic leukemia cell line, after a 24-h incubation with 100 μg LDL [29]. Here, we reported that adding increasing LDL concentrations to culture media led to a dose-related increase in ABCB1 expression after 48-h incubations. LDL concentrations above 0.85 mg/ml in the media also increased ABCC1 and LRP expression.

Several *in vitro* studies have reduced cell cholesterol concentrations by depleting plasma membrane cholesterol with cyclodextrin. It has been demonstrated that, under those conditions, cholesterol depletion of the plasma membrane inhibited transport of the ABCB1 substrate, rhodamine 123 [22]. Others showed that modulating membrane cholesterol under those same conditions had only a negligible effect on the MDR1 multidrug transporter [24,36]. However, the lack of effect was attributed to low basal ABCB1 activity in cells or to low membranous cholesterol content in these cells; consequently, those cells had lower susceptibility to membrane cholesterol depletion. However, the experiments were performed acutely, only 30 min after cyclodextrin addition. Acute depletions of cholesterol appear to impact ABCB1-mediated drug transport in a substrate- and cell-type-specific manner [37]. Interestingly, it has been shown that the cyclodextrin-mediated alterations in membrane ABCB1 expression or function could not be reversed by reloading cholesterol back into cells previously deprived of cholesterol [1]. On the other hand, it is not known whether cyclodextrin also leads to cellular damage [17]; therefore, ABCB1 may be inhibited as a result of a toxic side effect of cyclodextrin treatment.

In this report, we found that low concentrations of HDL increased ABCB1 expression, but HDL levels above 0.85 mg HDL protein/ml reduced ABCB1 expression. HDL metabolism is complex, and it remains incompletely understood. HDL is known to remove excess cholesterol from peripheral tissues and deliver it to liver and steroidogenic tissues. In addition, HDL can be taken up by cells through endocytosis [3]. In our experiments, the variations in results were small compared to those observed with LDL. Thus, our results gave rise to the hypothesis that, in starving cells, low HDL concentrations might be taken up by cells, but physiological or higher HDL concentrations might promote its known action of removing cholesterol from cells. However, this hypothesis remains to be investigated. In contrast, ABCC1 expression was not affected by HDL.

Serum-deprived media led to a strong increase in ABCB1 expression after 24-h incubations, followed by reduced expression for periods up to 48 h. The same trend was observed in ABCC1 expression, although on a smaller scale. LRP expression did not change. Although currently unproven, one hypothesis that might explain the observed effects would be that, in response to serum starvation, an increase in HMGCoA reductase expression [18] could increase the cell concentration of cholesterol, which in turn, could promote increases in ABCB1 and ABCC1 expression. With longer periods of starvation, cell cholesterol levels and ABCB1 and ABCC1 expression would return to basal levels. However, this hypothesis remains to be investigated.

Modulations in membrane cholesterol content can significantly alter MDR function, but the relationship between cholesterol content and MDR function may be complex. Thus, membrane lipid constituents other than cholesterol might also play a role in the above effects. In future studies, it may be particularly important to distinguish between direct cholesterol effects on MDR protein modulation, particularly ABCB1, with cholesterol depletion or saturation, and potentially indirect effects that might accompany these treatments; for example, inconspicuous changes in membrane permeability [38].

We do not know whether, physiologically, circulating LDL- and HDL-cholesterols contribute to the MDR phenotype. However, our study has indicated that, *in vitro*, ABCB1 expression in this sarcoma cell line was partially dependent on the presence of exogenous LDL cholesterol. This suggested a role for LDL cholesterol in the fine tuning of ABCB1 homeostasis. Our results have given rise to the question of how does the availability of cholesterol influence ABCB1 expression. This question may be addressed in future studies.

This study could be of clinical importance, due to its implications for diseases associated with the MDR phenotype. Our results have suggested an important link between the phenomenon of drug transport, cholesterol metabolism, and cancer.

Conflict of interest

None.

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