Correlation between cholesterol esterification, MDR1 gene expression and rate of cell proliferation in CEM and MOLT4 cell lines

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Abstract. A positive correlation between cholesterol esterification and growth rate potential was previously found in our laboratory during the growth of CEM and MOLT4 lymphoblastic cells. In the current study, we investigated whether the rates of cholesterol esters synthesis correlate with changes of acyl-CoA:cholesterol acyltransferase (ACAT) mRNA levels and of other genes implied in cholesterol biosynthesis and uptake, such as 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase and low density lipoprotein (LDL) receptor. The results showed that the more rapid growing CEM cells had lower levels of expression of HMGCoA-reductase and LDL receptors compared to MOLT4. By contrast, ACAT mRNA levels were higher in CEM cells, further supporting the concept of a possible involvement of cholesterol esters in the regulation of cell growth and division. In this study, high levels of cholesterol esterification and of expression of ACAT gene were also associated with a markedly increased expression of multidrug resistance (MDR1) gene, suggesting that MDR1 activity might contribute to regulate the rate of cell growth and division by modulating intracellular cholesterol ester levels.

Cholesterol metabolism has been studied extensively in our laboratory in different experimental models of cell proliferation (Dessì *et al* 1984, 1986, 1992a), as well as in several types of human neoplasm (Dessì *et al* 1991, 1992b, 1994). A constant finding of these studies has been that proliferative processes are characterized by a specific pattern of cholesterol metabolism, namely an increase in cholesterol synthesis and an accumulation of cholesterol esters in proliferating cells. These events are associated with a decrease of high density lipoprotein (HDL) cholesterol in the plasma compartment (Dessì *et al* 1990, 1992a,b, 1994). A major function attributed to HDL is to maintain normal cell cholesterol homeostasis by removing excess cholesterol from intracellular pools. On the basis of this notion, we have suggested that during growth the observed decrease in HDL and the concomitant accumulation of cholesterol esters may be due to a reduction of cholesterol efflux from proliferating cells presumably to prevent loss of intracellular cholesterol needed for new membrane biogenesis.

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To evaluate this hypothesis further, in a more recent study we have examined cholesterol synthesis, esterification and efflux during exponential and stationary growth of two different cell lines (CEM and MOLT4) isolated from patients with lymphoblastic T-cell leukaemia.

These two cell lines were selected because their cholesterol metabolism appears to be different: the activity 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase (Harwood *et al.* 1991) and the efficiency in synthesising cholesterol (Buttke & Folks 1992) being higher in MOLT4 compared to CEM cells.

The results obtained (Dessi *et al.* 1997) have shown that, although free cholesterol synthesis is initially higher in MOLT4, CEM cells exhibit higher baseline levels of acylCoA-cholesterol acyltransferase (ACAT) activity, the enzyme which catalyses the esterification of cellular cholesterol, coupled with an increased content of cellular cholesterol esters. The greater capacity to esterify and accumulate cholesterol in CEM cells was associated with a higher growth rate. We suggested that an increased capacity to make and store cholesteryl esters may facilitate new membrane biogenesis required for cell proliferation, and consequently a more prompt cell re-entry into the cell cycle.

In the current study we investigated, during subsequent replicative cycles of CEM and MOLT4 cells, whether the rate of cholesterol esters synthesis correlated with changes in mRNA levels of ACAT and of other genes implied in cholesterol biosynthesis and uptake, such as HMGCoA reductase and LDL receptor.

It has been demonstrated previously that cholesterol esterification depends on the availability of cholesterol substrate in the endoplasmic reticulum (ER) and that cholesterol is transported by an energy-dependent pathway from the plasma membrane to the ER where it is esterified by ACAT (Lange & Steck 1994, 1997). Thus, an important aspect that remains to be clarified is the mechanism(s) by which cholesterol is transported from the plasma membrane to ER and how this process is regulated.

Recently, a model in which multidrug resistance (MDR) p-glycoproteins are required for transport of free cholesterol from the plasma membrane to the ER has been proposed (Metherall *et al.* 1996a,b, Debry *et al.* 1997). This model implicates MDR activity as being involved in a general cellular process that is required for esterification. They demonstrated a direct correlation between cholesterol esterification and vinblastine accumulation suggesting that an isoform capable of catalysing drug efflux, such as the p-glycoprotein encoded by the MDR1 gene responsible for the multidrug resistance phenomenon (Bellamy 1996), may be involved. On the basis of this notion, we deemed it also important to test whether variations in MDR1 gene expression were also present during the growth of CEM and MOLT4 cell lines.

MATERIALS AND METHODS

Cell culture conditions

The cell lines CCRF-CEM, and MOLT4 isolated from acute human T-cell leukaemia patients and the murine leukaemia cell line L1210 were obtained from the American Type Culture Collection (ATCC, Rockville, MD).

Stock cultures were maintained in exponential growth (between 1×10^5 cells/ml and 1×10^6 cells/ml) at 37°C, 5% CO₂, in T-75 cm tissue culture flasks (Falcon, MI9, Milan, Italy) containing RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), streptomycin (100 µg/ml) and penicillin (100 U/ml) (growth medium). At the start of the experiments, cells from the stock cultures were diluted and seeded at a density of $\approx 1 \times 10^5$ cells/ml (5×10^4 for L1210) in growth medium. The cells were harvested at 0, 24, 48, 72, 96 and 120 h after seeding.

Cell growth measurement

At indicated times, cell number was determined with a Coulter counter and corrected for viability, as determined by the trypan blue dye exclusion test. Furthermore [^3H]-thymidine (185 kBq/ml) incorporated into DNA was also determined during the growth of CEM and MOLT4 cell lines as previously described (Dessi *et al* 1997). Briefly, after incubation the cells were rinsed twice with PBS, and DNA was precipitated by adding 5% trichloroacetic acid at 4°C. Cells were digested with 1 M NaOH at room temperature. Aliquots were assayed for [^3H]-thymidine radioactivity and protein content.

Cholesterol synthesis

To determine the rate of cholesterol synthesis on cells cultured as described above, cells were incubated for 3 h with 185 kBq/ml of sodium [^{14}C]-acetate (DuPont, Cinisello Balsamo, Milan, Italy; NEN specific activity 185 GBq/mmol). After incubation, 1×10^6 cells were separated by centrifugation and collected. Cellular lipids were extracted with acetone and neutral lipids were separated by thin layer chromatography (TLC) on kieselgel plates (Merck, Darmstadt, Germany) using a solvent system containing *n*-heptane/isopropyl ether/formic acid (60/40/2, v/v/v). Free cholesterol and cholesteryl ester bands were identified by comparison with standards that were run simultaneously on one side of the plate and visualized using iodine vapour. For counting, the silica was added directly to the counting vials containing 10 ml of Econofluor (DuPont NEN) liquid scintillation fluid.

Cholesterol esterification

Cells were incubated for 4 h in medium containing [^{14}C]-oleate bound to bovine serum albumin (BSA). To prepare the Voleate-BSA complex, 3.7 MBq of [^{14}C]-oleic acid in ethanol (Dupont, NEN specific activity 2.035 GBq/mmol) was mixed with 1.4 mg KOH and the ethanol evaporated. PBS (1.5 ml) without Ca and Mg containing 4.24 mg BSA (essentially fatty acid-free, Sigma) was added and the mixture shaken vigorously. This solution was added to each well at a final concentration of 74 kBq/ml. After incubation cells were washed with PBS and extracted with acetone. Lipid subclasses were separated by TLC as described above and incorporation of [^{14}C]-oleate into cholesterol esters was measured. Incorporation of [^{14}C]-oleate into esterified cholesterol represents esterification by ACAT enzyme.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cultured cells by the guanidine isothiocyanate phenolchloroform extraction method (Chomczynsky & Sacchi 1987). The RNA concentration was measured spectrophotometrically at 260 nm, and 260/280 ratio was evaluated to check for protein contamination. Equal amounts of total RNA (1 µg) were reverse transcribed into cDNA. cDNA was subsequently amplified by the polymerase chain reaction (PCR) in the presence of specific primers according to the instructions provided by the manufacturer (GeneAmp RNA PCR Kit, Perkin-Elmer Cetus, Monza, Milan, Italy). Primers, step cycles and expected length of the amplification products used in this study are summarized in Table 1. Preliminary experiments demonstrated that these PCR conditions were within the linear range of amplification of gene fragments. Under the conditions reported in Table 1, PCR products separated on agarose and stained with ethidium bromide, showed a major band of the predicted size (Figs 3, 4, 5 & 6, panel A).

During PCR reaction the nonradioactive label Digoxigenin-11-dUTP (DIG) (Boehringer, Mannheim, Germany) was incorporated, and the DNA fragments separated by electrophoresis in agarose were blotted onto a nylon membrane for 16 h in $10 \times \text{SSC}$. The

Table 1. Primer sequences and sizes of polymerase chain reaction (PCR) products

Gene	PCR primers	PCR protocol (number of cycle)	Size expected of PCR products (bp)
HMG-CoA reductase	5'-TACCATGTCAGGGGTACGTC-3' 5'-CAAGCCTAGAGACATAATCATC-3'	95°C-60 s/60°C-45 s/ 72°C-45 s (35)	246
LDL-receptor	5'-CAATGTCTCACCAAGCTCTG-3' 5'-TCTGTCTCGAGGGGTACCTG-3'	95°C-60 s/60°C-45 s/ 72°C-45 s (35)	258
ACAT	5'-AGCAGAGGCAGAGGAATTGA-3' 5'-GCACACCTGGCAAGATGGAG-3'	95°C 30 s/58°C-50 s/ 72°C-60 s (40)	958
MDR1	5'-CCCATCATTGCAATAGCAGG-3' 5'-GTTCAAACCTTCTGCTCCTGA-3'	94°C-30 s/55°C-60 s/ 72°C-60 s (35)	167
β -actin	5'-AGGGGCCCGAAGCTCGTCATACT-3' 5'-GGCGGCACCACCATGTACCCT-3'	96°C-30 s/60°C-59 s/ 72°C-45 s (20)	202

blot was exposed to X-ray film (Kodak X-OMAT, Sigma, Milan, Italy) for 2–10 min in an X-ray cassette at room temperature. A Kodak Digital Science BandScanner Image Analysis System containing HP ScanJet, ID Image analysis Software, assessed the intensity of the bands in the autoradiographs.

β -actin cDNA was amplified as an internal control. Since a low yield of PCR products is often obtained when cDNA segments were coamplified with an internal standard gene in the same tube (Noonan *et al.* 1990). The relative levels of gene expression have therefore been determined by comparing the PCR products of the target cDNA and β -actin gene in separate tubes. Target gene/ β -actin ratio was taken to represent the relative expression of the genes studied.

Chemical analyses

Proteins were determined by the method of Lowry *et al.* (1951), and using bovine albumin as standard.

Statistical analysis

Means $\pm$ SEM for triplicate determinations are presented. Statistical significance of difference was determined by a nonparametric Mann–Whitney *U*-test. $P < 0.05$ was the criterion for significance. All experiments were repeated at least twice.

RESULTS

Growth rate and cholesterol metabolism of CEM and MOLT4 cells

According to previous results (Buttke & Folks 1992, Dessì *et al.* 1997), the level of [14 C]-acetate incorporation into total cholesterol increased sharply during the first 72 h of culture and dropped at 96 h, in both CEM and MOLT4 cell lines. Total cholesterol synthesis was initially higher in MOLT4 cells, whereas during times of exponential growth (48–72 h) the levels were similar in the two cell lines (Fig. 1).

The doubling time of the two cell lines was significantly different (MOLT4: 26.6 ± 0.7 h vs. CEM: 19.5 ± 0.5 h). As shown in Fig. 2a, CEM cells grew at a faster rate than MOLT4, reaching the saturation density about 24 h earlier than MOLT4 (MOLT4: 96 h vs. CEM: 72 h) (Fig. 2a). Similarly, the rate of [3 H]-thymidine incorporation was consistently higher throughout the experimental time in CEM cells than in MOLT4 cells (data not shown).

Figure 1. Total cholesterol synthesis in CEM and MOLT4 cell lines. 1×10^6 cells/ml were grown in RPMI supplemented with 10% FCS. Three hours before the indicated times, cells were labelled with [¹⁴C]-acetate (185 kBq/ml). Data represent means $\pm$ SEM for triplicate determinations of a representative experiment. $P < 0.05$ vs CEM.

Consistent with previous studies (Dessi *et al.* 1997), cholesterol esterification was significantly higher in CEM compared to MOLT4 at all time point considered including 4 h and 120 h (Fig. 2b). However, it has to be noted that in both cell lines, cholesterol esterification reached

Figure 2. Proliferation and cholesterol esterification of CEM and MOLT4 cell lines. 1×10^5 cells/ml were grown in RPMI supplemented with 10% FCS. At the indicated times, cell numbers were determined with a Coulter counter and corrected for viability by the Trypan blue dye exclusion method (a). For cholesterol esterification, cells were labelled with $[^{14}\text{C}]$ -oleateBSA complex (74 kBq/ml) for 4 h (b). Data represent means $\pm$ SEM for triplicate determinations of a representative experiment. * $P < 0.05$ vs. CEM.

the peak during the stationary growth phase (96–120 h), and that the transition to active growth following cell dilution was associated with a marked decrease in cholesterol esterification (24 h). The values obtained at 4 h likely reflected the residual metabolic activities of the stationary phase cultures (120 h). Thus, the comparison of cholesterol synthesis (Fig 1) and cholesterol esterification (Fig 2b) during cell growth (Fig 2a) suggested that, irrespective to the cell line considered, the cholesterol esters accumulated during stationary growth phase (96–120 h) could be readily utilized for cholesterol needs at the initial phases of active growth (0–24 h). At this time, in fact, cholesterol biosynthesis started to increase.

It is noteworthy that similar results were obtained in the murine leukaemia L1210 cells that were chosen among others because of their particularly short doubling time (12 h). As a matter of fact, in these fast growing cells, cholesterol esterification was consistently higher than in both CEM and MOLT4 cells (data not shown).

Measurements of HMGCoA reductase, LDL receptors and ACAT mRNA levels in CEM and MOLT4

Subsequently, the expression of the main genes implicated in the regulation of intracellular cholesterol metabolism—HMGCoA reductase, LDL receptor and ACAT, were analysed during exponential and stationary growth phases of CEM and MOLT4 cells.

Figures 3–6 (panel A) illustrates the PCR products of the target genes mRNAs separated on gel agarose stained with ethidium bromide. The figures indicate that the length of the amplification products obtained was as expected (see Table 1) and that β -actin bands remained relatively stable over this time course in both cell lines.

Autoradiographs of PCR-amplified mRNA of HMGCoA reductase (Fig 3, panel B), indicated that HMGCoA-R gene expression was initially higher in MOLT4 cells than in CEM cells whereas during more active growth phases (48–72 h) the levels were similar in the two cell lines. The differences of the two cell lines in the HMGCoA-R gene expression were obtained in three independent time courses, relative data of which are reported in Fig 3, panel C. The apparently contradictory patterns of HMGCoA-R mRNA levels (Fig 3c) and [14 C]-acetate incorporation (Fig 1) likely reflected the fact that gene expression start to be down regulated when its gene product is still at the maximum activity.

Higher levels of LDL receptor mRNA were also observed in MOLT4 cells compared to CEM cells, at least during the first 48 h of incubation (Fig 4, panels a, b, c).

By contrast, ACAT mRNA levels were higher in CEM cells (Fig 5, panels a and b) and quantitative densitometric determination of the bands after normalization with β -actin confirmed that the mRNA levels were higher in CEM cells at all time points considered (Fig 5, panel c).

Measurement of MDR1 mRNA levels in CEM and MOLT4 cell lines

Since a direct correlation between MDR activity and cholesterol esterification has been previously found in a number of cultured human cell lines (Field *et al* 1995, Metherall *et al* 1996a,b), we also analysed MDR1 mRNA levels during growth of CEM and MOLT4 cell lines.

The results obtained (Fig 6, panels a, b and c) showed that CEM cells overexpressed MDR1 gene not only during exponential growth but also under stationary culture conditions. In MOLT4 cells, the expression of this gene was instead not detectable by the ethidium bromide staining (Fig 6, panel a) with exception for a very thin band at 0 h and only autoradiographs of the stained gel revealed the presence of very low levels of MDR1 mRNA (Fig 6, panels b and c).

Figure 3. Time course of HMGC0A reductase mRNA levels of CEM and MOLT4. Cells were cultured as indicated in Figure 1. At the indicated times, total cellular RNA was isolated and mRNA levels were determined by RT-PCR analysis, as described under 'Materials and methods'. β -actin was used as an internal standard. (a) Representative PCR products of HMGC0A-reductase (top half) and β -actin (bottom half) separated in ethidium bromide-stained agarose gel. (b) Representative chemiluminescent images of PCR amplified HMGC0A-reductase mRNA (top half) and β -actin (bottom half). (c) Values are represented as the ratio of HMGC0A reductase/ β -actin (%) and are the means $\pm$ SE of three independent experiments. * $P < 0.05$ vs. CEM.

Figure 4. Time course of LDL receptor mRNA levels of CEM and MOLT4. Cells were cultured and treated as indicated in Figs 1 & 3. (a) Representative PCR products of LDL receptor (top half) and β -actin (bottom half) separated in ethidium bromide-stained agarose gel. (b) Representative chemiluminescent images of PCR amplified LDL receptor mRNA (top half) and β -actin (bottom half). Values in panel C represent the ratio of LDL receptor/ β -actin (%) and are the means $\pm$ SE of three independent experiments. * $P < 0.05$ vs. CEM

Figure 5. Time course of ACAT mRNA levels of CEM and MOLT4. Cells were cultured and treated as indicated in Figs 1 & 3. (a) Representative PCR products of ACAT (top half) and β -actin (bottom half) separated in ethidium bromide-stained agarose gel. (b) Representative chemiluminescent images of PCR amplified ACAT mRNA (top half) and β -actin (bottom half). Values in panel (c) represent the ratio of ACAT/ β -actin (%) and are the means $\pm$ SE of three independent experiments. * $P < 0.05$ vs. CEM.

Figure 6. Time course of MDR1 mRNA levels of CEM and MOLT4. Cells were cultured and treated as indicated in Figs 1 & 3. (a) Representative PCR products of MDR1 (top half) and β -actin (bottom half) separated in ethidium bromide-stained agarose gel. (b) Representative figure of chemiluminescent images of PCR amplified MDR1 mRNA (top half) and β -actin (bottom half). Values in (c) represent the ratio of MDR1/ β -actin (%) and are the means $\pm$ SE of three independent experiments. * $P < 0.05$ vs. CEM.

DISCUSSION

Cholesterol homeostasis utilizes a complex network of reactions that allow an adequate supply of cholesterol while avoiding overaccumulation that may be toxic to the cells. In the majority of cells cholesterol supply depends on the balance between endogenous synthesis, under the control of HMGCoA reductase, and exogenous cholesterol uptake involving endocytosis of the LDL-receptor complex (Brown & Goldstein 1979). Two cellular processes avoid excess free cholesterol toxicity: cholesterol efflux via HDL (Oppenheimer, Oram & Birman 1987) and cellular cholesterol esterification by activation of ACAT (Suckling & Stange 1985).

Under pathological conditions, the accumulation of cholesterol esters is a constant finding in atherosclerosis (Ross 1986) as well as in rapidly proliferating tissues including tumour tissues (Dessi *et al.* 1984, Gebhard *et al.* 1987, 1992a,b). In both processes cholesterol ester accumulation is accompanied by a strong decrease in HDL cholesterol levels in the plasma compartment raising the question why free cholesterol is preferentially stored as cholesteryl esters, via ACAT, rather than being released from the cells to circulating HDL.

The data reported in the present study, further supporting our hypothesis on the role of cholesterol esterification during growth, give a possible explanation for the matter. Our previous results (Dessi *et al.* 1997) showed that an increased ability of cells to esterify cholesterol is associated with an increased growth rate (CEM cells vs. MOLT4 cells). We now show that, compared to MOLT4, CEM cells have higher baseline levels of cholesterol esterification coupled with higher levels of ACAT mRNA. On the contrary, the absolute levels of cholesterol synthesis and cholesterol uptake do not appear to be directly correlated with growth rate in MOLT4 and CEM cells. The slower growing MOLT4 cells have in fact consistently higher baseline levels of both HMGCoA reductase and LDL receptor mRNA as well as of cholesterol synthesis, than CEM cells. Therefore, we can speculate that a larger store of cholesteryl esters within the cells could allow for a more rapid mobilization and utilization of cholesterol for new membrane biogenesis and consequently for a more prompt cell re-entry into the cell cycle.

We also found that in CEM cells the higher levels of cholesterol esterification and of expression of ACAT gene are associated with a higher expression of the MDR1 gene. The physiological role of the MDR1 gene product in cholesterol transport and esterification recently proposed by Metherrall *et al.* (1996a,b), together with the higher MDR1 gene expression found by us in CEM compared to MOLT4 cells, would point to the involvement of the MDR1 gene in the regulation of cell growth and division by modulating the intracellular cholesterol ester levels.

In summary, although these studies suggest that ACAT and MDR1 gene expression may be involved in cell proliferation, further studies are necessary to determine their exact role in this process. Experiments perturbing cell growth, either by lowering rates of cholesterol esters synthesis and by overexpressing MDR1 gene, are under current investigation in our laboratory.

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