Role of cholesterol synthesis and esterification in the growth of CEM and MOLT4 lymphoblastic cells

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CEM and MOLT4 are human T-cell lines isolated from patients with acute cell leukaemia. In culture they show important differences in cholesterol metabolism, CEM being less efficient at synthesizing cholesterol and having a lower activity of 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) reductase. To investigate further the relationship between regulation of intracellular cholesterol metabolism at various steps and rate of cell growth, cholesterol synthesis, esterification and efflux were evaluated in CEM and MOLT4 cells at different times during exponential and

stationary growth *in vitro*. It was shown that, although CEM cells have a lower rate of cholesterol synthesis, they grow at a faster rate than MOLT4 cells. However, CEM cells exhibit an increased capacity to esterify cholesterol associated with a decreased efflux of newly synthesized cholesterol into the medium. These results provide evidence for an association between the capability to synthesize and retain cell cholesterol esters and the growth rate potential.

INTRODUCTION

Regulation of cholesterol homeostasis is of vital importance for mammalian cell structure and function. It is achieved through a series of cellular responses which allow an adequate supply of cholesterol while avoiding overaccumulation, which may be toxic to the cells. The key regulatory steps of intracellular cholesterol homeostasis rely on endogenous synthesis, exogenous uptake via the low-density lipoprotein (LDL) receptor, storage as cholesterol esters and efflux via high-density lipoprotein (HDL) [1–4].

Based on the notion that activation of cell proliferation imposes an additional need for cellular cholesterol to support membrane biogenesis, a potential mechanism involved in growth regulation might reside in an altered cellular sterol balance and/or metabolism. In fact, among the cellular events that occur in response to a mitogenic stimulus are those involving modifications in cholesterol synthesis and metabolism [5–10].

Rapidly proliferating cells and tissues have been reported to show a high rate of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase activity, the rate-limiting enzyme of cholesterol biosynthesis [5,6], and an increase in the number of LDL receptors per cell [7,8], suggesting that an increased demand for free cholesterol derived from both exogenous and endogenous sources is part of the cellular response to a mitogenic stimulus.

However, it is not clear as yet to what extent cholesterol *per se* is required for mammalian cell growth, since other non-sterol isoprenoid moieties derived from early stages of the cholesterol-biosynthetic pathway, such as farnesyl pyrophosphate, also appear to be essential for mammalian cell proliferation [9,10].

Cholesterol metabolism has been extensively studied in our laboratory in different experimental models of cell proliferation [11–13], as well as in several types of human neoplasms [14–16]. 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 in HDL cholesterol in the plasma compartment [17,18]. A major function attributed to HDL is the maintenance of a 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 may be due to a reduction in cholesterol efflux from proliferating cells presumably to prevent loss of intracellular cholesterol needed for new membrane biogenesis, as indicated by the accumulation of cholesterol esters in proliferating cells.

To evaluate this hypothesis further, in the present study we 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 previous studies have shown that their cholesterol metabolism appears to be different, the activity of HMGCoA reductase [19] and the efficiency in synthesizing cholesterol [20] being higher in MOLT4 than in CEM cells. The results show that, although free cholesterol synthesis is initially higher in MOLT4 cells, as previously reported [19,20], the rate of growth is faster for CEM cells than MOLT4. However, CEM cells exhibit a higher rate of cholesterol esterification coupled with a lower degree of cholesterol efflux. The positive correlation between the cell capability to synthesize and retain cholesterol esters and the growth rate potential of CEM and MOLT4 cells suggests that the intracellular levels of cholesterol esters may be important in growth regulation by providing the appropriate amounts of cholesterol for membrane synthesis and assembly.

MATERIALS AND METHODS

Cell and culture conditions

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

Abbreviations used: HMGCoA, 3-hydroxy-3-methylglutaryl-CoA; LDL, low-density lipoprotein; HDL, high-density lipoprotein; FCS, fetal calf serum; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Stock cultures of CEM and MOLT4 were maintained in stationary suspension at 37 °C, 5% CO₂, in T-75-cm² tissue culture flasks (Falcon, MI9) containing RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), streptomycin (100 µg/ml) and penicillin (100 units/ml) (growth medium). Fresh medium was added every other day so as to maintain the cells at a density of about 1.2×10^6 cells/ml. At the start of experiments, cells from the stock cultures were diluted and seeded at a density of 1×10^5 cells/ml in growth medium. The cells were harvested at 0, 24, 48, 72 and 96 h after seeding.

Cell growth measurements

At the indicated times, cell number was determined with a Coulter counter and corrected for viability, as determined by Trypan Blue dye exclusion. Furthermore cell growth and viability were also determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [21]. The method is based on the cleavage of MTT into a blue product, formazan, which takes place only in living cells. The amount of formazan produced is proportional to the number of cells present [21].

[³H]Thymidine-incorporation studies

At the time points considered, cells were incubated in triplicate for 3 h with [³H]thymidine (5 µCi/ml). 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 in cells cultured as described above, two different methods were used. In method A cells were incubated for 3 h with 5 µCi/ml sodium [¹⁴C]acetate (DuPont-NEN; specific radioactivity 50 mCi/mmol). After incubation one group of cells was separated by centrifugation and collected (pulse). Another group of cells was washed, resuspended in growth medium and incubated for an additional 3 h, after which time cells were separated and collected (chase). In method B cells were incubated for 6 h with ³H₂O (10 mCi/ml; DuPont-NEN) and after incubation separated and collected.

Cellular lipids were extracted with acetone and neutral lipids were separated by TLC on kieselgel plates (Merck, Darmstadt, Germany) using a solvent system containing *n*-heptane/isopropyl ether/formic acid (60:40:2, by vol.). 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.

Efflux of endogenously synthesized cholesterol

Cells were incubated for 6 h with ³H₂O. After incubation, cells and media were separated and collected. Lipids in cells and media were extracted with chloroform/methanol (2:1, v/v) and separated as described above. Efflux of endogenously synthesized cholesterol was expressed as the percentage of total ³H₂O-labelled cholesterol (cells plus medium) recovered from the medium.

Cholesterol esterification

To determine the rate of cholesterol esterification, cells were

incubated for 6 h in medium containing [¹⁴C]oleate bound to BSA. To prepare the [¹⁴C]oleate-BSA complex, 100 µCi of [¹⁴C]oleic acid in toluene (Amersham; 60 mCi/mmol) was mixed with 1.4 mg of KOH and the toluene evaporated. PBS (1.5 ml) without Ca²⁺ and Mg²⁺ containing 4.24 mg of BSA (essentially fatty acid-free; Sigma) was added and the mixture was shaken vigorously. This solution was added to each well at a final concentration of 5 µCi/ml. After incubation, cells were washed with PBS, and lipid was extracted with acetone. Lipid subclasses were separated by TLC as described above, and incorporation of [¹⁴C]oleate into cholesterol esters was measured.

Chemical analyses

Cell cholesterol mass was determined by the method of Bowman and Wolf [22] using cholesterol and cholesterol palmitate as standards. Proteins were determined by the method of Lowry et al. [23] using BSA as standard.

Statistical analysis

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

RESULTS

Growth rate of CEM and MOLT4 cells

In the first series of experiments, we tested the growth rate of CEM and MOLT4 cells cultured in medium containing 10% FCS at a density of 10^5 cells/ml.

As shown in Figure 1, during the first 24 h the doubling time of the two cell lines was not significantly different. However,

Figure 1 Growth curves of CEM (■) and MOLT4 (□) cell lines

The cells were seeded at a density of 1×10^5 cells/ml and grown in RPMI supplemented with 10% FCS. At the indicated times, cell numbers were determined with a Coulter counter and were corrected for viability by Trypan Blue dye exclusion. Data represent means $\pm$ S.E.M. for five separate experiments. **P* < 0.05 compared with CEM cells.

Figure 2 MTT assay in CEM (■) and MOLT4 (□) cell lines

The cells were grown as described in Figure 1. At the indicated times cells were incubated for 4 h with MTT (0.5 mg/ml) and the absorbance of the blue formazan produced was measured at 560 nm. Data represent means $\pm$ S.E.M. for three separate experiments. * $P < 0.05$ compared with CEM cells.

Table 1 [^3H]Thymidine incorporation into DNA in CEM and MOLT4 cell lines

The cells were grown as described in Figure 1. At 3 h before the indicated times, cells were labelled with [^3H]thymidine (5 $\mu\text{Ci}/\text{ml}$). After incubation, the cells were rinsed with PBS and the [^3H]thymidine incorporated into DNA was determined as described in the Materials and methods section. Data represent means $\pm$ S.E.M. for triplicate determinations of a representative experiment. * $P < 0.05$ compared with CEM cells.

Time (h)	[^3H]Thymidine incorporated into DNA (c.p.m./ μg of cell protein)	
	CEM	MOLT4
3	53.1 $\pm$ 3.0	33.0 $\pm$ 1.2*
24	59.0 $\pm$ 1.5	47.2 $\pm$ 0.9*
48	48.3 $\pm$ 1.0	21.5 $\pm$ 0.6*
72	51.1 $\pm$ 1.2	22.3 $\pm$ 0.6*
96	39.2 $\pm$ 1.0	20.1 $\pm$ 0.4*

after this time CEM cells grew at a faster rate than MOLT4 cells, reaching the saturation density of approx. 1.3×10^6 cells/ml about 24 h earlier than MOLT4. Differences in growth rate of these two cell lines were also evident when cell proliferation was measured by the MTT test (Figure 2). Similarly, the rate of [^3H]thymidine incorporation was consistently higher throughout the experimental time in CEM cells than in MOLT4 cells (Table 1).

Cholesterol metabolism of CEM and MOLT4 cells

To test whether different growth rates of CEM and MOLT4 were associated with different endogenous synthesis of cholesterol, the incorporation of sodium [^{14}C]acetate into total cholesterol was determined. In addition, the incorporation of $^3\text{H}_2\text{O}$ into both free and esterified cholesterol was estimated.

Figure 3 [^{14}C]Acetate incorporated into total cholesterol (TC) in CEM and MOLT4 cell lines

The cells were grown as described in Figure 1. At 3 h before the indicated times, cells were labelled with [^{14}C]acetate (5 $\mu\text{Ci}/\text{ml}$) (pulse) or incubated for an additional 3 h in growth medium alone (chase). The radioactivity in total cholesterol was determined as described in the Materials and methods section. Data represent means $\pm$ S.E.M. for triplicate determinations of a representative experiment.

In one series of experiments, cells were incubated for 3 h in the presence of [^{14}C]acetate (pulse). At the indicated times (Figure 3) sets of cells from both groups were collected, while another set was washed and reincubated in fresh medium without radioactive precursor for an additional 3 h period (chase).

As evidenced by both the 3 h pulse and subsequent 3 h chase period, the level of [^{14}C]acetate incorporation into total cholesterol increased sharply during the first 48 h of culture, after which it declined, reaching the lowest value at 96 h in both cell lines (Figure 3). However, pulse experiments indicated that the rate of cholesterol synthesis was initially higher in MOLT4 cells, in agreement with the data of others [19], whereas during more active growth phases (48–72 h) the levels were higher in CEM cells (Figure 3). Results obtained after chase periods indicate that the levels of [^{14}C]acetate incorporation into cholesterol were similar to the corresponding pulse values for CEM cells, whereas they were greatly reduced at all experimental time points in MOLT4 cells (Figure 3), suggesting that CEM cells have a greater ability to retain newly synthesized cholesterol than MOLT4 cells.

In order to characterize this aspect better, in subsequent experiments cultures were incubated with $^3\text{H}_2\text{O}$ for 6 h before being harvested at 24 h intervals. Incorporation of $^3\text{H}_2\text{O}$ into free and esterified cholesterol was evaluated.

The $^3\text{H}_2\text{O}$ incorporation into intracellular free cholesterol in CEM and MOLT4 cells displayed a pattern similar to that found with [^{14}C]acetate-incorporation experiments (Figure 4). However, the synthesis of cholesterol esters was significantly higher in CEM than in MOLT4 cells throughout the experimental time examined (Figure 5). Conversely, the percentage of total cholesterol efflux into the medium was generally higher in MOLT4

Figure 4 $^3\text{H}_2\text{O}$ incorporated into free cholesterol (FC) in CEM (■) and MOLT4 (□) cell lines

The cells were grown as described in Figure 1. At 6 h before the indicated times cells were incubated with $^3\text{H}_2\text{O}$ (10 mCi/ml). The radioactivity in free cholesterol was determined as described in the Materials and methods section. Data represent means $\pm$ S.E.M. for triplicate determinations of a representative experiment. * $P < 0.05$ compared with CEM cells.

cells than in CEM cells (Figure 6), again suggesting that the latter have an increased ability to retain newly synthesized cholesterol. Efflux was maximal in both cell lines after 96 h of culture, i.e. during the stationary growth phase (Figure 6).

Figure 5 $^3\text{H}_2\text{O}$ incorporated into cholesterol esters (CE) in CEM (■) and MOLT4 (□) cell lines

The cells were grown and the radioactivity determined as described in Figure 1. Data represent means $\pm$ S.E.M. for triplicate determinations of a representative experiment. * $P < 0.05$ compared with CEM cells.

Figure 6 Cholesterol efflux from CEM (■) and MOLT4 (□) cell lines

The cells were grown in 10% FCS/RPMI containing $^3\text{H}_2\text{O}$ (10 mCi/ml). Efflux, determined as described in the Materials and methods section, was expressed as a percentage of the total radioactivity recovered in the medium. Data represent means $\pm$ S.E.M. for triplicate determinations of a representative experiment. * $P < 0.05$ compared with CEM cells.

Next we investigated cholesterol esterification in CEM and MOLT4 cells during 96 h of culture, by measuring cellular [^{14}C]cholesterol ester accumulation in cells exposed to medium containing [^{14}C]oleate-BSA complex for 6 h before harvest at the time points indicated in Figure 7. As shown, during more active growth (24–48 h), [^{14}C]oleate incorporation into cholesterol esters was lower in both cell lines.

In CEM cells, cholesterol esterification was highest during the stationary growth phase (96 h), and transition to active growth was associated with a large decrease in the ability to esterify cholesterol. This was already evident after 6 h of culture and continued until 24 h, after which a significant increase in cholesterol esterification was evident. Cholesterol esterification was significantly lower in MOLT4 than in CEM cells throughout the experiment (Figure 7), consistent with the results presented above.

The increased ability to esterify cholesterol in CEM compared with MOLT4 cells was also reflected in a higher content of cholesterol esters in the former compared with the latter (Figure 8). In CEM cells, cholesterol ester levels were highest during the stationary growth phase and decreased sharply during the more active growth phase (48–72 h).

In contrast, free cholesterol content was initially higher in MOLT4 cells; however, it decreased to levels comparable with those of CEM cells after 24 h of culture and remained relatively stable in both cell lines throughout the experiment (Figure 9).

DISCUSSION

The results of these studies can be summarized as follows: (i) CEM cells grow at a faster rate than MOLT4 cells; (ii) CEM cells have higher baseline levels of cholesterol esterification as well as

Figure 7 Cholesterol esterification in CEM (■) and MOLT4 (□) cell lines

The cells were grown as described in Figure 1. At 6 h before the indicated times cells were labelled with [¹⁴C]oleate-BSA complex and the amount of [¹⁴C]oleate incorporated into cholesterol esters (CE) was determined as described in the Materials and methods section. Data represent means ± S.E.M. for triplicate determinations of a representative experiment. **P* < 0.05 compared with CEM cells.

Figure 8 Cholesterol ester (CE) content in CEM (■) and MOLT4 (□) cell lines

The cells were grown as described in Figure 1. Cellular lipids were extracted and processed as described in the Materials and methods section. Data represent means ± S.E.M. for triplicate determinations of a representative experiment. **P* < 0.05 compared with CEM cells.

higher levels of cholesterol ester content. In contrast, MOLT4 cells display higher levels of cholesterol synthesis.

A number of observations in the literature point to the existence of an intimate relationship between cell proliferation and the cholesterol-biosynthetic pathway and/or LDL-mediated endo-

Figure 9 Free cholesterol (FC) content in CEM (■) and MOLT4 (□) cell lines

The cells were grown as described in Figure 1. Cellular lipids were extracted and processed as described in the Materials and methods section. Data represent means ± S.E.M. for triplicate determinations of a representative experiment. **P* < 0.05 compared with CEM cells.

cytosis. This conclusion is based on the observations that, in response to mitogenic stimuli, cells exhibit an increase in cholesterol synthesis [5,6] as well as in the number of LDL receptors [24,25]. On the other hand, cholesterol deprivation inhibits the ability of cells to respond to mitogens [26]. In addition, farnesyl residues, generated via the cholesterol-biosynthetic pathway, are important regulators of the activity of proteins involved in cell cycle progression [9,10].

Besides *de novo* biosynthesis and/or LDL-receptor-mediated endocytosis, cholesterol esterification and de-esterification are additional mechanisms by which cells can regulate their free cholesterol content [25]. HDL contributes to the maintenance of normal cell cholesterol homeostasis by promoting the efflux of excess cholesterol from peripheral tissues [3,4]. *In vivo*, accumulation of cholesterol esters is a normal cellular process in lipoprotein-secreting tissues, such as intestine and liver, and in steroid-hormone-secreting organs [27], whereas excess storage in other tissues can lead to certain pathologies. As is well known, the accumulation of cholesterol esters within macrophages and smooth-muscle cells is a characteristic feature of early lesions of human atherosclerotic plaque [28]. However, the levels of cholesterol esters greatly increase in developing [29] as well as in rapidly proliferating tissues [11,12,30]. In addition, accumulation of cholesterol esters is also a consistent finding in human cancers, as shown by us [15,16] and others [31]. In this context, Gebhard et al. [31] have reported that free cholesterol in human clear cell carcinoma of the kidney is preferentially stored as cholesterol esters via acyl-CoA-cholesterol acyltransferase, the intracellular esterifying enzyme of cholesterol, rather than being released from the cells to circulating HDL, irrespective of whether it is derived from endogenous synthesis or external uptake. Similarly, numerous studies from our laboratory have shown stimulation of the cholesterol-biosynthetic pathway, accompanied by ac-

cumulation of cholesterol esters in tumour tissues and a concomitant decrease in HDL cholesterol in the plasma compartment in human cancer patients [15,16] as well as in experimental models of neoplasms [13,17]. The significance of this alteration is currently under investigation. Our present results suggest that an increased ability to accumulate cholesterol esters is associated with an increased growth rate and decreased lag phase. CEM cells showed higher baseline levels of cholesterol esterification and an increased content of cholesterol esters compared with MOLT4 cells. The greater capacity to esterify and accumulate cholesterol in CEM cells was associated with a higher growth rate and a shorter lag phase after seeding. Given that cholesterol is essential for membrane biogenesis, a larger store of cholesterol esters in these cells when compared with MOLT4 could in fact allow more rapid mobilization and utilization and a more prompt re-entry into the cell cycle. On the other hand, Kraemer et al. [32] have reported that cholesterol ester enrichment of smooth-muscle cells by treatment with LDL-cholesterol or 25-hydroxy-cholesterol, an inducer of cholesterol esterification, may be a basic mechanism for smooth-muscle hyperplasia during atherogenesis since it prolonged basic fibroblast growth factor production in the atherosclerotic lesion.

In our study, the absolute levels of total cholesterol synthesis did not appear to be directly correlated with growth rate in MOLT4 and CEM cells. The slower growing MOLT4 cells had in fact significantly higher baseline levels of cholesterol synthesis than CEM cells.

In summary, the results reported in this study add new insights on the possible role of cholesterol metabolism during proliferating processes. The regulatory role of the cholesterol-biosynthetic pathway in the response of cells to mitogenic stimuli is well established [5,6,9,10]. In addition the present data suggest a possible involvement of cholesterol ester content in regulating growth. It appears that cells can re-enter the cell cycle earlier if they have sufficient cholesterol ester stores that can be rapidly mobilized for metabolic needs, in particular membrane biogenesis.

Investigations on expression of the acyl-CoA-cholesterol acyltransferase gene and other genes related to cholesterol metabolism in different growth conditions should provide further insights on the possible role of cholesterol esters during cellular growth.

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