EUROPEAN JOURNAL OF CANCER 46 (2010) 1735–1743

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Severe hypocholesterolaemia is often neglected in haematological malignancies

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ARTICLE INFO

Article history:
Received 18 December 2009
Received in revised form 25 March 2010
Accepted 31 March 2010
Available online 29 April 2010

Keywords:
Cancer
Hypocholesterolaemia
Leukaemia
Lymphoma
Mevastatin

ABSTRACT

Aim of the study: It is generally believed that high levels of cholesterol (hypercholesterolaemia) are life-threatening, while low levels seem to be positive. Unfortunately this assumption is far from true, and can be indicative of an underlying serious medical condition in most of the cases (i.e. cancer). However, the biological role of severe hypocholesterolaemia is poorly understood. Here, the possible biological process is being investigated. Cholesterol plays a key role in cell proliferation, hence it has been suggested that low cholesterol levels are probably linked to the high cellular cholesterol demands from neoplastic cells.

Summary of the methods: We used serum and isolated T-lymphocytes from patients with acute lymphoblast leukaemia and human lymphoblast cell line to test this hypothesis.

Results: We found that patients with low serum cholesterol levels have instead high levels of cholesterol in lymphocytes. These data were supported with *in vitro* studies. In fact we have demonstrated that low cholesterol level in the culture medium was related to the neoplastic cellular growth, suggesting a greater use by lymphoma cells for their proliferation. Therefore by inhibiting cholesterol synthesis by mevastatin, *in vitro*, we showed that cholesterol levels did not change significantly in culture medium and the cellular growth was inhibited.

Concluding statement: Following these preliminary results, blood cholesterol levels could be potentially considered a good biological marker to follow up the neoplastic process.

1. Introduction

High levels of blood cholesterol (CHO) have always been an interesting and highly speculated issue for both public and scientific community,[1] given their association with increased risk of cardiovascular disease. Conversely low levels of CHO (hypoCHO) are often neglected. However, there is evidence of hypoCHO in patients with solid tumours and haematological malignancies,[2–4] although the reason and the biological role of hypoCHO in patients with cancer are not known.

To understand the possible relation between CHO and tumour, it is important to look inside the CHO metabolism and how this could potentially drive the tumour growth. It is generally known that CHO and its precursor mevalonate

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doi:10.1016/j.ejca.2010.03.041

play an essential role for cellular growth and division.[5,6] While high CHO levels have been observed during cell growth,[7,8] CHO synthesis inhibitors arrest the cell cycle progression,[9–11] modify molecular mechanisms for cell differentiation[12] and/or apoptosis[13,14] and inhibit angiogenesis.[15]

Moreover CHO has also an important role in regulating the synthesis of *invadopodia*: protrusion in the cell membrane of some cells, displaying the properties of sphingomyelin (SM) and CHO-enriched membranes or lipid rafts frequently seen in invasive and metastatic cancer cells that invade the surrounding tissues.[16] CHO depletion impairs the formation and persistence of invadopodia and this process is efficiently reverted by simply restoring the CHO demand.[17] It has been in fact demonstrated that lipid rafts are implicated in tumour growth and aggressiveness.[18] It has been reported that in human colon cancer cells cisplatin induces apoptosis with a redistribution of Fas, FADD and procaspase-8 into membrane lipid rafts and consequent early small GTPase RhoA activation which stimulates actin microfilaments' remodelling.[19]

Evidences regarding the important role that CHO plays in the tumour process come also from epidemiological studies, suggesting that the modern Western diet, which contains substantial levels of CHO and other fatty substances, facilitates prostate cancer progression. On the other hand prolonged inhibition of CHO synthesis, by pharmacologic intervention, has been associated with the reduction of risk for advanced prostate cancer.[18]

These are evidences on how CHO and its metabolism have an impact on the tumour process. Studies looking at the link between CHO and tumours are endless shifting from breast cancer resistance protein[20] to chemotherapy resistance.[21] Therefore the biological mechanism behind the role of CHO levels is probably linked to the high demand for cell growth. It has been reported that cancer patients had low density lipoprotein (LDL) and high density lipoprotein (HDL), which are essential for cell proliferation.[22] A study of 203 patients with altered lipid profile showed that 79 patients had serum LDL levels < or = 70 mg/dl while 124 patients had levels >70 mg/dl. The first group demonstrated increased odds of haematological cancer by more than 15-fold.[23] In addition in 102 patients with multiple myeloma (MM) the levels of total CHO and LDL were significantly lower than those of the controls and their value was higher in the patients with stage I than those of stages II and III, suggesting an increased LDL clearance and utilisation of CHO by myeloma cells.[24]

In support of this hypothesis we would probably expect a further reduction in serum CHO with a corresponding increase in neoplastic cells.

To the best of our knowledge, no indication was reported about the possible relation between low CHO blood level and high CHO cell amount in cancer so far. The aim of this study was to understand how neoplastic cells could benefit from CHO by studying (1) the CHO level in serum and isolate lymphocytes from patients with acute lymphoid leukaemia (ALL); (2) the CHO role on lymphoblastic lymphoma cell growth *in vitro* and (3) the ensuing modification of cancer cells with mevastatine, daunorubicine or 25-hydroxy-cholesterol (25-OH-CHO).

2. Materials and methods

2.1. Chemicals

Bovine serum albumin, CHO, 25-OH-CHO, phosphatidylcholine (PC), phosphatidylethanolamine (PE), SM, phosphatidylinositol (PI), phosphatidylserine (PS), phenylmethylsulphonylfluoride, IgG peroxidase conjugate and polyclonal anti-PKCzeta antibody were obtained from Sigma Chemical Co. (St. Louis, MI, USA). Thin layer chromatography (TLC) plates (silica Gel G60) were from Merck, Darmstadt, Germany. Radioactive [Me-^{14}C] SM (54.5 Ci/mol, 2.04 GBq/mmol), [Me-^{3}H] (L-3-phosphatidyl [N-Me-^{3}H] choline 1,2 dipalmitoyl, 81.0 Ci/mmol, 3.03 TBq/mmol) and [^{3}H]-thymidine were from Amersham Pharmacia Biotech (Rainham, Essex, UK), Ecoscint A from National Diagnostic (Atlanta, GA, USA). Polyclonal anti-Bax, anti-cyclinD1, anti-Signal Transducer and Activator of Transcription-3 (anti-STAT3), anti-Peroxisome Proliferator-Activated Receptor Gamma (anti-PPARgamma) and monoclonal anti-RNA polymerase II antibodies were obtained from Santa Cruz Biotechnology Inc. (CA, USA).

2.2. Patients

Blood samples from patients affected by ALL, diagnosed from the 'Laboratorio Centralizzato di analisi chimico-cliniche Ospedale Silvestrini, Perugia', were collected over a 24-month period. Young and without poor nutrition state or extremely dietary control patients were chosen as experimental model. From all patients (75), only those with a low level of CHO (12 patients) were analysed. The population was composed of 7 males and 5 females and average age was 33 years (range 20–55 years). Eight of 12 patients presented low level of CHO (range = 100–110 mg/dl) and 4 very low level of CHO (range = 65–100 mg/dl) referred to the laboratory analysis study. The control group was composed of 15 healthy blood donors: 10 males and 5 females and average age was 38 years (range 18–58 years) with normal serum CHO levels. CHO and CHO esters were measured in serum samples and in lymphocyte T isolated as reported by Boyum.[25]

2.3. Cell culture and treatments

Human T-lymphoblast cells (line SUP-T1) were grown in DMEM supplemented with 10% foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 g/ml streptomycin and 2.5 g/ml amphotericin B (fungizone). The cells were maintained at 37 °C in a saturating humidity atmosphere containing 95% air 5% CO_2. To analyse cell growth, the cells were counted until day six; the vitality of SUP-T1 cells were determined by trypan blue staining.

Increasing concentrations of CHO (200–1000 nM), mevastatin and/or daunorubicin (1–20 μM) were added to the cultures.

2.4. Homogenate preparation of cell culture

The cells were washed two times with PBS and centrifuged at 800*g* for 10 min. The pellets were suspended in buffer containing 1.5 M sucrose, 3 mM $CaCl_2$, 2 mM Mg acetate, 0.5 mM dithiothreitol + 1 mM PMSF, 3 mM Tris–HCl, pH 8.0 (1 ml/

10^3cells) and gently homogenised by a tight-fitting teflon-glass homogeniser.

2.5. Biochemical determinations

Protein, DNA and RNA contents were determined as previously reported.[26]

2.6. Lipid analysis

Lipids were analysed as previously reported.[4] The lipids were extracted from patients' serum, culture medium and cells and the phospholipids' (PLs) amount was measured by evaluating inorganic phosphorus. Either PLs or CHO and CHO esters were separated on TLC. The lipids were detected with iodine vapour by using the standards as reference and scraped into test tubes for amount determination.

2.7. DNA synthesis

The DNA synthesis was studied by evaluating the incorporation of ^{3}H-tymidine in the nuclear DNA. SUP-T1 cells were plated in 10 cm dishes and cultured with DMEM containing 0.2% FBS for further 24 h to induce cell synchronisation. After this time, the cells were cultured with complete medium supplemented with 10% FBS and examined at different times from 0 to 48 h. 1 μCi of ^{3}H-tymidine was added to the medium 2 h before the analysis. The cells were washed twice with PBS and centrifuged at 800*g* for 10 min. The pellet was re-suspended in 0.1 M Tris, pH 7.6, and used for nuclei purification. The DNA was extracted and used in part for DNA amount determination[24] and in part for radioactivity evaluation by diluting the samples in counting vials with 10 ml of Ecoscint A and 1 ml of H_2O and measuring the values with a Packard liquid scintillation counter. The effect of CHO, mevastatin and/or daunorubicin on DNA synthesis was evaluated at 24 h of cell culture. The experiment was repeated with 500 nM 25-OH-CHO, alone or in combination with 800 nM CHO.

2.8. Electrophoresis and Western blot analysis

Thirty micrograms of protein were used for SDS–PAGE electrophoresis as previously described[24] in 8% polyacrylamide slab gel for RNA polymerase II, Stat 3, PKCzeta, Cyclin D1, PPAR-gamma and 12% for Bax detection with specific antibodies diluted 1:1000. The apparent molecular weight of the proteins was calculated according to the migration of molecular size standard. The area density of the bands was evaluated by densitometry scanning and analysed with Scion Image.

2.9. Neutral-sphingomyelinase assay

Neutral-sphingomyelinase (N-SMase) activity was performed, at the optimal conditions, as reported for melanoma cells.[27]

2.10. Determination of ^{3}H-sphingomyelin level

The cells were incubated with 1 Ci/ml of ^{3}[H] palmitic acid, diluted with cold palmitic acid to a final concentration of 20 nM in culture medium containing 10% FBS for 24 h as previously reported.[28] The lipids were extracted and SM was separated by TLC. The spot was scraped and suspended in counting vials with 10 ml Ecoscint A and 1 ml water and the radioactivity was measured with a Packard liquid scintillation analyser.

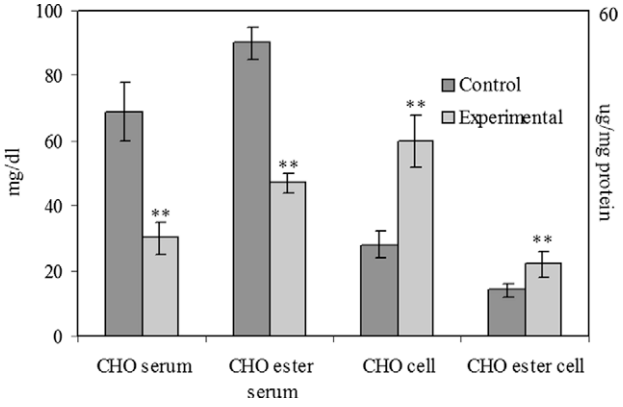

Fig. 1 – Cholesterol and cholesterol ester levels in serum and T-cells of LLA patients. The data are expressed as mg/dl and represent the average ± S.D. of four experiments performed in duplicate. Significance **$P < 0.001$ versus control sample. CHO, cholesterol.

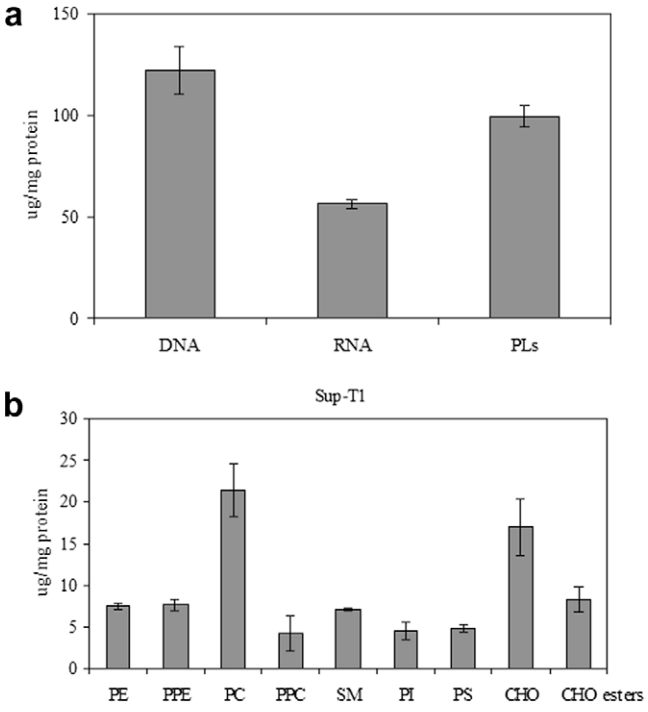

Fig. 2 – SUP-T1 composition. (a) DNA, RNA and phospholipid contents and (b) lipid fraction composition. The data are expressed as μg/mg protein and represent the average ± S.D. of four experiments performed in duplicate. PE, phosphatidylethanolamine; PPE, plasmalogen of phosphatidylethanolamine; PC, phosphatidylcholine; PPC, plasmalogen of phosphatidylethanolamine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; CHO, cholesterol.

2.11. Statistical analysis

Data are expressed as mean ± S.D. ANOVA or t-test was used for statistical analysis when appropriate.

3. Results

3.1. Cholesterol level in cancer patients

ALL patients with very low level of CHO (65–80 mg/dl) in serum were chosen as experimental model. The analysis of the serum lipid fraction showed that in the healthy blood donors (control sample) CHO and CHO esters were 31 ± 5 and 68 ± 6 mg/dl, respectively, whereas in all patients (experimental sample) the values were 12 ± 5 and 23 ± 8 mg/dl. Since the total value of CHO plus CHO esters was lower than that reported in the laboratory analysis, the lipids were extracted three times as reported in Section 2. After this treatment the CHO level was 69 ± 9 and 30 ± 6 mg/dl whereas CHO esters level was 99 ± 5 and 47 ± 7 mg/dl in the control and experimental samples, respectively (Fig. 1). This treatment has been used for all successive experiments. The data showed that in the experimental sample the CHO was reduced 56.5% and CHO esters 52.5% with respect to the control sample. The CHO and CHO esters in isolated lymphocytes of LLA patients increased 2.1-folds and 1.57-folds in comparison with lymphocytes isolated from healthy blood donors. The data did not change significantly after adjustment for age (range 20–30, 30–40, 40–55 years), family history of lipid profile and body mass index.

3.2. SUP-T1 composition

The SUP-T1 protein content was 480 ± 35 μg/10^6 cells. The RNA content was 2.15-folds and PL content was 1.23-folds lower than that of DNA (122.13 ± 11.4 μg/mg protein, Fig. 2a). To study the importance of CHO in SUP-T1 cells, the basal lipid composition was analysed in whole cells, thus including cellular membrane and cytoplasm. The results showed that the content of CHO and CHO ester was 16.69 ± 3.34 and 8.33 ± 1.15 μg/mg protein, respectively. The value of PC was 21.43 ± 3.21 μg/mg protein whereas that of PE, plasmalogen-PE (PPE), SM was about 7 μg/mg protein and that of plasmalogen-PC (PPC), PI and PS was about 4 μg/mg protein. The data indicated that SUP-T1 cells were very rich in CHO content (Fig. 2b).

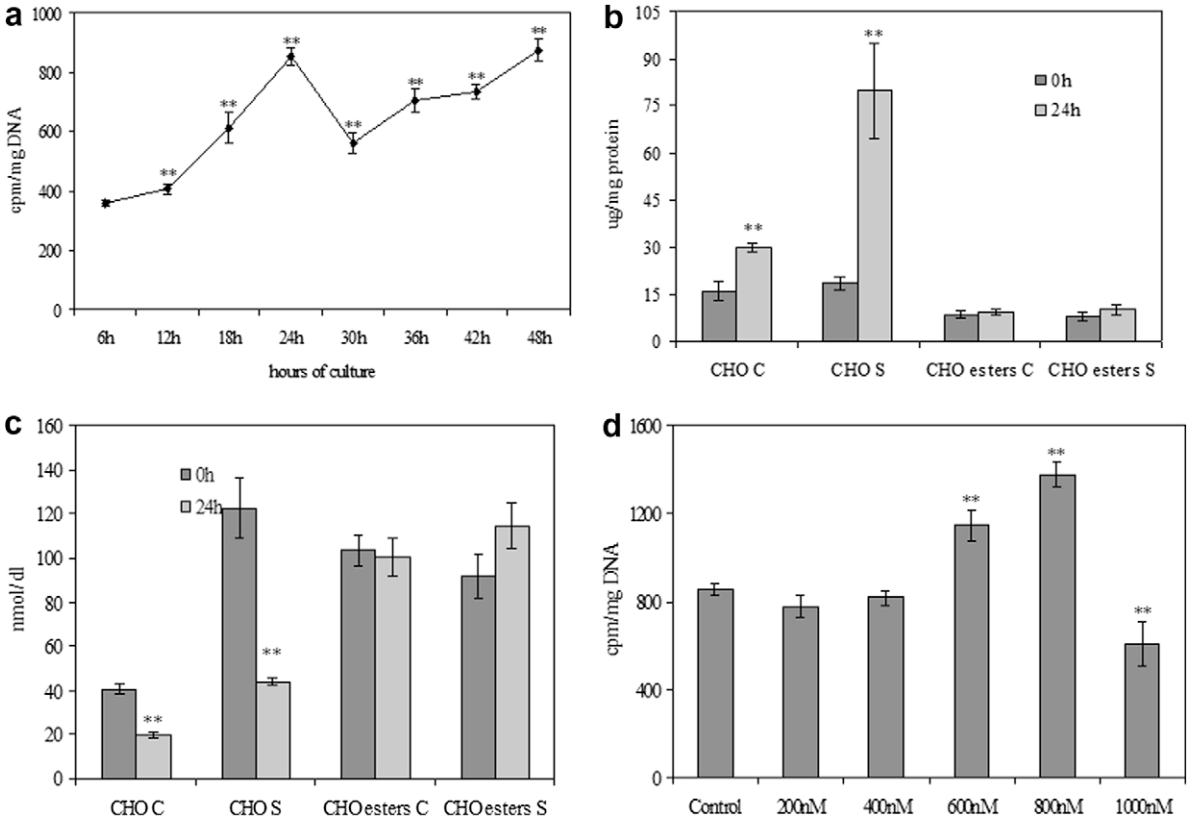

Fig. 3 – Lymphoblastic lymphoma cells: Role of cholesterol levels on DNA synthesis. (a) The experiment was performed at different hours of culture in synchronised cells by using radioactive ^{3}H-thymidine to study the peak of S-phase of the cell cycle. The data are expressed as cpm/mg DNA and represent the average ± S.D. of five experiments performed in duplicate; (b) after 24 h of incubation with high cholesterol (CHO) levels: DNA synthesis is stimulated, the data are expressed as cpm/mg DNA and represent the average ± S.D. of five experiments performed in duplicate; (c) the CHO levels decrease significantly in the medium, the data are expressed as nmol/dl and represent the average ± S.D. of four experiments performed in duplicate; and (d) the CHO levels increase in the cells, the data are expressed as μg/mg protein and represent the average ± S.D. of four experiments performed in duplicate. Significance **$P < 0.001$ versus 0 h.

3.3. *Effect of cholesterol in SUP-T1 cell growth and survival*

Cell growth was studied by the analysis of DNA synthesis performed in synchronised cells. The results showed that in the SUP-T1 cells the specific activity of the DNA, calculated as cpm/g DNA, was very low at 6 and 12 h, increased at 18 h and reached a peak at 24 h, which correspond to the S-phase of the cell cycle (Fig. 3a). Increasing concentrations of CHO (from 200 nM to 1000 nM) were added to the culture medium at 0 h and its effect was evaluated at 24 h. At 0 h the culture medium contained about 40 nmol/dl of CHO and 123 nmol/dl of CHO ester and therefore the CHO concentration increased to 60, 80, 100, 120 and 140 nmol/dl. Only 600 and 800 nM CHO stimulated the DNA synthesis 1.34× and 1.60×, respectively, whereas higher concentration had inhibitor effect and lower concentration did not have effect (Fig. 3b). This biphasic phenomenon of DNA synthesis which correlated with CHO concentration supports the previous observations reported in statin-treated cell experiments[29] and *in vivo* animal models.[30] It was evident that the optimal concentration was 800 nM and therefore it was used to evaluate the incorporation of CHO in the cells. After 24 h the CHO present in the culture medium was reduced 52% in the control sample (without CHO addition) and 64% in experimental sample (with 800 nM CHO, Fig. 3c). On the other hand the CHO present in the cells increased 1.87-fold in control sample and 4.30-folds in experimental sample (Fig. 3d). In another group of experiments the proteins involved in cell survival of tumour cells such as Cyclin D1 and STAT3,[31] RNA polymerase II,[32] PKCzeta[33] and PPARgamma[34,35] or in apoptosis such as Bax[36] were analysed. As shown in Fig. 4a the band of RNA polymerase II corresponding to 240 kDa apparent molecular weight, that of STAT3 corresponding to 90 kDa apparent molecular weight, that of PKCzeta corresponding to 80 kDa apparent molecular weight, that of PPARgamma corresponding to 68 kDa apparent molecular weight and that of Cyclin D1 corresponding to 38 kDa apparent molecular weight increased whereas band density of Bax, corresponding to 23 kDa apparent molecular weight, appeared reduced with 800 nM CHO. The analysis of band density demonstrated that RNA polymerase II increased 1.5×, STAT3 1.8×, PKCzeta 1.2×, PPARgamma 2.2×, Cyclin D1 1.9× whereas Bax decreased 1.3× (Fig. 4b).

To study the specific role of CHO on cancer cell growth, mevastatin and daunorubicin as anticancer drugs that act on CHO metabolism[37,38] were used. The results showed that 1 μM and 2 μM mevastatin did not reduce cell growth, 4 μM concentration reduced cell growth with very low level of dead cells (Fig. 5a). Differently, to obtain the same effect the optimal dose of daunorubicin was 2 μM (Fig. 5a). Higher concentrations of the two drugs increased the level of dead cells (Fig. 5a). These data were supported by comparing the effect of increasing mevastatin or daunorubicin concentrations on the DNA synthesis peak (Fig. 5b) and by cell counting (Fig. 5c). The incubation in the presence of 800 nM CHO reversed the effect of the two drugs (Fig. 5c). Since the mevastatin is a competitive inhibitor of HMG-CoA reductase, key enzyme for CHO synthesis that synthesises mevalonic acid, it is possible that the effect of the mevastatin on DNA synthesis was due to the inhibition of CHO synthesis. To support this observation, the cells were incubated with 25-OH-CHO, a potent inhibitor of HMG-CoA reductase.[39,40] The results showed that treatment with 25-OH-CHO alone induced a reduction of DNA synthesis equivalent to 46%, but the presence of CHO undid the effect of 25-OH-CHO and increased DNA synthesis 1.54× (Fig. 6). In addition, to document the effect of the mevastatin or daunorubicin on cell growth via CHO, the level of

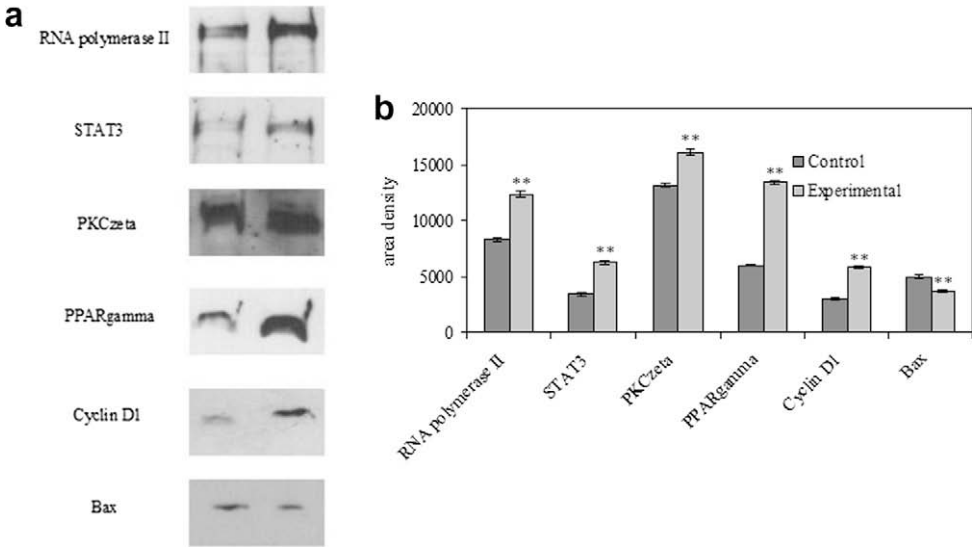

Fig. 4 – Effect of cholesterol on RNA polymerase II, STAT3, PKCzeta, PPARgamma, Cyclin D and Bax. (a) The position of the 240 kDa protein for RNA polymerase II, 90 kDa for STAT3, 80 kDa for PKCzeta, 68 kDa for PPARgamma, 38 kDa for Cyclin D and 23 kDa for Bax was indicated in relation to the position of molecular size standards and (b) The area density was evaluated by densitometry scanning and analysis with Scion Image; the data represent the mean ± S.E.M. of four separate experiments. **$P < 0.001$ versus control sample.

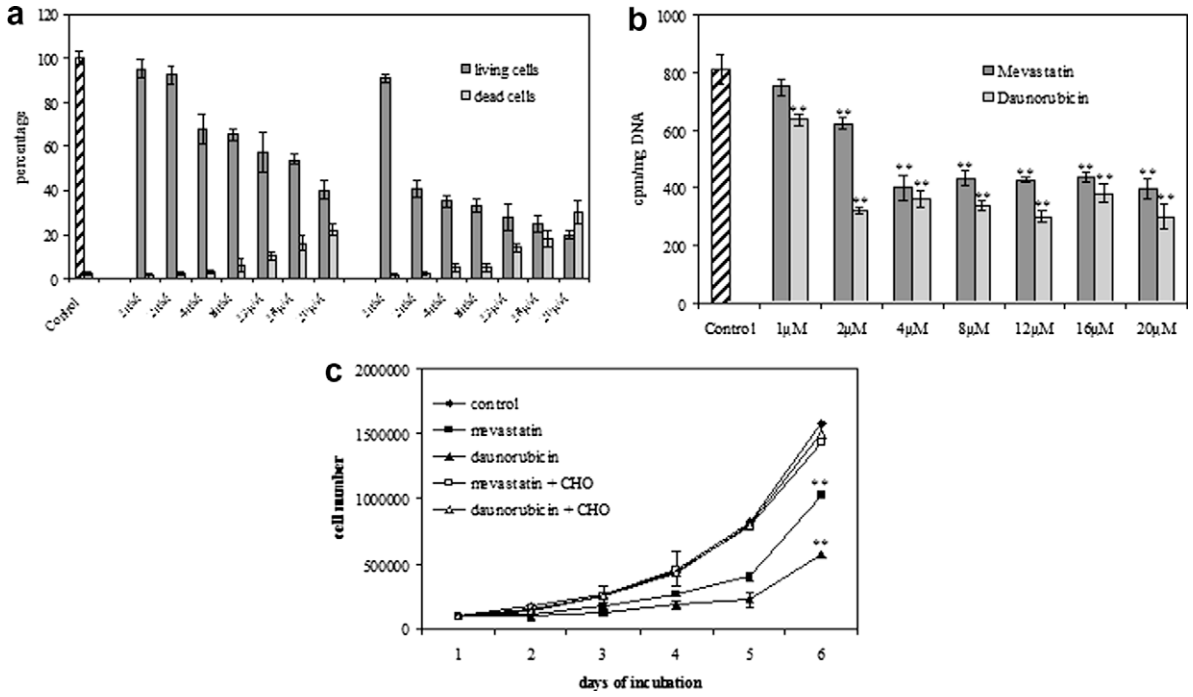

Fig. 5 – Effect of mevastatin or daunorubicin in SUP-T1 cells. (a) Living and dead cell numbers with increasing concentration of drugs, the data are expressed as percentage with respect to the control sample and represent the average ± S.D. of four experiments performed in duplicate; (b) DNA synthesis after 24 h of incubation with increasing concentration of drugs, the data are expressed as cpm/mg DNA and represent the average ± S.D. of four experiments performed in duplicate and (c) cell growth in the presence of 4 μM mevastatin or 2 μM daunorubicin or 4 μM mevastatin ± 800 nM cholesterol (CHO) or 2 μM daunorubicin ± 800nM CHO. **P < 0.001 versus control sample.

CHO and CHO esters in culture medium and in SUP-T1 cells was measured after 24 h of culture. As shown above, at this time the amount of control sample CHO decreased in culture medium and increased in the cells with respect to the sample at 0 h by stimulating cell growth. With either mevastatin or daunorubicin the translocation of CHO from culture medium to cells was inhibited (Fig. 7a and b). In addition the level of CHO in the cells was reduced with mevastatin treatment (Fig. 7b).

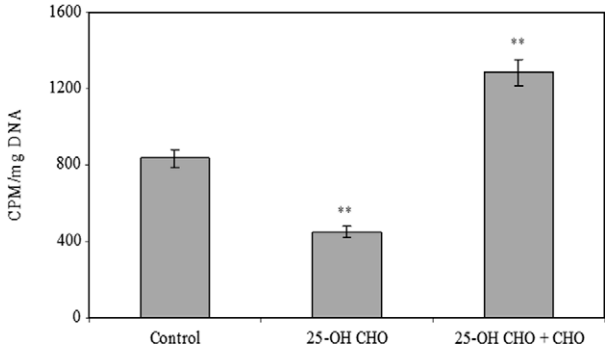

Fig. 6 – Effect of 25-hydroxy-cholesterol on DNA synthesis. The analysis was performed in synchronised cells cultured for 24 h in the presence of 500 nM 25-hydroxy-cholesterol (25-OH-CHO) or 500 nM25-OH-CHO + 800 nM cholesterol (CHO). The data are expressed as cpm/mg DNA and represent the average ± S.D. of three experiments performed in duplicate. **P < 0.001 versus control sample.

Because we had previously shown the relation CHO-SM regulated by N-SMase to address the cell to proliferation or apoptosis,[41] to more clarify the possible mechanism that uses CHO in SUP-T1 cells, the activity of N-SMase was assayed in the cells cultured for 24 h in the presence of CHO or mevastatin or daunorubicin. The results demonstrated that N-SMase activity was inhibited 1.84-fold with CHO whereas it was stimulated 1.42- and 1.49-fold with mevastatin or daunorubicin, respectively (Fig. 7c). The incorporation of labelled palmitic acid in SM supported these data (Fig. 7d).

4. Discussion

Our paper indicated clearly the importance of CHO in tumour cell growth. Cancer cells were hungry of CHO incorporated from serum with avidity and used to favour the expressions of proteins involved in cell proliferation such as RNA polymerase II, STAT3, PKCz and Cyclin D1. In addition CHO stimulated PPARgamma which represents a ligand-dependent nuclear receptor family that regulates multiple metabolic processes associated with CHO transport[42] and has a regulatory role in the proliferation, survival and differentiation of malignant B cells.[43] Our data obtained *in vitro* showed that a low increase in CHO concentration in the culture medium with respect to the normal level stimulated neither the incorporation of CHO into the cells nor cell growth whereas when the

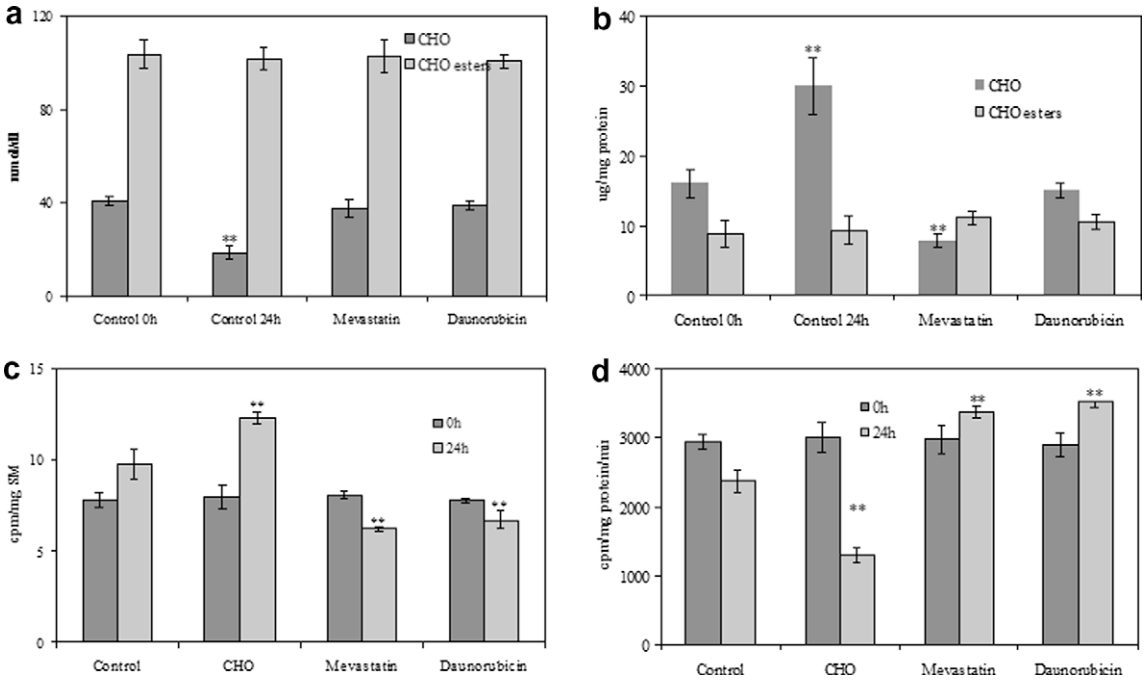

Fig. 7 – Cholesterol and sphingomyelin in SUP-T1 cell. (a) Cholesterol (CHO) and CHO esters in culture medium after 24 h of incubation without or with mevastatin or daunorubicin, the data are expressed as nmol/dl and represent the average ± S.D. of four experiments performed in duplicate; (b) CHO and CHO esters in the cells after 24 h of incubation without or with mevastatin or daunorubicin, the data are expressed as μg/mg protein and represent the average ± S.D. of four experiments performed in duplicate; (c) neutral sphingomyelinase activity, the data are expressed as pmol/mg protein/min and represent the average ± S.D. of five experiments performed in duplicate; and (d) ^{3}H-palmitic acid incorporation in sphingomyelin (SM), the data are expressed as cpm/mg SM and represent the average ± S.D. of five experiments performed in duplicate. **P < 0.001 versus control sample.

CHO concentration increased to 280 mg/dl, a strong CHO intake was detected and cell growth was significantly stimulated. These data could suggest that if the blood CHO concentration in patient with initial tumour is normal or slightly increased, the effect of CHO in tumour growth is low whereas the hyperCHO facilitates the entry of CHO in cells stimulating cell growth and inducing severe hypoCHO. Therefore the hyperCHO influences the development of tumour and the severe hypoCHO is a sign of tumour progression. Di Vizio et al. have reported epidemiological evidences suggesting that the modern Western diet, which contains substantial levels of CHO and other fatty substances, influences prostate cancer.[18]

We showed here that the anticancer drugs act on tumour cell CHO. In particular, the CHO synthesis is inhibited by mevastatin and 25-OH-CHO, both inhibitors of HMG-CoA reductase, whereas no effect is obtained with daunorubicin. Differently, either mevastatin or daunorubicin prevents the incorporation of CHO in tumour cells and induces them to apoptosis via N-SMase. These data suggest that hypoCHO originated from statin-related status is not correlated with the increase in CHO content in cancer cells as that occurs in hypoCHO of cancer patients. In addition mevastatin treatment regulates the CHO level in the blood to normal values and does not induce severe hypoCHO as in cancer. Therefore only severe hypoCHO status with an increase in CHO content in cancer cells was responsible for neoplastic proliferation. On the other hand in lymphocytes isolated from patients with AML there was an elevated receptor-mediated uptake of LDL with a decrease in the plasma CHO level.[3] After chemotherapic treatment, the leukaemic cells disappeared and the plasma CHO levels returned to normal values.[3] Considering our data that indicate the importance of blood CHO level in the patients with the initial tumour, we suggest that the therapy acting via CHO may need to be tailored for individual patients with tumour. Since our results indicated that during tumour process it is important the entry of CHO in the cell, it is possible to suppose that it could modify the structure/function of cell membrane modifying the specialised domains such as lipid rafts rich in CHO and SM that act as platform for different proteins involved in cell signal.[44] It has been proposed that cancer progression involves dysregulation of lipid raft-resident signalling complexes.[18] On the other hand death-inducing signalling complex clusters in lipid raft aggregates were reported as a supramolecular and physical entity responsible for the induction of apoptosis in leukaemic cells by the antitumour drug such as edelfosine.[45]

In conclusion we propose to use the level of CHO as a good biological marker for following up (low level) the neoplastic process.

Conflict of interest statement

None declared.

Acknowledgements

We thank Remo Lazzarini for the technical assistance. We also acknowledge the financial support through the contract grants Ministero dell' Università e Ricerca (PRIN project) and the Fondazione Cassa di Risparmio di Perugia.

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