Perturbation of polyamine metabolism and its relation to cell death in human colon cancer cells treated by 7ß-hydroxycholesterol and 7ß-hydroxysitosterol

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Abstract. 7ß-OHsitosterol and 7ß-OHcholesterol are natural compounds of plant and animal cells with high structural similarity. Recently it was reported that both compounds induced apoptosis on human colon cancer cells by targeting different signalling pathways. Our study aimed at comparing their effects on polyamine metabolism and its relation to apoptosis. When human colon cancer cells were exposed to 7ß-OHsitosterol and to 7ß-OHcholesterol at concentrations inhibiting growth by the same degree, both compounds caused a reduction of polyamine biosynthetic enzyme activity, of the polyamine pools, and an increase of N^1-acetylspermidine concentration indicating the enhancement of polyamine catabolism. Exogenous putrescine did not prevent cell death caused by 7ß-OHsitosterol, whereas 7ß-OHcholesterol-induced apoptosis was inhibited. MDL 72527, an inhibitor of polyamine oxidase, an enzyme of the polyamine catabolic pathway, potentiated the antiproliferative effects of 7ß-OHcholesterol by increasing the N^1-acetylspermidine pool and enhanced the accumulation of apoptotic cells. In contrast, MDL 72527 did not change the apoptosis rate and the N^1-acetylspermidine content in cells treated with 7ß-OHsitosterol. These data indicate that polyamine metabolic perturbations triggered by 7ß-OHcholesterol but not by 7ß-OHsitosterol are related to cell death.

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Introduction

Phytosterols are only present in plants, fruits and vegetables. Among the phytosterols, sitosterol, campesterol and stigmasterol are the most abundant (1). The cholesterol lowering effect of phytosterols is well documented and numerous studies on atherogenenesis have focused on their potential to reduce cholesterol absorption in the small intestine and cholesterol uptake by LDL (2). As for cholesterol, plant sterols undergo oxidation during storage and cooking. The 7ß-hydroxy form of phytosterols and cholesterol is abundant and has also been detected in human serum (3,4). Several studies have reported on the cytotoxic effects of 7ß-hydroxycholesterol (7ß-OHchol) on cancer cells (3,5), but few data exist on the anti-cancer properties of 7ß-hydroxysitosterol (7ß-OHsito) (6,7). The difference between these molecules is the presence of an ethyl group on 24' 7ß-OHsito (Fig. 1). Their structural similarities have led to the assumption that a functional similarity exists between 7ß-OHsito and 7ß-OHchol.

We have previously shown that 7ß-OHsito and 7ß-OHchol cause apoptosis on human colonic cancer cells (Caco-2). However, in spite of their structural similarities the two compounds targeted different cell death pathways (8). To gain more insight into the mechanisms involved in their apoptotic effects we studied the impact of the two hydroxysterols on intracellular pathways related to apoptosis and on polyamine metabolism in Caco-2 cells which is up-regulated in this cell line (9).

The natural polyamines are multifunctional constituents of all eucaryotic cells. They are involved in cell proliferation and the maintenance of cell viability. A characteristic of polyamine metabolism is its sophisticated regulation and its perturbation may induce dysfunction and cell death. Growth factors, hormones and polyamines themselves regulate key biosynthetic enzymes such as ornithine decarboxylase and S-adenosylmethionine decarboxylase, and catabolic enzymes such as spermine/spermidine N^1-acetyltransferase and polyamine oxidase as well as their uptake and release (10).

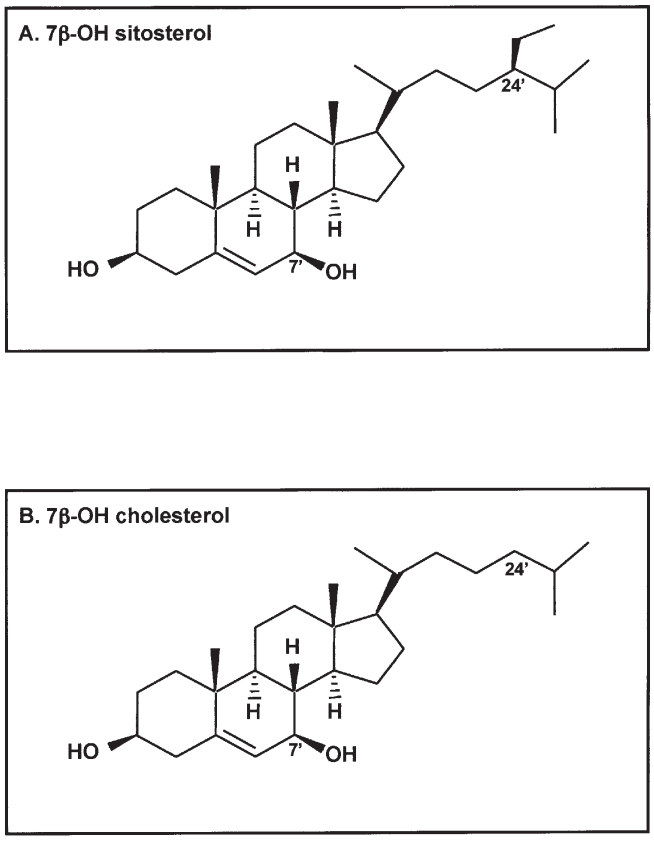

Figure 1. Structure of 7ß-OH sitosterol (A) and 7ß-OH cholesterol (B).

Recently, much attention has focused on the relationship between polyamine metabolism and programmed cell death with many contradictory results. In some cases apoptosis was activated due to the selective depletion of intracellular polyamine pools by using selective inactivators of key enzymes (11). Polyamine depletion has also been achieved by using structural analogues which mimic polyamines in regulatory pathways (12). In return, few data of apoptosis activation during increase of polyamine concentration have been described (13). There is no doubt about the link between the natural polyamines and several intracellular pathways controlling apoptosis and many schemes and hypotheses have been elaborated (10,14).

Our objectives were to compare the effects of 7ß-OHsito and 7ß-OHchol on polyamine metabolism in the human colon cancer Caco-2 cells and their relationship to cell death.

Materials and methods

Reagents. 7ß-OHsito and 7ß-OHchol were obtained from oxidation of the ß-sitosterol and cholesterol respectively. ß-sitosterol was purified by preparative adsorption chromatography starting from a commercial mixture of phytosterols (unsaponifiable fraction of Soya oil). The purity of the ß-sitosterol was >95%. The impurities consisted of traces of campesterol. Cholesterol was a Sigma-Aldrich product (Sigma-Aldrich, Steinheim, Germany). ß-sitosterol and cholesterol were oxidized as described previously (15). After purification on silica gel column, 7ß-OHsito and 7ß-OHchol were obtained at approximately 95% purity. Hydroxysterols were dissolved with ethanol 100% and for the control group the ethanol did not exceed 0.5% (v/v) in the culture medium. MDL 72527 [N^1,N^4-bis(2,3-butadienyl)-1,4-butanediamine dihydrochloride] was synthesized as described previously (16). Putrescine was purchased from Sigma-Aldrich. N^1-acetylspermidine was synthesized as described previously (17).

Cell culture. Human colon adenocarcinoma cells (Caco-2) were obtained from the European Collection of Animal Cell Culture (ECACC, Salisbury, UK). They were cultured in 75-cm^2 Falkon flasks in Dulbecco's modified Eagle's medium containing 25 mM glucose (DMEM) and supplemented with 10% heat-inactivated horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% non-essential aminoacids (Gibco, Invitrogen Corp., Cergy Pontoise, France). The use of horse serum avoids oxidative deamination of spermidine and spermine by serum amine oxidase. Cells were incubated at 37˚C in a humidified atmosphere with 5% CO_2 and subcultured after trypsinization (0.5% trypsin/2.6 mM EDTA). For all experiments, cells were seeded at 0.6-1x10^6 cells in culture dishes (10-cm diameter) or at 4500 cells in 96-well plates. They were cultured in DMEM supplemented with 3% heat-inactivated horse serum, 5 μg/ml transferrin, 5 ng/ml selenium and 10 μg/ml insulin (Gibco).

ODC and AdoMetDC assays. Cells (0.6x10^6/plate) were seeded and exposed to different compounds (60 μM 7ß-OHsito and 30 μM 7ß-OHchol) 24 h after seeding and further incubated for different times. At each time point, cells were harvested by scraping, washed twice in PBS and stored at -80˚C until analyses were carried out. Cells were homogenized by sonication in 100 mM Tris-HCl buffer, pH 7.4 containing 1 mM EDTA, 1 mM dithiothreitol, 0.5 μM leupeptin and 0.5 mM phenylmethylsulfonyl fluoride. After centrifugation at 33,000 x g for 25 min at 4˚C, ODC and AdoMetDC assays were performed in the supernatants. ODC activity was measured by the rate of $^{14}CO_2$ formation from [1-^{14}C] L-ornithine (55 mCi/mmol, Amersham Biosciences, France) and AdoMetDC activity by measuring the rate of $^{14}CO_2$ formed from [1-^{14}C] S-adenosylmethionine (60mCi/mmol, Amersham Biosciences) (18). Results were expressed as pmol/h/mg protein.

Determination of polyamine concentration. Cells (0.6x10^6/plate) were seeded and 24 h later they were incubated in the presence or absence of 60 μM 7ß-OHsito, 30 μM 7ß-OHchol, 50 μM MDL 72527 and combinations of MDL/hydroxysterols for different time points. Cells were collected as described above. The cell pellets were homogenized by sonication in perchloric acid (200 mM), and centrifuged at 3000 x g for 10 min after standing for 16 h at 2˚C. The acid-insoluble pellets were used for protein determination and the clear supernatants for the determination of putrescine, spermidine, spermine and N^1-acetyl spermidine by separation of the ion pairs formed with *n*-octanesulfonic acid, reaction of the column effluent with *o*-phthalaldehyde/2-mercaptoethanol reagent. The primary amino groups form fluorescent 1-alkylthio-2-alkylisoindole derivatives, which were determined by continuous monitoring of fluorescence intensity (19). Results were expressed as pmol/mg protein.

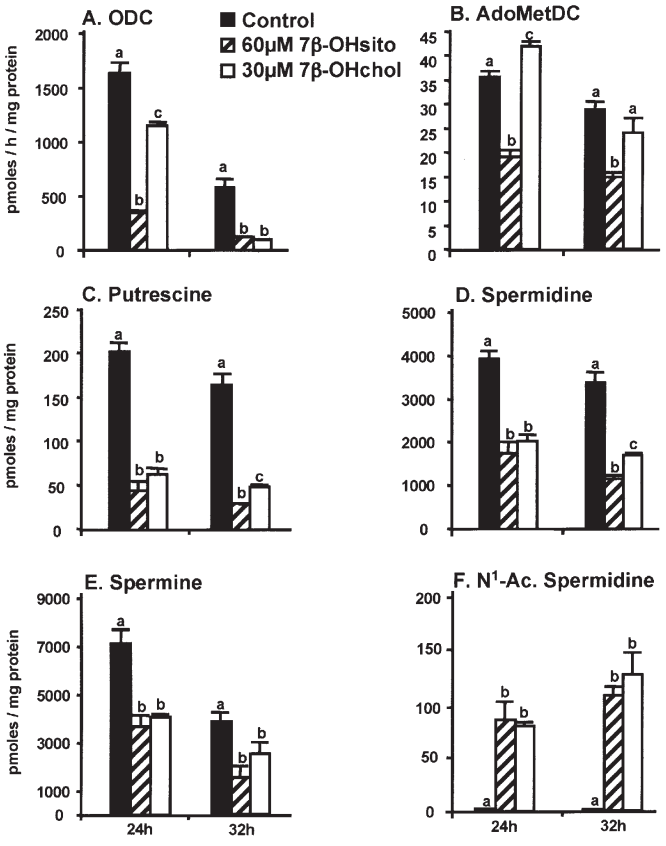

Figure 2. Effects of 7ß-OH sitosterol (7ß-OHsito) and 7ß-OH cholesterol (7ß-OHchol) on ornithine decarboxylase (ODC) and S-adenosyl methionine decarboxylase (AdoMetDC) activity and on intracellular polyamine concentration. Cells were grown in 0.5% ethanol (control)-containing DMEM, 60 μM 7ß-OHsito and 30 μM 7ß-OHchol for 24 and 32 h. A and B, ODC and AdoMetDc activity (pmol CO_2/h/mg protein); C, putrescine; D, spermidine; E, spermine; F, N^1-acetylspermidine (pmol/mg protein) in the absence (control) and in the presence of 7ß-OHsito and 7ß-OHchol. Data are the mean ± SE of at least 3 separate experiments. Statistical differences: a≠b≠c, p<0.05.

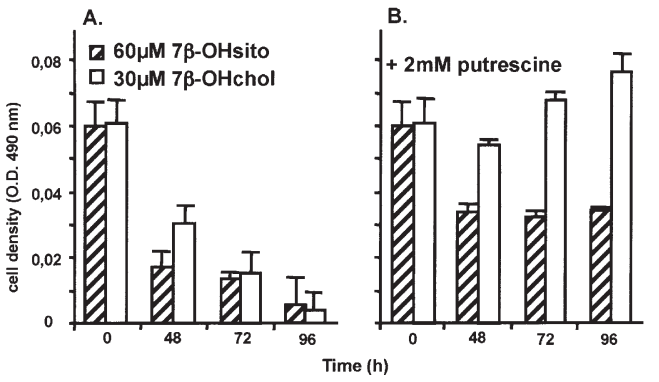

Figure 3. Effect of exogenous putrescine on Caco-2 cell growth after 7ß-OH sitosterol (7ß-OHsito) and 7ß-OH cholesterol (7ß-OHchol) treatment. A, cells were treated with 60 μM 7ß-OHsito and 30 μM 7ß-OHchol for 48, 72 and 96 h. B, 24 h after hydroxysterol treatment, 2 mM of putrescine was added. Columns represent cell growth at different time points. Data are presented as the mean ± SE of at least 3 separate experiments.

Effect of putrescine on Caco-2 cell growth. Cells (4500/well) were seeded in 96-well plates and exposed to hydroxysterols. Twenty-four hours after hydroxysterol treatment, 2 mM putrescine (Sigma-Aldrich) was added to the culture medium. Culture medium was changed every 48 h and fresh solutions of hydroxysterols and putrescine were prepared. Cell culture was stopped at different time points by the addition of 50 μl trichloroacetic acid (50%, v/v) and proteins were stained with 100 μl sulforhodamine B diluted in 1% acetic acid (0.4%, w/v). Cells were rinsed 3 times with 1% acetic acid and 200 μl/well of 10 mM Tris-HCl (pH 10.5) was added. Absorbance was measured at 490 nm (20). The relationship between cell number (protein content/well) and absorbance is linear from 0 to 200,000 cells/well.

Flow cytometry. Cells (1x10^6/plate) were exposed to different compounds 24 h after seeding and incubated for different time periods. Cell cycle distribution and percentage of hypodiploid DNA cells were analysed by labelling cells with propidium iodide and assays were carried out by flow cytometry (21). After incubation in the dark at 37˚C for 30 min, the fluorescence of 10,000 cells was analysed using a FACScan flow cytometer and CellQuest software (Becton Dickinson, San Jose, CA, USA).

Statistical analysis. All experiments were performed at least 3 times. Data are reported as mean ± SE. Statistical differences between groups were evaluated by one-way ANOVA and specific differences were identified using the Student-Neuman-Keuls multiple comparison test or the Student's t-test.

Results

Alterations of polyamine metabolism by 7ß-OHsito and 7ß-OHchol. Cells were exposed to 60 μM 7ß-OHsito and to 30 μM 7ß-OHchol for 24 and 32 h. Both compounds inhibited similar cell growth (8). As shown in Fig. 2A an ~70% reduction of ODC activity was observed in cells treated for 24 h with 7ß-OHsito whereas only a 30% reduction of enzyme activity was obtained with 7ß-OHchol. At 32 h both compounds reduced ODC activity similarly (Fig. 2A). In contrast, only 7ß-OHsito treatment led to a significant (45%) decrease of AdoMetDC activity (Fig. 2B).

As shown in Fig. 2C and E, cells exposed to both hydroxysterols exhibited a depletion of the polyamine pool, compared to untreated controls. Putrescine was reduced by 70%, and spermidine and spermine by 40-45%. This was accompanied by a significant increase in the intracellular content of N^1-acetylspermidine (Fig. 2F), an indicator of enhanced polyamine catabolism. N^1-acetylspermidine was not detected in untreated Caco-2 cells.

Effect of exogenous putrescine on cell growth and death. Putrescine was added to the culture medium 24 h after starting treatment with the hydroxysterols and it was again added together with the hydroxysterols at each media change. As represented in Fig. 3, inhibition of cell growth observed with 7ß-OHchol was prevented with 2 mM putrescine. In cells exposed to 7ß-OHsito the effect of putrescine was less important.

Flow cytometry analysis of hypodiploid cells was used in order to assess the effect of exogenous putrescine on cell

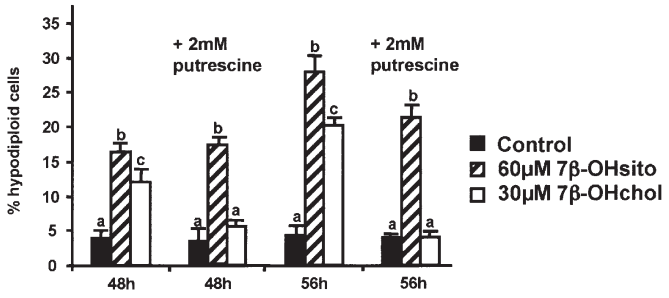

Figure 4. Determination of hypodiploid cells after addition of exogenous putrescine to 7ß-OH sitosterol (7ß-OHsito) and 7ß-OH cholesterol (7ß-OHchol) treated Caco-2 cells. Cells were grown in DMEM in the presence of 0.5% ethanol, 60 μM 7ß-OHsito and 30 μM 7ß-OHchol. Exogenous putrescine (2 mM) was added 24 h after the hydroxysterols. Cells were harvested at 48 and 56 h after hydroxysterol treatment, stained with propidium iodide and analyzed by flow cytometry. Columns represent the % of hypodiploid cells. Data are the mean ± SE of at least 3 separate experiments. Statistical differences: a≠b≠c, p<0.05.

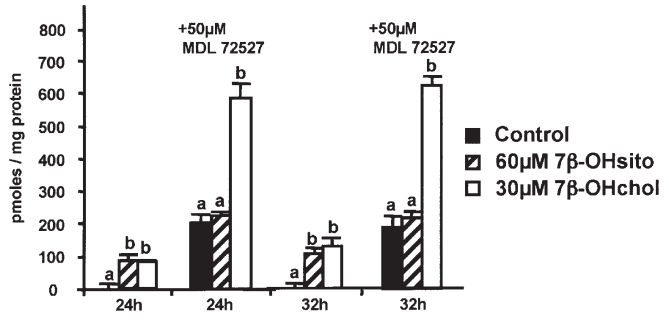

Figure 5. Determination of N^1-acetylspermidine in Caco-2 cells exposed to hydroxysterols and MDL 72527. Caco-2 cells were cultured in DMEM containing 0.5% ethanol in the absence (control) or presence of 60 μM 7ß-OHsito, 30 μM 7ß-OHchol and 50 μM MDL 72527. At 24 and 32 h cells were isolated and prepared for the determination of N^1-acetylspermidine (pmoles/mg protein). All data are the mean ± SE of at least 3 separate experiments. Statistical differences: a≠b, p<0.05.

death after treatment with hydroxysterols (Fig. 4). Cells were exposed to hydroxysterols for 24 h and then exogenous putrescine was added to cells. The proportion of hypodiploid cells was determined at 24 and 32 h after putrescine addition. These time periods corresponded to 48 and 56 h of culture in the presence of the hydroxysterols. In the absence of putrescine the amount of hypodiploid cells was significantly increased by hydroxysterol treatments compared to controls. In the presence of putrescine the number of hypodiploid cells was significantly reduced in 7ß-OHchol-treated cells as compared to cells exposed to 7ß-OHsito. These data are in accordance with data shown in Fig. 3B where cell growth was inhibited by exogenous putrescine in the case of 7ß-OHchol.

Effect of MDL 72527 and N^1-acetylspermidine on hydroxysterol-mediated cell death. As shown in Fig. 5, N^1-acetylspermidine accumulated in cells in the presence of the PAO inhibitor (50 μM MDL 72527). A 3-fold increase of N^1-acetylspermidine was observed in cells treated with 7ß-OHchol and MDL 72527, whereas no changes were observed in cells exposed to a combination of 7ß-OHsito and MDL 72527.

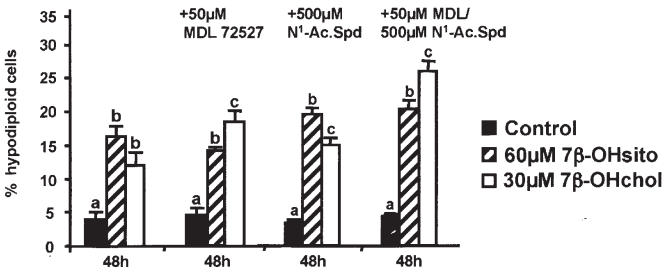

Figure 6. Effect of hydroxysterols, MDL 72527 and N^1-acetylspermidine on hypodiploid cell formation. Caco-2 cells were treated with DMEM containing 0.5% ethanol (control), 60 μM 7ß-OHsito and 30 μM 7ß-OHchol in the absence or presence of 50 μM MDL 72527, 500 μM N^1-acetyl spermidine (N1-AcSpd) and MDL/N^1-AcSpd. Cells were harvested at 48 h, stained with propidium iodide and hypodiploid cells were determined by flow cytometry. Columns represent the % of hypodiploid population. Data are the mean ± SE of at least 3 separate experiments. Statistical differences: a≠b≠c, p<0.05.

The number of apoptotic cells was significantly increased after a combined treatment with 7ß-OHchol/MDL72527 when compared to cells exposed to 7ß-OHsito in the presence or absence of MDL 72527 (Fig. 6). These data suggest that the accumulation of N^1-acetylspermidine caused by MDL 72527 is correlated to the apoptotic effect of 7ß-OHchol. Moreover, when cells were exposed to 500 μM of exogenous N^1-acetylspermidine the amount of apoptotic cells was further enhanced when the acetylated polyamine was associated with MDL 72527 but only in the presence of 7ß-OHchol and not in the presence of 7ß-OHsito (Fig. 6).

Discussion

We have previously reported that 7ß-OHsito at 60 μM and 7ß-OHchol at 30 μM similarly inhibited Caco-2 cell growth and induced apoptosis as it was demonstrated by DNA fragmentation and caspase-9 activation (8). This study demonstrates that both compounds impair polyamine biosynthesis; 7ß-OHsito was more potent than 7ß-OHchol by inhibiting the activity of both polyamine biosynthetic enzymes, ODC and AdoMetDC. A high expression of ODC and AdoMetDC is characteristic of cancer cells (22). Polyamine metabolism is a target in colon cancer chemoprevention and an increase of polyamine catabolism is usually associated with a decrease of polyamine concentration (9).

The decrease in the activity of polyamine biosynthetic enzymes and in the intracellular polyamine content seem not to be directly related to apoptosis induced by 7ß-OHsito since cell growth inhibition was not prevented by exogenous putrescine. In contrast, we found that 7ß-OHchol-mediated apoptosis was significantly reduced by putrescine. This suggests that in 7ß-OHchol-mediated apoptosis, the perturbation of polyamine metabolism plays a more important role than in 7ß-OHsito-induced apoptosis.

Our data show that both hydroxysterols caused an activation of intracellular polyamine catabolism as indicated by the enhanced accumulation of acetylated polyamines. Two key enzymes are involved in polyamine catabolism: spermine/

spermidine acetyl transferase (SSAT) and PAO. Using spermine and spermidine as substrates, SSAT generates the formation of N^1-acetyl polyamines which are substrates of PAO (23). Inactivation of PAO by MDL 72527 is followed by a decrease of putrescine and spermidine pools and an increase of acetylated polyamines (24).

We report that, combined with hydroxysterols, MDL 72527 reduced the polyamine content of Caco-2 cells. However the concentration of N^1-acetylspermidine and the number of apoptotic cells were increased when 7ß-OHchol was associated with MDL 72527. Similar effects were not observed in the presence of 7ß-OHsito. Since MDL 72527 inactivates PAO, the observed increase of N^1-acetylspermidine may be caused by the prevention of its degradation. Alternatively, an activation of SSAT cannot be excluded. Furthermore, we found that these effects could be further enhanced by exogenous N^1-acetylspermidine, indicating a direct involvement of acetylated polyamines on cell death in the presence of 7ß-OHchol. These effects were not observed with 7ß-OHsito.

Depletion of acetyl-CoA due to the massive formation of N^1-acetylpolyamines may contribute to growth inhibition (25,26) and one may speculate about the possibility that the accumulation of intracellular N-acetylpolyamines affects histone acetylation by competing with acetylCoA:spermidine N^8-acetyltransferase, an enzyme that has also histone acetylating properties (27). However, these and related effects are more likely to be observed in situations of excessive SSAT induction than under the conditions of the present study, although they cannot be excluded at present.

We have previously shown that 7ß-OHsito and 7ß-OHchol induced apoptosis on colon cancer cells by targeting different pathways in spite of their structural similarity (8). In the case of 7ß-OHsito, a caspase-dependent pathway is activated whereas, in the case of 7ß-OHchol, the process is more complex and involves various caspase-independent factors. These differences may be related to the alkyl side chain present at position 24' for 7ß-OHsito. Cholesterol oxides are well-known modulators of cholesterol metabolism which affect cell proliferation and death (3), and may interact with polyamine metabolism. However, it was shown that plant sterols have no effect on cholesterol metabolism due to the presence of the alkyl side chain which prevents any interaction with molecules implicated in cholesterol biosynthesis (28). However, no data exist for plant sterol oxides. It was previously reported that the alkyl side chain renders the molecule more hydrophobic and alters the composition of cell membrane lipids leading to the activation of cell factors and pathways implicated in cell viability and death (29).

The present report indicates that 7ß-OHchol and 7ß-OHsito both alter polyamine biosynthesis and catabolism but to a different degree. Moreover, alterations in polyamine metabolism initiated by 7ß-OHchol have a direct incidence on Caco-2 cell death, a situation which was not observed for 7ß-OHsito.

Acknowledgements

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