

# Effect of $7\beta$ -hydroxycholesterol on astrocyte primary cultures and derived spontaneously transformed cell lines

## Cytotoxicity and cholesterogenesis

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Received 14 January 1991

The correlation between the lethal effect of  $7\beta$ -hydroxycholesterol ( $7\beta$ -OH-CH) on spontaneously transformed cell lines derived from rat astrocyte primary cultures (normal cells) and de novo cholesterogenesis was investigated. Both  $7\beta$ -OH-CH and 7-keto-CH were not cytotoxic on normal cells but  $7\beta$ -OH-CH affected markedly the viability of the transformed cells. The use of [ $^{14}$ C]acetate or [ $^{14}$ C]mevalonate indicated that 7-keto-CH inhibits de novo cholesterogenesis upstream of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) in both cell types whereas  $7\beta$ -OH-CH also inhibits downstream of HMGR. The accumulation of two radiolabelled products  $X_1$  and  $X_2$  between mevalonate and CH was found in unsaponifiable neutral lipids extracted from  $7\beta$ -OH-CH treated transformed cells. HPLC and GC-MS revealed that  $X_1$  and  $X_2$  are not lanosterol and 24,25-epoxylanosterol, respectively. Incubation of the transformed cells with  $X_1$  and  $X_2$  did not affect their viability. Our data demonstrate that, under our experimental conditions,  $7\beta$ -OH-CH cytotoxicity is not linked to the inhibition of de novo cholesterogenesis in cultured glial transformed cells.

Astrocyte culture;  $7\beta$ -Hydroxycholesterol; Cytotoxicity; Cholesterogenesis; 3-Hydroxy-3-methylglutaryl CoA reductase

## 1. INTRODUCTION

The  $7\beta$ -hydroxycholesterol ( $7\beta$ -OH-CH) is a metabolite of cholesterol (CH), present in minute amounts in human plasma and biliary fluid [1,2] and secreted by rat liver epithelial cell lines [3]. In vitro studies have shown that  $7\beta$ -OH-CH exert a cytotoxic activity on proliferating and tumoral cells (for review see [4]) such YAC-1 and RDM-4 lymphomas, HTC cells, ZHC cells, heart fibroblasts and liver epithelial cells [5-8]. The lethal effect consisted either of rapid lysis or loss of adhesion and 'necrosis' of the cells.

With regard to the action mechanism of this compounds, two hypotheses prevail: (i) the incorporation of  $7\beta$ -OH-CH in the plasma membrane of transformed and tumoral cells may affect its fluidity, function and stability [9]; (ii)  $7\beta$ -OH-CH, as other oxysterols, inhibits 3-hydroxy-3-methylglutaryl-CoA reductase activity [10] and thus affects de novo cholesterogenesis required for cell proliferation at G1 and/or M phases [11,12].

Recently, we have demonstrated that  $7\beta$ -OH-CH was cytotoxic to spontaneously transformed cell lines derived from neonatal rat astrocyte, but not to the mother cells [13]. Further investigations indicated that the decline in plasma membrane CH/phospholipid (PL) is

not the unique cause of  $7\beta$ -OH-CH cytotoxicity. We have also demonstrated that the transformed cells esterified  $7\beta$ -OH-CH on C-3-OH by naturally occurring fatty acids and that the extent of esterification correlates with the degree of  $7\beta$ -OH-CH cytotoxicity [13-15]. However, the effect of  $7\beta$ -OH-CH on de novo CH biosynthesis in our biological model remained unclear and thus, the relationship between  $7\beta$ -OH-CH cytotoxicity and CH biosynthesis pathway has been addressed in this report.

## 2. MATERIALS AND METHODS

### 2.1. Primary cultures of astrocytes (normal cells)

Astrocyte primary cultures derived from neonatal rat brain hemispheres were obtained according to Sensenbrenner et al. [16]. The cells were seeded at a density of  $2 \times 10^5$  on 35 mm diameter culture dishes (Falcon 3001, USA). Culture medium (2 ml) (Dulbecco's Modified Eagles, DMEM; Gibco, Grand Island, NY) was supplemented with 10% fetal calf serum (FCS; Gibco). Cells were grown in a 95% air/5%  $\text{CO}_2$  humid atmosphere at 37°C and medium was changed at 5 days intervals.

### 2.2. Spontaneously transformed cell lines (transformed cells)

Spontaneously transformed cell lines were derived from the primary cultures by repetitive subculturing as reported previously [17]. To each subculture a passage number (P) was attributed and P101, P102, P103, P105, P106 and P117 were obtained by trypsinization of the confluent previous passages with 0.05% trypsin and 0.04% Tyrode-KCl. The harvested cells were seeded at a density of  $10 \times 10^4$  cells per 35 mm culture dishes in DMEM containing 10% FCS. Cells were grown as described for the primary cultures.

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### 2.3. Morphological studies and cell viability

Cells treated with 7-keto-cholesterol (7-keto-CH) or with 7 $\beta$ -OH-CH were observed with a Nikon phase-contrast microscope and photographed with a Nikon camera. The cell viability was tested by the trypan blue method and the cell number was determined as previously described [18].

### 2.4. Effect of 7-keto-CH and 7 $\beta$ -OH-CH on 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) activity

Growing or confluent cell cultures (13 or 19 days in culture for normal cells, respectively, and 2 or 5 days in culture for transformed cells, respectively) were treated with 30  $\mu$ M 7-keto-CH (Sigma, USA) or 30  $\mu$ M 7 $\beta$ -OH-CH (Sigma, USA) dissolved in 8  $\mu$ l ethanol. After 24 h, cells were incubated either with 20  $\mu$ Ci of sodium [2- $^{14}$ C]acetate (53 mCi/mmol; Amersham, UK) in 100  $\mu$ l H<sub>2</sub>O or with 5  $\mu$ Ci of DL-dibenzylethylendiamine [2- $^{14}$ C]mevalonate (44 mCi/mmol; Amersham, UK) in 100  $\mu$ l NaCl 9% for 2 h. Cells were washed with 9% NaCl, detached from the culture substratum by means of a rubber policeman and submitted for radioactive steroids analysis. When experiments were undertaken for detecting radioactive accumulation products between mevalonate and CH, transformed cell cultures were incubated with 1  $\mu$ Ci [2- $^{14}$ C]mevalonate for 16 h.

### 2.5. Lipid extraction and radioactive steroid analysis

Lipids were extracted according to Folch et al. [19] and submitted to bidimensional thin-layer chromatography (TLC) on precoated silica 60 plates (Merck, Germany) and to autoradiography as reported previously [13,20]. Silica gel areas corresponding to the radioactive spots were scraped in 500  $\mu$ l of H<sub>2</sub>O, dissolved in 10 ml scintillator (Biofluor, NEN, USA) and counted in a scintillation counter (United Technologies Packard Tri-Carb 4000 series, USA). For the detection of radioactive metabolites accumulating downstream of HMGR, the lipids were extracted in the presence of 0.08% butylated hydroxytoluene and submitted to saponification in 0.2 ml of a 0.5 M NaOH ethanolic solution for 30 min at 60°C; 0.2 ml of H<sub>2</sub>O was added and the unsaponified lipids extracted twice with 1 ml of hexane. The organic phase was washed with 0.8 ml of 48% ethanol containing 0.25 M NaOH and evaporated under a nitrogen stream. The dry residue was submitted to monodimensional TLC; the solvent system was petroleum ether (b.p. 40–60°C)/acetone(8:2 v/v).

### 2.6. HPLC

HPLC (Waters Associates) was performed with a 5  $\mu$ m silica liichrosorb reversed-phase column (3.9 mm  $\times$  15 cm) using methanol (Merk, Germany) as eluent with a flow rate of 1 ml/min and a pressure of 1000 p.s.i. The labeled compounds were detected at 220 nm by a Spectrachrom 100 spectrophotometer (Spectra, USA) and by a Flo-One counter (Radiomatic Instruments, USA) coupled to the column output. Retention times of 25-OH-CH (Sigma, USA), 7-keto-CH, 24,25-epoxylanosterol (a generous gift from Dr F. Schuber), desmosterol (Sigma, USA), lanosterol (Sigma, USA) and CH (Sigma, USA) were 2.10, 3.10, 3.10 and 6.50, 7.55 and 9.05 min, respectively.

### 2.7. GC-MS spectrometry

Mass spectra were obtained on a GC-MS (Finnigan-Mat, Model 4021, USA) operated in the electron impact mode with an electron energy of 70 eV. Injector, separator, transfer line and ion source temperatures were 300, 270, 270 and 250°C respectively. Gas chromatography was performed with a 25 m (0.32 mm internal diameter) fused silica capillary column coated with SE54 liquid phase (Film 0.2  $\mu$ m, methylsilicone). Helium N55 grade (Air Liquide, France) was used at a pressure of 1 bar as carrier gas. The column temperature was 220°C for 0.1 min, programmed from 220 to 290°C (3°C/min) and maintained at 290°C for 1 min. The undervivified samples were injected using a ROS moving needle injector. The injection solvent was chloroform and samples were eluted free from contaminants at 245°C.

Protein determination was performed according to Lowry et al. [21].

## 3. RESULTS

### 3.1. Cytotoxic effect of 7 $\beta$ -OH-CH on cultured transformed cells

Neither the viability, nor the overall morphology of 13 and 19 days cultured normal cells were affected by 30  $\mu$ M 7 $\beta$ -OH-CH. In contrast, this treatment affected 2 days in culture transformed cells: after 24 h cell bodies retracted progressively and, after 48 h, started to detach from the culture substratum (cell number and protein content were reduced by 60%). The lethal effect was delayed by 24 h when 5 days in culture transformed cells were treated with 30  $\mu$ M 7 $\beta$ -OH-CH. Replacement of 7 $\beta$ -OH-CH by 30  $\mu$ M 7-keto-CH was not lethal on both normal and transformed cell types. This model which demonstrates that neither 7 $\beta$ -OH-CH, nor 7-keto-CH are cytotoxic on normal cells and that 7 $\beta$ -OH-CH exclusively affected the transformed cells allowed us to investigate the relationship between 7 $\beta$ -OH-CH cytotoxicity and cholesterologenesis blockage.

### 3.2. Effect of 7 $\beta$ -OH-CH on HMGR activity

Endogenous HMGR activity was assayed by evaluating the incorporation of either radiolabelled [ $^{14}$ C]acetate (upstream of HMGR) or [ $^{14}$ C]mevalonate (downstream of HMGR) in de novo synthesized CH; the difference in percent inhibition of radioactivity incorporated in CH between cells incubated either with the former or with the latter radioactive precursor measures the extent of HMGR activity inhibition. Control experiments indicated that the extent of [ $^{14}$ C]acetate and [ $^{14}$ C]mevalonate uptake in the total lipid extract, which reached a plateau and 2 h incubation, was not modified by the addition of 7 $\beta$ -OH-CH and 7-keto-CH (7% and 0.2% of the added radioactivity for acetate and mevalonate, respectively). It is noteworthy that incorporation of [ $^{14}$ C]acetate and [ $^{14}$ C]mevalonate in CH by dividing transformed cell cultures was about 3–4-fold higher than when the cultures reached confluency (legend to Table I).

Table I shows that treatment of normal, dividing and quiescent cells, and of dividing transformed cells with 30  $\mu$ M 7-keto-CH reduces essentially the incorporation of [ $^{14}$ C]acetate in CH. In contrast to 7-keto-CH, 7 $\beta$ -OH-CH affects the incorporation of both [ $^{14}$ C]acetate and [ $^{14}$ C]mevalonate in CH; however, the extent of inhibition of label incorporation in CH is higher when the cells were incubated with [ $^{14}$ C]acetate than with [ $^{14}$ C]mevalonate (Table I). It is noteworthy that for confluent transformed cell cultures both 7-keto-CH and 7 $\beta$ -OH-CH act in a similar way: the percent inhibition of [ $^{14}$ C]acetate incorporation in CH was equal to that of [ $^{14}$ C]mevalonate.

These findings indicate that (i) 7-keto-CH and 7 $\beta$ -OH-CH inhibit de novo CH synthesis in normal and transformed cultured glial cells, (ii) 7-keto-CH acts mainly at HMGR activity step whereas 7 $\beta$ -OH-CH af-

Table 1

Inhibition of de novo cholesterol biosynthesis in rat astrocyte primary cultures and derived spontaneously transformed cell lines treated either with  $7\beta$ -OH-CH or 7-keto-CH

		$7\beta$ -OH-CH (30 $\mu$ M)		7-keto-CH (30 $\mu$ M)	
		$[^{14}\text{C}]$ acetate	$[^{14}\text{C}]$ mevalonate	$[^{14}\text{C}]$ acetate	$[^{14}\text{C}]$ mevalonate
Normal cells (a)	DC	95%	60%	90%	20%
	CC	80%	36%	90%	15%
Transformed cells (b)	DC	95%	70%	90%	25%
	CC	63%	75%	80%	80%

The cells were treated as described in Materials and Methods and the data is expressed as percent inhibition of radioactivity found in cholesterol. The radioactivity (cpm/mg protein) found in CH extracted from controls was 7000, 9300, 39462 and 7100 for normal, dividing and confluent, cultures and transformed, dividing and confluent, cultures respectively when treated with  $[^{14}\text{C}]$ acetate; in the case of  $[^{14}\text{C}]$ mevalonate the radioactivity found in CH was 700, 1050, 3200 and 620, respectively. (a) Normal cells treated at 13 and 19 days in culture. (b) Transformed cells treated at 2 or 5 days in culture (P102, P103 and P105). DC = dividing cultures; CC = confluent cultures;  $7\beta$ -OH-CH =  $7\beta$ -hydroxycholesterol; 7-keto-CH = 7-keto-cholesterol. These values are means of two independent determinations for normal cells and three for transformed cells carried out in duplicate, for which standard errors  $\leq 10\%$ .

fects also cholesterologenesis downstream of HMGR.

Thus we may infer that  $7\beta$ -OH-CH cytotoxicity does not correlate with cholesterologenesis inhibition since (i) 7-keto-CH and  $7\beta$ -OH-CH do not alter the development of the cultured normal cells but inhibit CH biosynthesis, (ii) 7-keto-CH which is not lethal to transformed cells inhibits CH biosynthesis.

### 3.3. Effect of $7\beta$ -OH-CH downstream of HMGR activity in the transformed cells

Since  $7\beta$ -OH-CH inhibits also CH biosynthesis

downstream of HMGR in transformed cells (Table 1), we have concentrated on the accumulation of radioactive metabolites between mevalonate and CH. Autoradiography of TLC plates revealed the presence of two radioactive compounds ( $X_1$  and  $X_2$ ) when dividing transformed cells were treated with  $7\beta$ -OH-CH and incubated with  $[^{14}\text{C}]$ mevalonate. Treatment of the transformed cells with 7-keto-CH did not show a significant accumulation of  $X_1$  and  $X_2$  (Fig. 1). The  $R_f$  of  $X_1$  corresponds to lanosterol whereas that of  $X_2$  is slightly lower than that of 24,25-epoxylanosterol (Fig.

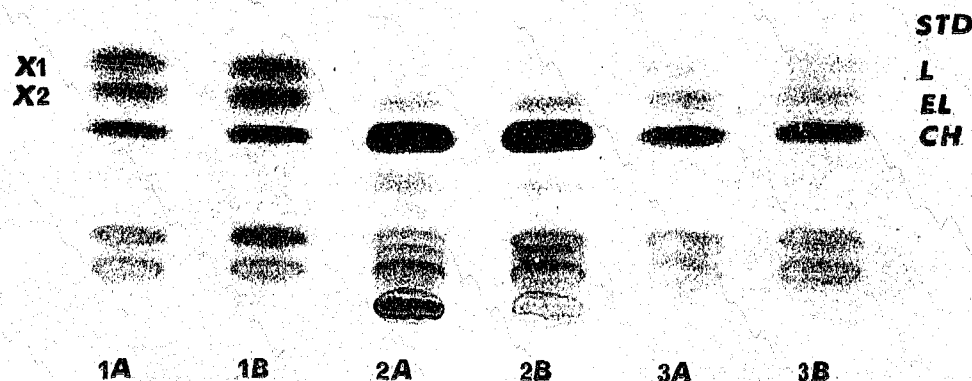


Fig. 1. Autoradiography of  $^{14}\text{C}$ -radiolabelled unsaponifiable neutral lipids extracted from dividing transformed cells (A: P106; B: P117) treated either with  $7\beta$ -OH-CH (lane 1) or with 7-keto-CH (lane 3) and with  $[^{14}\text{C}]$ mevalonate as described in Materials and Methods. The chromatogram was exposed to X-ray film for 4 days. (Lane 1A) 12 000 cpm; (lane 1B) 15 000 cpm; (lanes 3A) 11 000 cpm; (lane 3B) 11 000 cpm. Lanes 2A (30 000 cpm) and 2B (32 000 cpm) represent the autoradiography of the extracted, and chromatographed, unsaponifiable neutral lipids from cells treated with  $[^{14}\text{C}]$ mevalonate only. STD = standards; L = lanosterol; EL = 24,25-epoxylanosterol; CH = cholesterol.

1). In the case of confluent transformed cell cultures, the extent of  $X_1$  and  $X_2$  in control,  $7\beta$ -OH-CH, and 7-keto-CH treated cells was similar and low. HPLC and GC-MS analysis indicate that  $X_1$  and  $X_2$  are not eluted as lanosterol and 24,25-epoxylanosterol. The retention times under HPLC analysis are 4.10 min for  $X_1$  and 4.45 min for  $X_2$  whereas it is 3.10 min and 7.55 min for lanosterol and 24,25-epoxylanosterol, respectively. Under GC-MS analysis,  $X_1$  is eluted at 8.38 min and shows a major peak ( $m/e$ : 375) whereas lanosterol is eluted at 20.22 min with a characteristic molecular peak ( $m/e$ : 426). Thus  $X_1$  and  $X_2$  seem less polar than lanosterol and 24,25-epoxylanosterol under our experimental HPLC and GC-MS conditions.

These observations raised the question whether  $X_1$  or/and  $X_2$  are implicated in  $7\beta$ -OH-CH cytotoxicity. Extraction of  $X_1$  and  $X_2$  from the silica with chloroform/methanol/water (5:5:1 v/v) and incubation of the cultured cells with amounts of  $X_1$  and  $X_2$  (dissolved in 8  $\mu$ l ethanol) which were equivalent to those synthesized by a same quantity of cells did not alter their viability.

#### 4. DISCUSSION

Astrocyte cultures provide a valuable system for testing the cytotoxicity of  $7\beta$ -OH-CH and elucidating its action mechanism since this cell type possesses a proliferative potential which is involved in certain pathological processes such as reactive gliosis and the production of glioblastomas. We took advantage of the fact that neither  $7\beta$ -OH-CH nor 7-keto-CH are cytotoxic on the normal cells, on the one hand, and that, on the other hand,  $7\beta$ -OH-CH exclusively affects the transformed cells for examining the correlation between  $7\beta$ -OH-CH cytotoxicity and inhibition of de novo cholesterol synthesis. We focus particularly on HMGR, the rate-limiting enzyme of the sterol synthetic pathway, which catalyzes the conversion of HMG-CoA to mevalonate.

Our results indicating a high level of cholesterol synthesis in cultured dividing cells and the inhibition of de novo cholesterol synthesis by both  $7\beta$ -OH-CH and 7-keto-CH agrees with the findings of other authors [22,23]. We have also demonstrated a differential action of  $7\beta$ -OH-CH and 7-keto-CH on CH biosynthesis: 7-keto-CH acts on HMGR activity whereas  $7\beta$ -OH-CH affects also the pathway downstream of HMGR. Interestingly enough, that  $7\beta$ -OH-CH and 7-keto-CH affected CH biosynthesis downstream of HMGR activity in confluent transformed cell cultures may be attributed to the dynamic and physical state of the cultures growing as layers overlapping one another. Nevertheless, our findings demonstrate clearly that correlation between oxysterol cytotoxicity and de novo CH biosynthesis could not be established. Maltese et al. and Langan et al. have shown the relationship between

sterol biosynthetic pathway and DNA synthesis and cellular proliferation in developing normal and tumoral astrocyte cultures [11,24]; however, differences in the biological model as well as in the culture conditions should be kept in mind. This may also explain the fact that 25-OH-CH cytotoxicity on C6 cells corresponds to the decline of membrane CH/PL [24] whereas the cytotoxicity of  $7\beta$ -OH-CH on our transformed cells does not always correlate with CH/PL decrease [14]. Our model also seems useful for investigating the effect of oxysterols on HMGR activity; recently, Shaw et al. [25] have reported that the use of human hepatoblastoma cell line (Hep-G2) may not be the appropriate cell system for studying the inhibition of HMGR activity by CI-981, BMV-21950, lovastatin, pravastatin and fluvastatin.

The use of [ $^{14}$ C]mevalonate concomitantly with  $7\beta$ -OH-CH or 7-keto-CH revealed the accumulation of two radiolabelled compounds downstream of HMGR activity in transformed cells treated with  $7\beta$ -OH-CH only. In spite of  $R_f$  values similar to lanosterol and 24,25-epoxylanosterol according to TLC and autoradiography, HPLC and GC-MS indicated that  $X_1$  and  $X_2$  are not lanosterol and 24,25-epoxylanosterol, respectively. Gupta et al. [26] have demonstrated that the incubation of IEC-6 cultures with ketoconazole inhibits HMGR activity with the accumulation of lanosterol and 24,25-epoxylanosterol. Treatment of dividing transformed cells with  $X_1$  and  $X_2$  did not affect their viability. Moreover, treatment of confluent cultures with  $7\beta$ -OH-CH affected the cellular viability whereas  $X_1$  and  $X_2$  accumulate faintly.

These data which demonstrate the inhibition of de novo cholesterol synthesis downstream of HMGR activity, exclude the involvement of this phenomenon in the cytotoxic action mechanism of  $7\beta$ -OH-CH. These findings together with our previous report [15] strengthen the hypothesis that the esterification of  $7\beta$ -OH-CH on C-3-OH by Acyl-CoA: cholesterol acyltransferase activity (ACAT) in the transformed cells, is directly linked to the cytotoxic effect of  $7\beta$ -OH-CH. The fact that the esterification of  $7\beta$ -OH-CH occurs in the endoplasmic reticulum (ER) since ACAT is localised in this organelle, prompted us to study the effect of  $7\beta$ -OH-CH and/or of  $7\beta$ -OH-cholesteryl-3-esters on ER key functions, such as protein glycosylation. Our preliminary results (data not shown) indicate that treatment of transformed cells with  $7\beta$ -OH-CH reduced markedly the labelling of membrane proteins recognized by cerebellar soluble lectin (CSL), a lectin specific to mannose-enriched glycan residues [27]. In contrast, when cells were treated with 7-keto-CH or with  $7\beta$ -OH-CH and an inhibitor of ACAT, the decrease in membrane proteins labelled with CSL was not observed. Since the presence of dolichylphosphate (DP) in ER is a prerequisite for primary protein glycosylation, on the one hand, and DP, synthesized downstream of HMGR,

possesses its own regulation in the brain [28,29] on the other, we may hypothesize that the mechanism of action of  $7\beta$ -OH-CH is linked to its esterification on C-3 which in turn alters the glycosylation process of some native peptides on ER. Experiments are now underway to examine this possibility.

**Acknowledgements:** This work was partially funded by Grant 6282 from l'Association pour la Recherche contre le Cancer. We are pleased to thank Dr J.P. Zanetta for testing CSL and for helpful discussion, Mrs L. Schellenbaum and Mr. R. Bersuder for expert technical assistance and Mrs V. Ulrich for typing this manuscript.

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