Vol. 120, No. 2, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 30, 1984 Pages 657-664

ANTAGONISTIC ACTION OF CHOLESTEROL TOWARDS THE TOXICITY OF HYDROXYSTEROLS ON CULTURED HEPATOMA CELLS

Hélène HIETTER, Elisabeth TRIFILIEFF, Lysiane RICHERT[x], Jean-Paul BECK[x], Bang LUU, and Guy OURISSON.

Centre de Neurochimie du CNRS
[x]Institut de Physiologie et de Chimie Biologique
Université Louis Pasteur 67084 STRASBOURG (France)

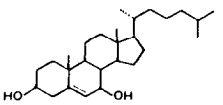
Received March 23, 1984

SUMMARY : The cytostatic and cytolytic action of 22R-hydroxydesmosterol on hepatoma cells cultured in a medium containing 10% newborn-calf serum can be reversed within certain concentration limits by adding cholesterol to the culture medium. In contrast, under the same conditions, the cytotoxicity of 7β-hydroxycholesterol could not be reversed, whatever the concentrations of cholesterol added. However, in a lipoprotein-poor and in a chemically defined medium, the cytolytic action of both hydroxysterols can be reversed by adding cholesterol, but growth inhibition cannot be suppressed. This demonstrates the importance of serum lipids and lipoproteins for the toxicity of the hydroxysterols and for the antagonistic effect of cholesterol. Our results suggest that the action mechanisms of 7β-hydroxycholesterol and 22R-hydroxydesmosterol on HTC hepatoma cells are not fully identical.

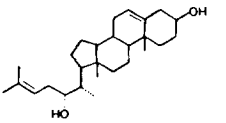
Several hydroxylated sterols, in particular 7β-OHC and 22R-OHD, are cytotoxic(1,2) towards HTC cells, a cell line derived from a rat Morris hepatoma(3). We have reported that these hydroxysterols interfere with the regulation of cholesterol biosynthesis, at least at two levels: the reduction of HMG-CoA(4) and the oxidative demethylation of lanosterol(5); nevertheless, no quantitative correlation could be established between the cytotoxicity of the hydroxysterols and the inhibition of these two steps of the biosynthetic pathway of cholesterol. It has been reported that the arrest of growth of human fibroblasts cultured in a sterol-free medium, which is attributed to the inhibition of HMG-CoA reductase by 20α-hydroxycholesterol, can be reversed by the addition of cholesterol to the culture medium(6). However, Yachnin et al.

Abbreviations used: 7β-OHC= 7β-hydroxycholesterol; 22R-OHD= 22R-hydroxydesmosterol; HMG-CoA= 3-hydroxy-3-methylglutaryl-coenzyme A; WM= whole medium; LPPM= lipoprotein poor medium; CDM= chemically defined medium; HMG-CoA reductase (EC 1.1.1.34); C = Cholesterol.

7β-hydroxycholesterol:



22R-hydroxydesmosterol:



0006-291X/84 $1.50

showed that the inhibition of cholesterol biosynthesis by several hydroxysterols could not be related directly to their biological effects on different cells (inhibition of human lymphocyte E-rosette formation(7), inhibition of human leukocyte chemotaxis(8), modification of red blood cells into echinocytes (9)). They assumed that the biological activity was related to the insertion of the hydroxysterols into the cell plasma membrane and showed that the serum lipoproteins modulate this insertion into human red globules and lymphocytes(10). Furthermore, we reported(11) that the cytotoxicity of 7β-OHC on hepatoma cells was about 10 times higher in a medium deprived of serum lipids and lipoproteins than in a medium containing whole serum. In the present paper, we describe the effect of the simultaneous addition of cholesterol and of the two cytotoxic hydroxysterols to HTC cells cultured in 3 different types of media: a medium containing 10% whole calf serum , a medium containing 10% lipid- and lipoprotein-deprived serum and a chemically defined medium, without calf serum.

MATERIAL AND METHODS

Chemistry: 7β-OHC was synthesised according to the procedure of Cheng(12), adapted to large scale synthesis by Rong(13), and 22R-OHD was synthesised according to Poyser et al.(14). Cholesterol, obtained from Carl Roth (Karlsruhe), was dissolved in ethanol (1g/100ml) at room temperature and precipitated by adding slowly the ethanolic solution in distilled water (300ml) under vigourous stirring. The suspension was then centrifuged at 3000 rpm for 10 mn in order to isolate the solid phase, which was lyophilised. The purity of samples was checked before each experiment by thin layer chromatography on silica gel (Precoated TLC Plates Silica Gel 60 F-254 Merck).
Cell culture conditions: HTC cells were grown in suspension in Swim's S77 medium (Gibco) containing either: (I) **10% newborn-calf serum**(Gibco batch n° 10Q0430; all experiments were run using the same batch of serum) and designated as "whole medium" (WM), or (II) **10% of the same newborn-calf serum deprived of lipids and lipoproteins** according to Watson et al.(15) and designated as "lipoprotein-poor medium" (LPPM). The determination of the lipid and cholesterol content of this serum was run by an automatised technique (CHOD -PAP Method from Boehringer-Mannheim). For this batch of serum, the concentration of total cholesterol was lowered from 2.07 mM in whole serum to 0.56 mM after delipidation, that of free cholesterol from 0.42 mM to 0.17 mM, of triglycerides from 0.44 mM to 0.04 mM and of phospholipids from 0.97 mM to 0.34 mM; or (III) **2% serum substitute Ultroser G** (IBF, Villeneuve-la-Garenne), designated as "chemically defined medium" (CDM) and containing only traces of lipids (0.16 mM triglycerides, 0.29 mM phospholipids and 0.17 mM total cholesterol). The hydroxysterol samples were dissolved in ethanol at concentrations ranging from 6.25 mg/ml to 50 mg/ml, and 100 µl of the ethanolic solutions were added to 75 ml cell suspensions under magnetic stirring. Control tests showed that ethanol at the concentrations used had no effect on culture development. For the experiments, cells were usually maintained in suspension in Swim's S77 medium containing 10% newborn-calf serum, and 5 h before the beginning of the experiment cells were centrifuged at 600 g for 5 mn and

resuspended either in WM, LPPM, or CDM. Cell concentration was adjusted to 10^5 cells/ml in each culture flask and the different sterol samples, dissolved in ethanol, were added to the cultures (cholesterol and 7β -OHC simultaneously or cholesterol and 22R-OHD simultaneously). Cell growth was evaluated every 24 h during 3 days from cell counts with a Neubauer microcytometer, and the number of living cells was determined by the Trypan blue exclusion test. Cell viability is given for different times as 100 x the ratio of living cells to the initial cells. Experiments were usually run 3 times, in duplicate.

RESULTS

1. EFFECT OF CHOLESTEROL ON HYDROXYSTEROL-TREATED CELLS, CULTURED IN WHOLE MEDIUM.

Figure 1a shows that 7β -OHC, at 80 μM, causes lysis of all cells within 2 days, at 60 μM within 3 days, and at 40 μM, it displays a delayed toxicity and cell viability remains 70% after 3 days. The toxicity of 7β -OHC is not significantly antagonised by cholesterol at equimolar or double concentration, at the 3 doses of hydroxysterol studied.

Figure 1b shows that 22R-OHD at high concentrations (80 μM, 60 μM) causes lysis of all cells within 24 h. At the lower concentration (40 μM) total lysis occurs within 3 days. At the higher concentrations of 22R-OHD no

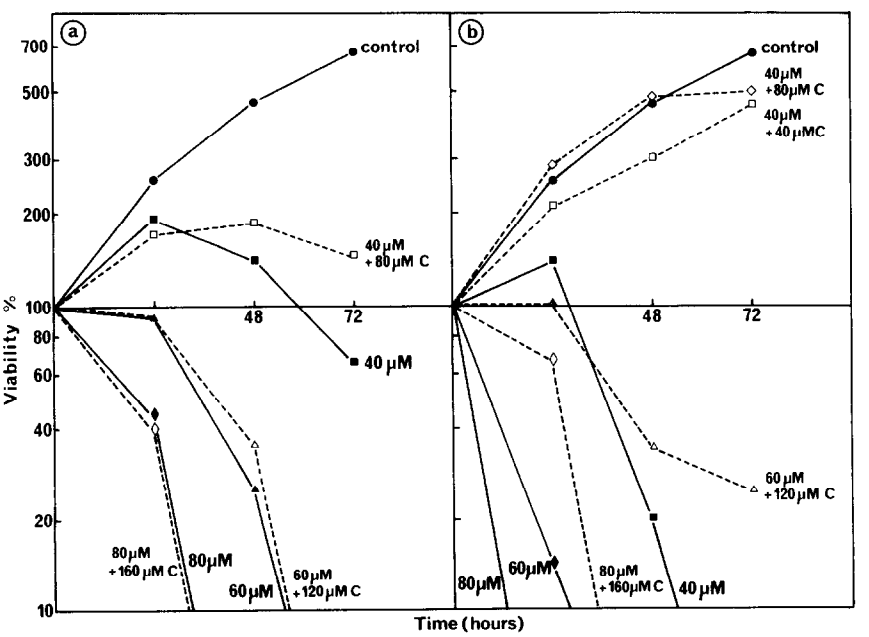


Figure 1 : Viability curves of HTC cells cultured in WM and treated with:
(a) various concentrations of 7β -OHC only (——) or with 7β -OHC and cholesterol added simultaneously (- - -)
(b) various concentrations of 22R-OHD only(——) or with 22R-OHD and cholesterol added simultaneously (- - -).

significant antagonism by cholesterol at equimolar or double concentration is observed, but at the lower concentration reversion of the cytolytic effect and recovery of normal cell growth are observed when equimolar concentrations of cholesterol are added; doubling the concentration of cholesterol slightly enhances the effect.

In conclusion, at the same molar concentration, 22R-OHD is more toxic than 7β-OHC and cholesterol antagonises only the cytotoxicity of 22R-OHD within certain limits of concentration.

2. EFFECT OF CHOLESTEROL ON HYDROXYSTEROL-TREATED CELLS, CULTURED IN LIPOPROTEIN-POOR MEDIUM.

Figure 2a shows that in LPPM medium, 7β-OHC at 20 μM is highly toxic and causes lysis of all cells within 24 h. At 10 μM of 7β-OHC, total cell lysis occurs after 3 days only. Reversion by cholesterol is observed only for low concentrations of 7β-OHC (10 μM). At least an equimolar dose of cholesterol

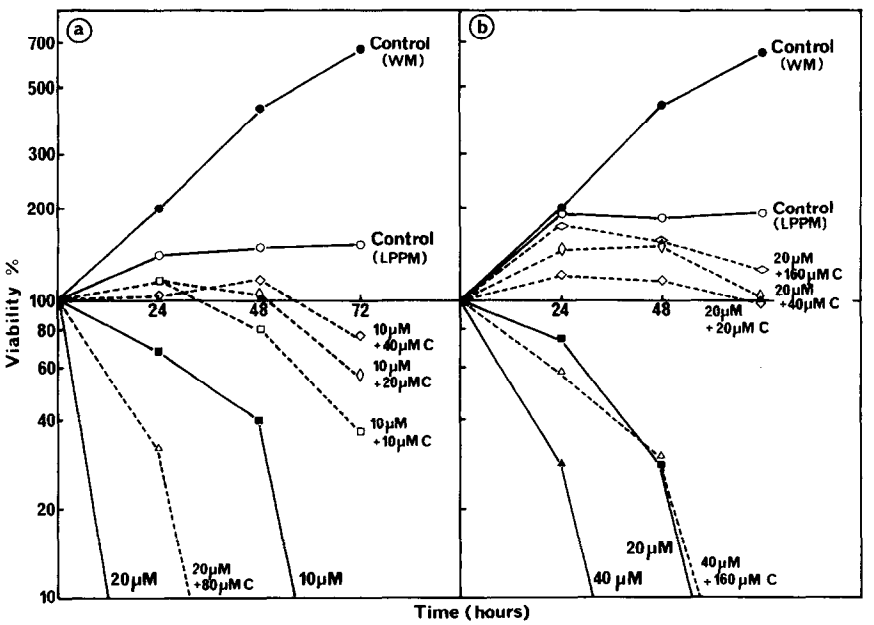


Figure 2 : Viability curves of HTC cells cultured in LPPM and treated with: (a) various concentrations of 7β-OHC only (——) or with 7β-OHC and cholesterol added simultaneously (- - -)
(b) various concentrations of 22R-OHD only(——) or with 22R-OHD and cholesterol added simultaneously (- - -).

is needed to antagonise partially the cytolytic effect and, at a double or fourfold concentration, cell viability remains about 70% after 72 h.

Cholesterol only impairs cell lysis, but does not restore normal cell growth.

In the presence of 22R-OHD, total cell lysis occurs within 48 h at 40 μM and within 72 h at 20 μM concentration. At high concentration of 22R-OHD, cholesterol has no effect when added simultaneously. At the lower concentration (20 μM) cytolysis is antagonised by cholesterol at equimolar concentration and cell viability remains about 100% within 3 days. When cholesterol is added at a double or even fourfold concentration, the antagonistic effect is not significantly enhanced.

We can conclude that, in spite of higher toxicity in LPPM, antagonism of the cytolytic effect of 7β-OHC and 22R-OHD by cholesterol is observed, within certain limits of concentration. It is also worth mentioning that, on cells cultured in LPPM, 7β-OHC is more toxic than 22R-OHD, while in WM, 22R-OHD is the most toxic compound.

3. EFFECT OF CHOLESTEROL ON HYDROXYSTEROL-TREATED CELLS, CULTURED IN CHEMICALLY DEFINED MEDIUM.

As shown on figure 2, after 24 h, in LPPM, there is no longer cell growth; it is likely that the medium shows some deficiency to allow optimal growth rate of HTC cells during 3 days. In consequence, and also because of the different results observed in WM and LPPM, we decided to culture HTC cells in CDM containing only traces of endogeneous cholesterol and we used Swim's S77 medium supplemented with 2% of serum substitute. In this medium, the growth rate of HTC cells is about the same as in WM, and, even within 3 days, no deficiency is observed. Furthermore, the variability due to the use of different serum batches and the possible denaturation of some constituents of the serum during delipidation can be avoided by using this CDM, the quality of which remains constant and allows optimal cell growth in the presence of only traces of lipids. Figure 3 shows that 7β-OHC and 22R-OHD have nearly the same toxicity in CDM as in LPPM. The antagonism obtained with cholesterol is also

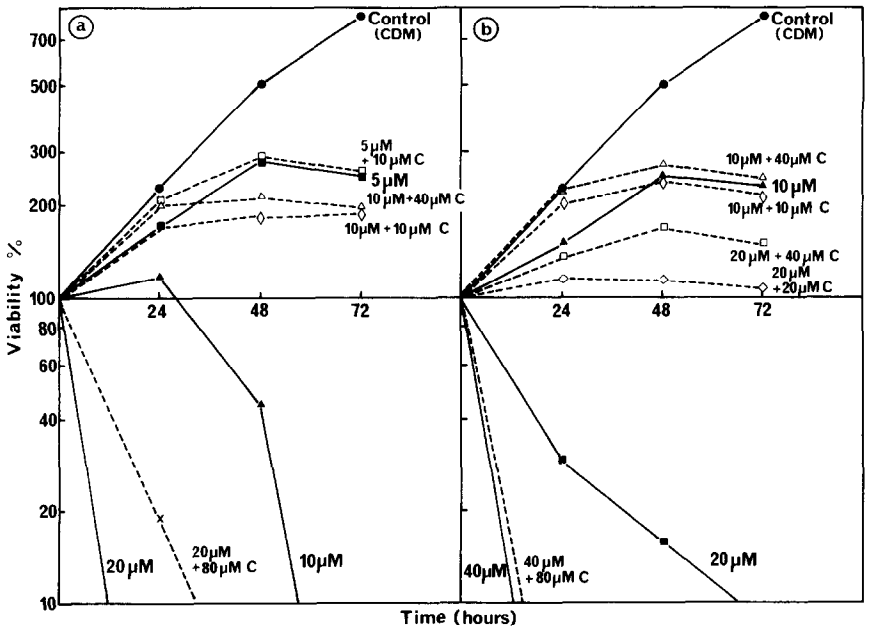


Figure 3 : Viability curves of HTC cells cultured in CDM and treated with:
(a) various concentrations of 7β-OHC only (——) or with 7β-OHC and cholesterol added simultaneously (- - -)
(b) various concentrations of 22R-OHD only(——) or with 22R-OHD and cholesterol added simultaneously (- - -).

comparable: within certain concentration limits (10 μM 7β-OHC and 20 μM 22R-OHD), cholesterol reverses cytolysis but does not restore normal cell growth.

In conclusion, cholesterol antagonises the toxicity of the two hydroxysterols present at fairly low doses, and the reversion of the cytotoxicity depends on the composition of the medium and more particularly on the presence or absence of lipids and lipoproteins.

DISCUSSION AND CONCLUSIONS

The major findings of this study are the following:

(I) In contrast to Yachnin et al.(10), who reported that lipoproteins but not free cholesterol could prevent the effects already cited in introduction, we show that free cholesterol antagonises the cytotoxic action of the two hydroxysterols studied.

(II) When, at high concentrations, 7β-OHC or 22R-OHD leads to very rapid cell lysis, cholesterol cannot antagonise this effect. This can be related to

an observation made by Bergmann (16): the growth of HTC cells cultured in WM and treated with 80 μM 7β -OHC can be restored if 7β -OHC is removed from the medium within 24 h by washing the cells; after this period of time, the cytotoxic effect can no longer be reversed.

(III) In several cases, one can dissociate the cytolytic effect of 7β -OHC and 22R-OHD from their inhibitory effect upon cell growth. In LPPM and CDM, only cell lysis can be inhibited by cholesterol but normal cell growth cannot be restored. If the hydroxysterols are added at cytostatic concentration, cholesterol has no effect, whereas in WM cytolytic action of 22R-OHD is antagonised by cholesterol and also inhibition of growth is suppressed.

(IV) Lipids and lipoproteins in the culture medium are important not only for the toxic effect of hydroxysterols but also for the antagonistic action of cholesterol. The variability in the range of toxicity of the two hydroxysterols and in the antagonism by cholesterol depends from the nature of the medium (and particularly from the presence or absence of lipids and lipoproteins). This can be related to some observations reported by Mummery et al.(17) who pointed out differences of cytotoxicity for several molecules depending from the presence or absence of serum in the culture medium. Dembinski et al.(18) also showed that the oestradiol sensitivity of cancerous cells is modulated by the serum concentration in the medium. Furthermore, as already mentioned, Yachnin et al.(10) also emphasised the importance of serum lipoproteins in the modulation of the insertion of hydroxysterols in cell plasma membrane; the insertion was shown to be responsible for biological effects such as echinocyte formation in red blood cells, altered cation (K^+, Rb^+) transport, etc... From our results, we assume that the mechanism of action of 7β -OHC and 22R-OHD cannot be identical as cholesterol antagonises differently the effects of the two molecules on HTC cells and as the relative toxicities change with the nature of the culture medium. Thus, the use of a chemically defined medium is very appropriate because it allows optimal growth while being very reproducible and containing only traces of lipids, which obviously play an important role in the mechanism of action of these

hydroxysterols. As a continuation of our study in this area we shall fractionate the lipids and lipoproteins removed from the total serum by centrifugation in order to establish which of them in particular antagonises the lytic effect of 7β -OHC on HTC cells.

Acknowledgements: We are grateful to Dr J.Borg for the determination of lipid and cholesterol contents and to Mrs M.T.Bohn for her technical assistance. This research was supported by grants from the Institut National de la Santé et de la Recherche Médicale (N° 832024).

REFERENCES

1. Cheng, K.P., Nagano, H., Luu, B., Ourisson, G.,.Beck, J.P.(1977) J. Chem. Research, S, 217; M, 2501-2521.
2. Nagano, H., Poyser, J.P., Cheng, K.P., Luu, B., Ourisson, G.,Beck, J.P.(1977) J. Chem. Research, S, 218; M, 2522-2571.
3. Thompson, E.B., Tomkins, G.M., Curran, J.F.(1966) Proc. Nat. Acad. Sci. USA, 56, 296-303.
4. Zander, M., Koch, P., LUU, B., Ourisson, G., Beck, J.P.(1977) J. Chem. Research, S, 219; M, 2572-2584.
5. Ortiz de Montellano, P.R., Beck, J.P., Ourisson, G.(1979) Biochem. Biophys. Res. Comm., 90, 897-903.
6. Chen, H.W., Kandutsch, A.A , Waymouth, C.(1974) Nature, 251, 419-421.
7. Streuli, R.A., Chung, J., Scanu, A.M., Yachnin, S.(1979) J. Immunol., 123, 2897-2902.
8. Gordon, L.I., Bass, J., Yachnin, S.(1980) Proc. Natl. Acad. Sci.USA, 77, 4313-4316.
9. Hsu, R.C., Kanofsky, J.R., Yachnin, S.(1980) Blood, 56, 109-117.
10. Yachnin, S., Chung, J., Scanu, A.M.(1982) Biochim. Biophys. Acta, 713, 538-546
11. Richert, L., Bergmann, C., Beck, J.P., Rong, S., Luu, B., Ourisson, G. (1983) Biochem. Biophys. Res. Comm., 117, 851-858.
12. Cheng, K.P.(1978) Doctoral Dissertation, University L. Pasteur, Strasbourg, France, p.11.
13. Rong, S.(1983) Doctoral Dissertation, University L. Pasteur, Strasbourg, France, p.30.
14. Poyser, J.P., Ourisson, G.(1974) J. Chem. Soc, Perkin I, 2061-2066.
15. Kisten, E.S., Watson, J.A.(1974) Biol. Chem., 249, 6104-6109.
16. Bergmann, C.(1982) Doctoral Dissertation, University L. Pasteur, Strasbourg, France, p.41.
17. Mummery, C.L., van den Bruick, S., van der Saag, P.T., de Laat, S.W.(1983) Toxicol. Lett., 18, 201-209.
18. Dembinsky, T.C., Green, C.D.(1983) Hoppe-Seyler's Z. Physiol. Chem., 364, 475-476.