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# Oxysterols and Apoptosis: Evidence for Gene Regulation Outside the Cholesterol Pathway

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## CONTENTS



## I. INTRODUCTION

Oxysterols are derivatives of cholesterol that contain additional oxygen atoms either on the steroid nucleus or the side chains. They were discovered by Kandutsch and Chen to be potent regulators of cholesterol synthesis and also to be inhibitory to growth of many cells.[1,2] Whether there is a singular direct linkage between their inhibition of cholesterol synthesis and their inhibition of cell growth remains to be seen. In considering how oxysterols regulate cholesterol synthesis it is noteworthy that they can do so without making use of the LDL:LDL receptor pathway.[3,4] Instead, oxysterols appear to be able to enter cells directly; they are also natural metabolic products. They have been shown to downregulate transcription of the LDL receptor, cholesterol synthase, hydroxymethylglutaryl coenzyme A (HMG CoA) reductase, and other key enzymes in the pathway.[5–7] In addition to their regulation of transcription, oxysterols have been shown to increase the rate of degradation of HMG CoA reductase or to control the level of HMG CoA reductase at a post-transcriptional level.[8–12] Thus, they may have more than one mechanism of action.

Sterol response elements have been identified in the promoters or regulatory regions of the genes controlled by oxysterols, and although a consensus sequence has been proposed which indeed can confer regulation upon a reporter gene and cotransfection experiments, careful mutational analysis of these sterol response elements (SREs) in several of the genes shows that they are not used identically.[13–15] Recently, progress has been made on identifying positive transcription factors which operate on these SREs, and the data suggest that the positive transcription factor precursor may be cytoplasmic. Therefore, sterols may be involved in preventing the proteolytic processing necessary to release the active transcription factor into the nucleus to transcriptionally activate the appropriate genes.[16]

Search for a cellular receptor specific for oxysterols thus far has led to the identification of only one protein, the oxysterol binding protein (OBP). Careful studies have shown that there is a close correlation between the potency of a wide variety of oxysterols in downregulating HMG CoA reductase and their affinity for OBP.[5] OBP has been cloned and sequenced, as well as characterized biochemically.[17–24] In biochemical studies it has been shown to exist as an oligomer with several other as yet unidentified proteins.[18] Its predicted amino acid sequence, derived from its cDNA coding sequence, shows that it lacks the characteristics of the receptors known to bind steroid hormones, and indeed competitive binding studies show no significant interaction between steroid hormones and OBP.[20,21] For that matter, cholesterol itself also shows little or no interaction for OBP. No well-known nuclear localization signals have been identified or predicted from the primary sequence of OBP, and in one paper studying its overexpression in CHO cells it appeared to be a cytoplasmic protein.[25] However, it is capable of binding DNA in a nonspecific fashion.

How do oxysterols inhibit cell growth, and what are the consequences of that inhibition? From original work by Chen et al. it was suggested that there was a close linkage between the regulation of cholesterol

synthesis and the regulation of DNA synthesis in lymphoid cells.[26] However, subsequent studies have challenged this point. Recent papers have suggested that lymphoid cell death brought about by oxysterols is of the type known as apoptosis.[27,28] Apoptosis is a morphologically distinct type of cell death, which is believed to be programmed and under the control of natural cell genes, as opposed to cell death induced in nonspecific ways by toxic substances. Cells undergoing apoptosis show distinctive morphologic changes, including shrinkage, membrane budding, condensation of cytoplasm and nuclei, a particular type of heterochromatization, and formation of apoptotic bodies, particles of condensed cellular materials that are often phagocytosed as such by surrounding cells.[29] Apoptosis can be initiated by internal messages or by extracellular signals. Glucocorticoids are well known to cause apoptosis, and we have studied this phenomenon extensively in the CEM line of human acute lymphoblastic leukemia cells and clones derived therefrom. We also have employed these cells to compare the apoptotic events induced by oxysterols as opposed to glucocorticoids. These experiments addressed several questions: Is there overlap between the apoptotic pathways activated by the two types of ligands? Is there correlation between apoptosis and binding to OBP? Is cessation of cholesterol synthesis the sole explanation for the apoptotic pathway activation brought about by oxysterols?

## II. CORRELATION BETWEEN OXYSTEROL BINDING TO OBP AND ACTIVATION OF APOPTOSIS

Using several clones of CEM cells grown in serum-free medium to avoid the possibility of involvement of LDL, we studied the dose response of cell kill to 25-hydroxycholesterol, in comparison with the affinity of the oxysterol for the oxysterol binding protein.[30] We found that the oxysterol causes inhibition of cell growth only after it has been present for 24 h or so, blocking the cell cycle in $G_0/G_1$. Actual cell death, apoptotic in appearance, followed cumulatively thereafter. In this timing sequence, death due to glucocorticoids and death due to oxysterols resemble each other. Selecting a four day interval as the time point by which most cell death had occurred, we found that there was correlation between lethal concentrations of the oxysterol and those which occupied OBP. An example of these data are presented in Figure 1. Less extensive studies with two other oxysterols, 20α-hydroxycholesterol and 7-ketocholesterol, were consistent with the same conclusion: occupancy of OBP correlates with apoptotic cell death. This conclusion was not only true for a particular clone of cells. The $K_d$ values for 25-hydroxycholesterol binding to OBP for several CEM clones correlated with the lethal dose of the oxysterol that kills 50% of those cells. Although these data are not yet complete for a large array of oxysterols, they support the view that OBP may be involved in the apoptotic process.

To test for cross resistance between glucocorticoids and oxysterols in their evocation of apoptosis, we employed clones of cells selected for resistance to high doses of either dexamethasone or 25-hydroxycholesterol. When these cells were tested with the alternate steroids no cross resistance was seen.[30] Figure 1, bottom panel, shows for example, that two glucocorticoid-resistant CEM clones are as sensitive to 25-hydroxycholesterol as is the glucocorticoid-sensitive clone. We also have selected for clones highly resistant to 25-hydroxycholesterol; these were completely sensitive to apoptosis following exposure to the glucocorticoid dexamethasone (unpublished results).

Thus, in this limited set of clones, no coincidence between the apoptotic pathways evoked has been discovered. This of course is not proof that there is no point at which the pathways evoked by the two types of steroid may converge. Some of the glucocorticoid-resistant mutants are known to be so due to altered glucocorticoid receptors, and it is unlikely that these proteins are involved in oxysterol action. The mechanism of resistance to either type of steroid of the other clones is unknown. More extensive surveys will be required to see whether doubly resistant cells can be found, but our recent discovery of similar biochemical events evoked by both types of steroid suggests that it should be possible to isolate such mutants (see below).

## III. INHIBITION OF CHOLESTEROL SYNTHESIS ALONE MAY NOT ACCOUNT FOR APOPTOSIS EVOKED BY OXYSTEROLS

When cells are inhibited by 25-hydroxycholesterol, a considerable interval occurs before they begin to die. During this 24 to 48 h "window", they appear normal, gradually arresting in the $G_1$ phase of the cell cycle. Cholesterol synthesis, however, is reduced quite rapidly, as HMG CoA reductase is dramatically reduced within the first few hours following administration of the oxysterol (Figure 2). We studied the ability of added cholesterol, the end product of the pathway, to restore viability or to prevent loss

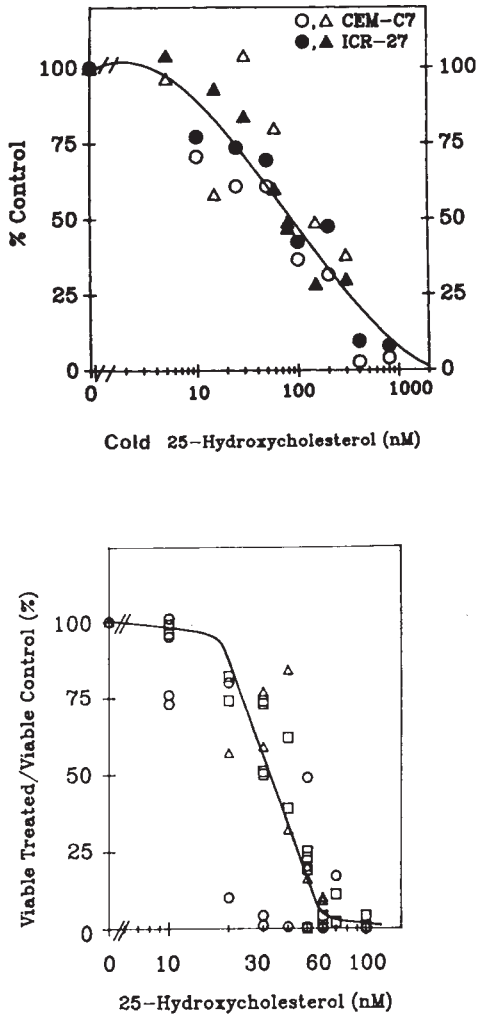


**Figure 1** Concentrations of 25-hydroxycholesterol that bind to OBP also are similar to those that cause apoptosis in CEM cells. (Top panel) Binding of $^3$H-25-hydroxycholesterol to OBP. Cells were harvested in late log growth, washed with serum-free medium, and resuspended in 1 ml aliquots of the medium with tritiated 25-hydroxycholesterol along with various concentrations of unlabeled oxysterol. After incubation a cytosolic extract of the cells was fractionated by velocity sedimentation on sucrose density gradients and the radioactivity in the ~7.5 S OBP peak quantitated. The results of four experiments on two clones are displayed. Open symbols represent OBP binding sites in CEM C7, glucocorticoid-sensitive cells; closed symbols, data from the glucocorticoid-resistant clone ICR-27 cells. (Bottom panel) Both glucocorticoid-sensitive and -resistant CEM clones are killed by 25-hydroxycholesterol with similar $LD_{50}$. Cells were plated at an initial concentration of 1–1.5 × $10^5$ viable cells/ml and exposed to various concentrations of 25-hydroxycholesterol. Control cells (ethanol vehicle only) grew logarithmically. After 4 days, viable cells were counted. Glucocorticoid-sensitive CEM C7 cells, squares; glucocorticoid-resistant CEM-4R4 cells, circles; and ICR-27 cells, triangles. To normalize for slight differences in cell numbers in control cells in several experiments, the data are shown as the percent of oxysterol-treated viable cells/ml per untreated viable cells. Error bars were omitted for clarity of data; for most points the standard error was <10%. The curve is a hypothetical best fit of the data. (From Bakos, J. T. et al., *J. Steroid Biochem. Mol. Biol.*, 46, 418, 1993. With permission.)

of viability in the treated cells. At a concentration of oxysterol which causes 75% cell death we found that only a vast excess of added cholesterol could prevent kill or "rescue" cells from apoptosis. At 200 n*M* 25-hydroxycholesterol, an 85-fold excess of cholesterol does not block cell death (Figure 3). It seems likely that some lethal process other than cholesterol synthesis is initiated by the oxysterol. The

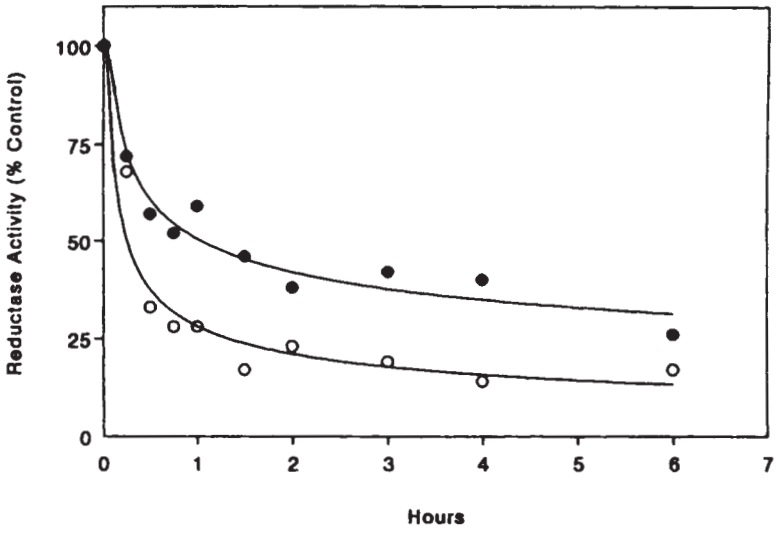


**Figure 2** Time course of the effect of 25-hydroxycholesterol on HMG CoA reductase activity in CEM C7 cells. Cells were grown in RPMI 1640 medium supplemented with 10% delipidated fetal calf serum plus 1 µg/ml (closed circles) or 2 µg/ml (open circles) of 25-hydroxycholesterol, added in ethanol and triturated with 5% bovine serum albumin (BSA). All flasks were incubated at 37°C, and cells from pairs of control and oxysterol-treated flasks were collected and assayed for HMG CoA reductase activity. The results were expressed as percentage of reductase specific activity in the cells from each treated flask compared to the time-matched control. The results are from two separate experiments.

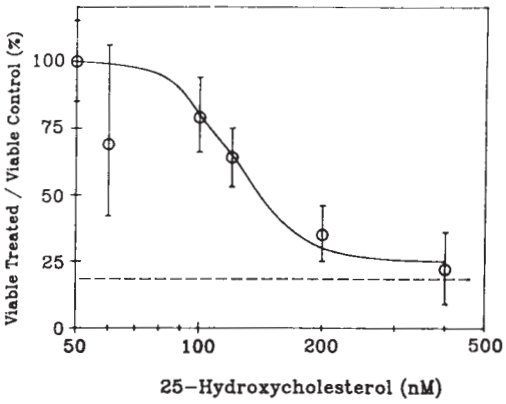


**Figure 3** Treatment of CEM C7 cells with increasing amounts of 25-hydroxycholesterol in constant cholesterol. Twelve hours after plating CEM C7 cells, cholesterol in 0.5% BSA was added to the wells to a final concentration of 17 µ*M*, followed at once by 25-hydroxycholesterol to the final concentrations indicated on the abscissa. Final counts and data analysis were as in Figure 1. The dashed line indicates percentage of viable cells treated with 60 n*M* 25-hydroxycholesterol without added cholesterol; n = 3. (From Bakos, J. T. et al., *J. Steroid Biochem. Mol. Biol.*, 46, 422, 1993. With permission.)

concentrations of cholesterol involved are those at which many known direct effects of cholesterol on membrane stability and membrane-associated enzymes have been observed.[31]

It is well known that the side pathways off the cholesterol synthesis path produce many products that may affect cell viability.[32–35] These include geranyl and farnesyl groups, dolichol and dolichol phosphate, ubiquinone, and isopentenyl adenine. The likelihood that cholesterol is supplying these factors, however, is remote, since the steps in the synthetic pathway are essentially irreversible. However, we also observed that high doses of mevalonate, which is the immediate product of HMG CoA reductase, could also prevent kill by oxysterols. Therefore, it is possible that in some fashion one or more of these by-products are involved in the apoptotic process.

## IV. GENES NOT INVOLVED IN THE CHOLESTEROL SYNTHESIS PATHWAY ARE REGULATED BY OXYSTEROLS AND MAY BE INVOLVED IN THEIR LETHAL EFFECT

Recently a few genes not involved in cholesterol metabolism have been shown to be regulated by oxysterols. One of these is cellular nucleic acid binding protein (CNBP). CNBP was originally cloned because it bound to single-stranded DNA containing SREs.[36] However, subsequent analysis did not show its correlation with specific gene regulation by oxysterols. The paper describing its original discovery presented suggestive evidence that CNBP was induced in HEPG2 cells by 25-hydroxycholesterol.[36] We therefore examined the regulation of CNBP in our clones of oxysterol-sensitive and -resistant CEM cells. Our data show that regulation of this gene correlates with oxysterol sensitivity in this cell system.[37] In CEM cells, however, unlike HEPG2 cells, oxysterols are potent *negative* regulators of CNBP. Figure 4 documents the reduction of CNBP by 25-hydroxycholesterol in sensitive CEM C7 cells, and the lack of reduction in oxysterol-resistant M10 cells. By 24 h after the addition of the sterol the levels of CNBP mRNA are reduced by 50%. The delay of at least 7 h in CNBP reduction suggests that it is a secondarily controlled event that must await some earlier oxysterol-regulated step. Response studies showed that this CNBP effect also correlates with concentrations of sterol known to occupy OBP, since the $K_d$ for CEM C7 cells for 25-hydroxycholesterol is 23.4 n*M* and CNBP reduction occurs at a similar oxysterol concentration (Figure 5). The oxysterol-resistant M10 cells required 17-fold greater concentrations of 25-hydroxycholesterol than wild-type CEM cells to undergo apoptosis.[30,37] When sufficient additional 25-hydroxycholesterol was given to the resistant cells and sufficient time allowed to pass, the cells did show cell death and, also, reduction in CNBP mRNA levels. However, no inhibition was seen at concentrations to which the cells were resistant and which were fully capable of killing wild-type cells (Figure 5). Both cell types contain the same quantities of OBP. Thus, we have identified regulation of

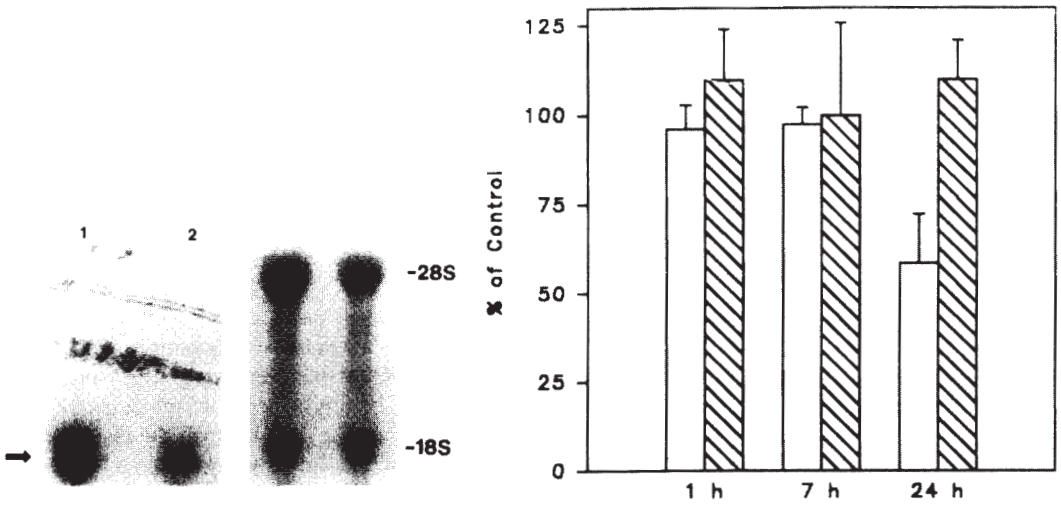


**Figure 4** Differential regulation of CNBP mRNA in oxysterol-sensitive CEM C7 cells, and oxysterol-resistant M10 cells. Northern blot analysis of CNBP mRNA levels after treatment with 25-hydroxycholesterol. (Left panel) Cells were cultured in RPMI 1640 medium with 5% delipidated serum and treated with either vehicle only (left side, lane 1) or 1 µ*M* 25-hydroxycholesterol (lane 2). Twenty-four hours later, total RNA was extracted and probed with a labeled CNBP cDNA probe. Arrow, CNBP signal. The left panel also shows (right side) ethidium bromide stained ribosomal RNA that had transferred to the same filter, indicating that nearly equal amounts of RNA were loaded and transferred in each lane. (Right panel) CEM C7 cells (open bars) and M10 cells (hatched bars) were exposed to either vehicle only (control) or to 300 n*M* 25-hydroxycholesterol, harvested for RNA extraction 1, 7, and 24 h later, and analyzed for CNBP mRNA. At each time point, the signal from the oxysterol-treated cells was compared to that from its control; the level of CNBP expression in the control cells did not vary significantly with time. The signals were normalized by reprobing the filters with ß-actin cDNA. Error bars indicate mean ± standard deviation of two to six determinations. For CEM C7 cells at 24 h $p \leq 0.05$ when values for the control and treated cells were compared by a paired Student's *t*-test. Other differences were not statistically significant. (From Ayala-Torres, S. et al., *J. Steroid Biochem. Mol. Biol.*, 48, 312–313, 1994. With permission.)

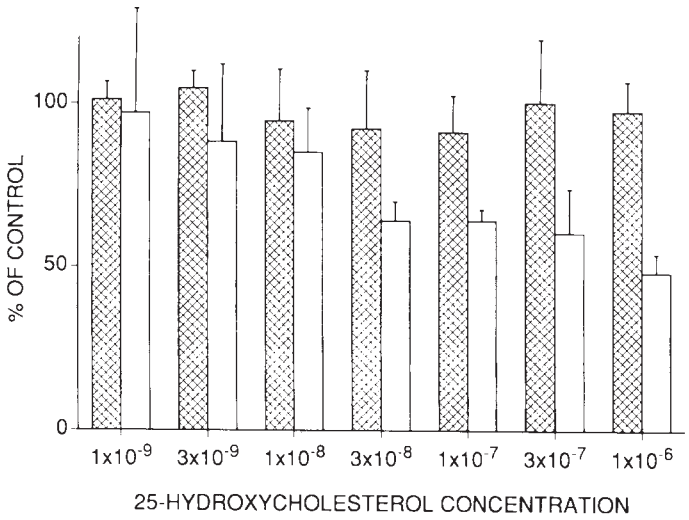


**Figure 5** Effect of various concentrations of 25-hydroxycholesterol on the level of CNBP mRNA in CEM C7 and M10 cells. Cells were cultured as in Figure 4 and exposed to either vehicle only (control) or to different concentrations of the oxysterol. After 24 h treatment, total RNA was extracted and analyzed by dot blotting. Hatched bars represent oxysterol-resistant M10 cells and open bars oxysterol-sensitive CEM C7 cells. Error bars indicate the mean ± standard deviation for at least three determinations. Values for the control and treated cells were significantly different at the 0.05 level for concentrations $\geq 3 \times 10^{-8}$ *M* 25-hydroxycholesterol when compared by a Student's *t*-test. (From Ayala-Torres, S. et al., *J. Steroid Biochem. Mol. Biol.*, 48, 313, 1994. With permission.)

a gene which is not part of the cholesterol synthesis pathway whose regulation correlates with sensitivity to oxysterol.

The function of CNBP is unknown. Its primary sequence predicts a zinc finger protein and therefore a possible regulatory factor.[35] Homologs to CNBP have been found in yeast and amphibians. In *Schizosaccharomyces pombe* it is involved in the *ras*1 signalling pathway that is employed during sporulation and conjugation.[38] An additional homolog was found in a *Xenopus* gene which is active in early embryo development.[39] Thus, the function of CNBP and its possible importance in regulation of cell viability remains intriguing.

We have recently noted regulation by oxysterols of an additional gene, c-*myc*, well known to be involved in maintenance of cell cycle and cell viability. Studies by a number of laboratories have shown that in lymphoid cells, negative c-*myc* regulation by glucocorticoids is a very early effect preceding accumulation of the cells in $G_1$ and subsequent apoptosis.[40-43] The c-*myc* gene of course is also well known as an early response gene and one which is essential for progression through the cell cycle. In a variety of cancers c-*myc* is translocated and overexpressed. In CEM cells we have shown that downregulation of c-*myc* by glucocorticoids correlates very tightly with cell death, and we have further implicated it as a critical factor by showing that if it is downregulated by antisense oligonucleotides, the cells die as if they had been treated with glucocorticoids. Conversely, if overexpression vectors are used to maintain c-*myc*, steroid-sensitive cells are rendered resistant.[42] The glucocorticoid repression of c-*myc* in CEM cells has been confirmed recently.[43] This work also shows that protein synthesis is required in addition to the c-*myc* effect.[43] Therefore, as we suggested,[42] it seems that c-*myc* probably controls a sequence of other genes which are essential for cell viability. Because of these findings with glucocorticoids, we investigated whether c-*myc* is also regulated by oxysterols in CEM cells. The data show (Figure 6) that oxysterols do in fact lower c-*myc* levels. By 24 h after addition of oxysterol, c-*myc* levels have been significantly reduced. Again, this downregulation does not occur in oxysterol-resistant clones. *Myc* is not known to function in cholesterol synthesis and, therefore, it represents a particularly intriguing gene for further study in the context of oxysterol regulation of apoptosis.

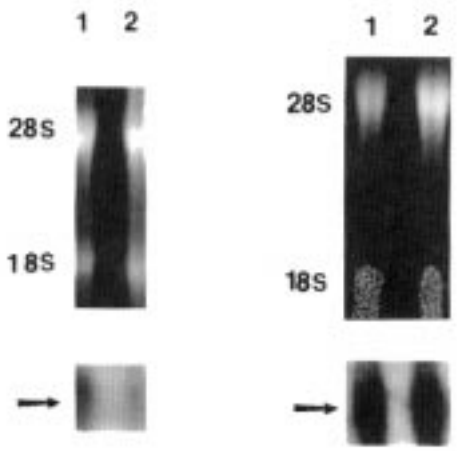


**Figure 6** Oxysterol reduces c-*myc* mRNA in CEM C7 but not in M10 cells. Cells were cultured and treated with 25-hydroxycholesterol as in Figure 5. Northern blot analysis of total RNA from 24 h cultures of control and treated cells is shown. Left panels, CEM C7 cells. Right panels, M10 cells. Lanes 1, control; lanes 2, oxysterol-treated. Arrows show the c-*myc* signal. Upper panels show the rRNA on the same filters, to indicate equivalent loading.

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