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Glucocorticoids, oxysterols, and cAMP with glucocorticoids each cause apoptosis of CEM cells and suppress c-*myc* ☆

E. Brad Thompson*, Rheem D. Medh, Feng Zhou, Sylvette Ayala-Torres, Naseem Ansari, Weiping Zhang, Betty H. Johnson

The Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX, 77555-0645, USA

Abstract

In clones of the CEM human acute lyumphoblastic leukemic cell line, glucocorticoids, oxysterols and activators of the cAMP pathway acting synergistically with glucocorticoids, each can cause apoptotic cell death. Morphologically and kinetically, these deaths resemble one another. The kinetics are striking: in each case, after addition of the lethal compound(s), an interval of approximately 24 h follows, during which cell growth continues unabated. During this "prodromal" period, removal of the apoptotic agent leaves the cells fully viable. We hypothesize that a sequence of biochemical events occurs during the prodrome which eventually results in the triggering of the full apoptotic response as evidenced by the activation of caspases and DNA fragmentation. At some point, the process is irreversible and proceeds relatively rapidly to cell death. Suppression of c-Myc seems a universal early event evoked by each of these lethal compounds or combinations, and we conclude that the negative regulation of this proto-oncogene is an important aspect of the critical pre-apoptotic events in these cells.

1. Introduction

CEM cells are a well-studied line of human acute lymphoblastic leukemic cells. Surface markers indicate that they are primitive T-lymphoblasts, and like other members of this class of lymphoid cells, they are sensitive to glucocorticoid-induced apoptosis. By cloning the original CEM cell line, we have obtained a number of valuable clones, which allow comparison and dissection of the effects of glucocorticoids and other agents. Recently we have shown that in addition to glucocorticoids, the class of steroids known as oxysterols can also cause apoptotic death of CEM cells. This finding has prompted a comparison of the pathways leading to death after the administration of each class of steroid. Furthermore, we have discovered that a clone of CEM cells that is resistant to glucocorticoids, although it possesses glucocorticoid receptors, can be killed apoptotically by a combination of forskolin, the activator of adenylate cyclase and dexamethasone, a synthetic glucocorticoid. In this paper we review our studies and present new data on apoptosis induced by these agents, with particular attention given to the timing of events in the cell death pathway. The effects of the agents on three major components in the apoptotic sequence, namely caspase activiation, DNA endonucleolysis, and suppression of the c-Myc protein, are addressed.

2. Materials and methods

The methods for determining cell viability, FACS analysis, TUNEL assay, Northern blotting, analyzing DNA lysis by standard and pulse field electrophoresis, all have been published [1–3]. Nuclear run-on experiments were done by the method described by Mahajan et al. [4]. Caspase activity was assayed by a

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* Corresponding author Tel.: 409-772-2271; Fax: 409-772-5159.
E-mail address: bthompso@utmb.edu (E.B. Thompson)

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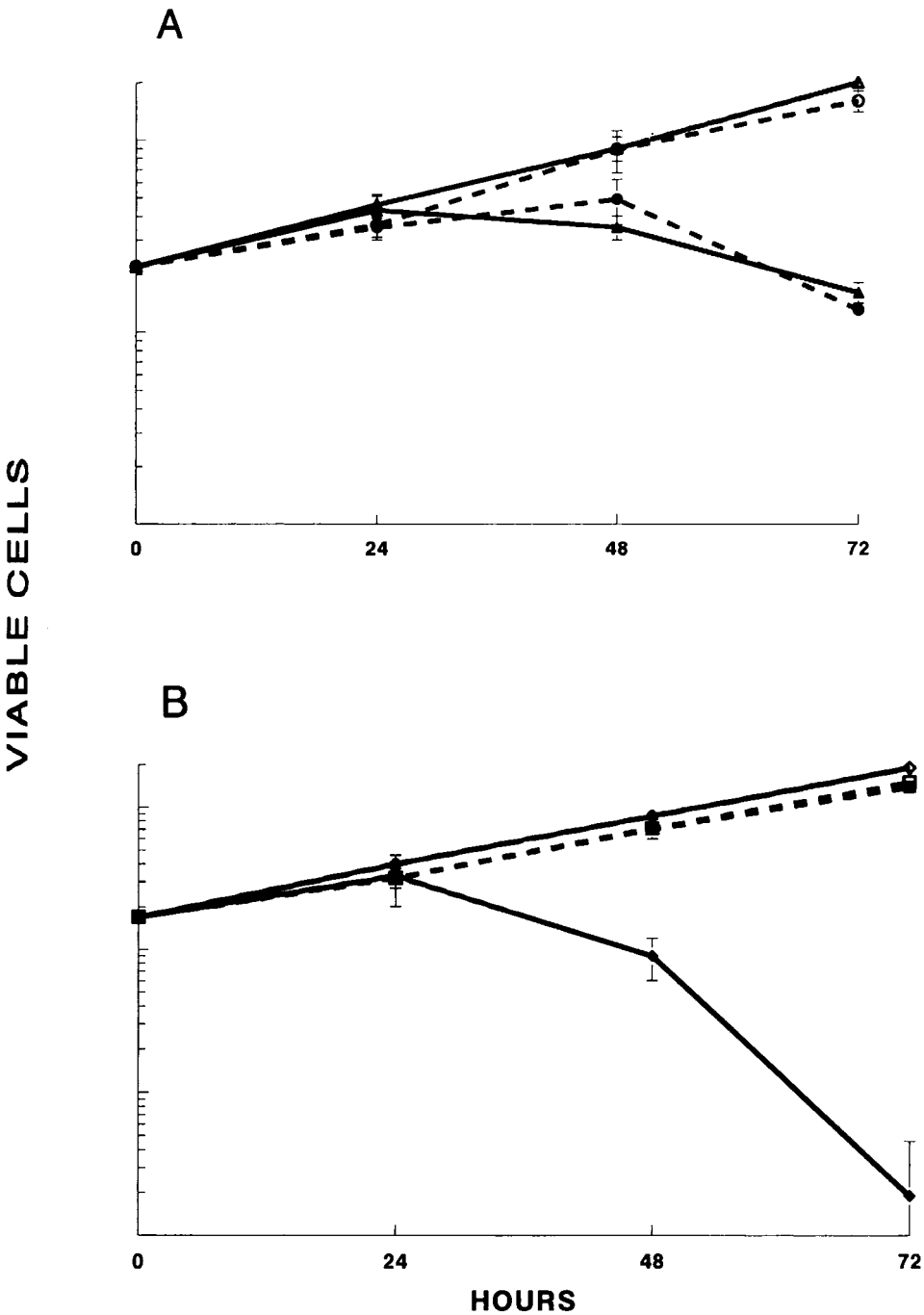

Fig. 1. Effects of glucocorticoid and oxysterol on the growth and viability of CEM-C7 and oxysterol-resistant subclone, M10R5 cells. (a) Glucocorticoid treatment: To cells in logarithmic growth 1 μM dexamethasone or < 1% ethanol vehicle was added and viable cells determined at the times shown. Triangles with unbroken lines = CEM-C7 cells, circles with broken lines = M10R5 cells. (b) Oxysterol treatment: To cells in logarithmic growth in medium with delipidated serum 1 μM 25-hydroxycholesterol was added and viable cells determined at the times shown. Diamonds with unbroken lines = CEM-C7 cells, squares with broken lines = M10R5 cells. (a and b) Open symbols = controls, closed symbols = steroid treated. Reprinted with permission from Ref. 1.

modification of the protocol described in Sarin et al. in which the caspase inhibitors were added during the cell culture incubations [5]. Messenger RNA half-life was determined by RNase protection assays performed according to the protocol from Ambion [Austin, TX].

3. Results

3.1. Steroids and apoptotic death of CEM cells

Cells of clone CEM-C7 and its subclone C7–14 are both glucocorticoid and oxysterol sensitive. These cells

contain approximately 11,000 glucocorticoid receptor binding sites per cell and are haploid for the active glucocorticoid receptor gene. The cells also contain high affinity binding sites specific for oxysterols, in the form of the oxysterol binding protein. Assays for binding of this class of steroid are best done in lysed cell extracts; therefore, sites per cell cannot be accurately estimated. However, in terms of binding affinity, an approximate K_d can be obtained. In tissue culture, as in vivo, K_d is dependent on the concentration of serum in the medium. It has been shown that most oxysterol is bound to serum albumin, reducing the free pool by as much as 75% of the total in serum [6]. In serum-free cultures of CEM-C7 cells, the approximate K_d for 25-hydroxycholesterol binding is 31 nM, and when partially purified oxysterol binding protein is used, a K_d of 7 nM is found [7,8].

Glucocorticoids are a classic cause of apoptosis in several kinds of lymphoid cells, and CEM cells display typical morphology after addition of quantities of glucocorticoids sufficient to bind to their receptors. We recently have demonstrated that these cells show virtually identical morphologic changes after treatment with 25-hydroxycholesterol in concentrations consistent with those binding to the oxysterol binding protein [2]. These changes include reduction in cell volume, loss of microvilli, and heterochromatization of DNA beginning with a centrifugal pattern in the nucleus followed by complete heterochromatization of the nuclear contents. Following these events in tissue culture, the cells eventually lyse into various apoptotic bodies. The concentrations of glucocorticoid that kill all the cells in a culture are consistent with those that fully occupy the glucocorticoid receptor in the cells. Complete cell death is seen at $\geqslant 10^{-7}$ M dexamethasone. In oxysterol under serum-free conditions approximately 60 nM 25-hydroxycholesterol is sufficient to kill the cells [7]. In 5% delipidated serum approximately 300 nM is required, while in whole serum 1 μM 25-hydroxycholesterol must be used. These results are consistent with the sequestration of oxysterol by albumin plus the partial protective effect of the cholesterol in whole serum [7].

3.2. Kinetics of cell death

After either steroid is given in sufficient quantity to cause apoptotic death, a striking time course is observed. When cell viability is followed after the addition of the steroid to cells in logarithmic growth phase, one sees no diminution of cell number for approximately 24 h (Fig. 1). After that, increasing numbers of cells demonstrate loss of cell viability, apoptotic morphology, and other dramatic changes as discussed below. Thus, the events that transpire after addition of a lethal concentration of either type of steroid can be considered in two time periods. The first is subsumed by the initial ~24 h period. During this time, removal of the steroid or addition of anti-steroid agents to block its effect allows the cells to continue growing logarithmically as if they had received no treatment. Beyond 24 h or so, removal of the steroid does not permit recovery of viability in those cells committed to die; so that by 36–48 h, removal of the steroid or the addition of an anti-steroid does not entirely prevent cell death of the culture. As time in the presence of steroids lengthens, fewer and fewer cells can be rescued, and several logs of kill are observed. We hypothesize that a sequence of biochemical events occurs in this initial time period that eventually results in irreversible changes which then rather abruptly cause apoptosis. It should be mentioned that this general kind of time course, with a prolonged interval preceding apoptosis after the addition of drug, is observed in most glucocorticoid sensitive transformed cell lines of mouse or human origin. For example, it has been seen in the mouse lines P1798, S49 and WEHI-7 [9–11]. Such a time course has been seen in Jurkat cells (when they are transfected with an expression vector carrying an active glucocorticoid receptor) [12]. These kinetics differ from those observed upon addition of steroid to freshly isolated rodent thymocytes. Those cells die much more quickly, within 24 h of addition of steroid. Peripheral blood lymphocytes on the other hand, are rather steroid insensitive, unless first stimulated to grow. They then show increased steroid sensitivity and a more extended time course of response analogous to the kinetics of the transformed cell lines. The details of the kinetics of cell death after addition of oxysterols to lymphocytes and thymocytes have not been so extensively studied.

In the CEM system we are testing our hypothesis by examining events that occur during the first 24 h and correlating these with later events and eventual cell death. Obviously, some early events will represent alterations in gene activity or other cell functions which are not relevant to the ultimate apoptosis. Other events will prove to be relevant and though reversible, may represent a sequence or cascade which eventually tips the scales of the molecular balance of power in the cells such that the apoptotic catastrophe is initiated.

3.3. Activation of caspases

The class of cysteine proteases known as caspases plays a prominent role in many types of apoptosis [13,14]. In general, three classes of caspases have been described based on sequence homology: ICE-like, CPP32-like, and Nedd2-like. The hypothesis of a sequential activation of caspases in a proteolytic cascade killing the cell stems from experiments done in a variety of systems. As yet, to our knowledge in no

single system has an entire proposed cascade been completely demonstrated. Various experiments suggest that caspases are constitutively expressed in cells and merely await activation by the appropriate signal. However, the signal that activates a given set of caspases obviously varies from system to system.

We have assayed for each of the major classes of caspases in extracts of CEM cells treated with glucocorticoid by use of the appropriate caspase class-specific substrates. The results give no evidence for the induction of ICE-like activity in these cells under these circumstances. However, there is activation of Nedd2 (class 2) and CPP32 (class 3), with onset of activity appearing about 24–36 h after addition of the steroid (Table 1). Similar caspase activation occurs following oxysterol addition (data not shown), and the general caspase inhibitor, Z-VAD.FMK significantly delayed apoptosis following the addition of 25-hydroxycholesterol or glucocorticoid. Thus, it appears that caspases are increasingly activated after a delay of about 24 h subsequent to the addition of steroid. The caspase activity probably is present in cells that have undergone or are about to undergo the relatively abrupt change to apoptosis. In the CEM system it has not been possible to distinguish whether caspases are activated in cells just before, simultaneously with, or after the beginning of morphologic signs of apoptosis. The postponement of apoptosis by the caspase inhibitor Z-VAD.FMK suggests that the initial activation preceeds irreversible apoptotic changes.

Table 1
1 μM Dexamethasone treatment induces caspase activities in CEM-C7-14 cells[a]

Time	Caspase activity (pmol of AFC[b]/h/10^6 cells)	
(h)	Control	Dexamethasone-treated
	Nedd2	
12	144	116
24	–	320
36	–	784
40	152	1592
48	–	5760
	CPP32	
12	300	276
24	–	116
36	228	2748
40	–	5480
48	–	21,530

[a] To logarithmically growing cultures of CEM-C7-14 cells, 1 μM dexamethasone in ethanol was added. Control cultures received an equal volume of ethanol. At the times shown, samples of cells were assayed for ICE-like, Nedd2-like, and CPP32-like caspase activity. No ICE-like activity was seen. Not shown: addition of Z-VAD.FMK at 24 h blocked the rise in Nedd2 and CPP32 activities in the dexamethasone-treated cells.

[b] AFC = 7-amino-4-trifluoromethylcoumarin (excitation 400 nm, emission 505 nm).

3.4. Endonucleolysis

One of the well-known concomitants of apoptosis in many systems is the lysis of the nucleosomes, which can be demonstrated by a variety of methods. DNA nicking can be detected by the TUNEL assay, which uses terminal deoxynucleotidyl transferase to label the ends of nicked DNA. Complete schism into pieces of double-stranded DNA may occur with two forms of products. Best known is the 180 base pair fragment resulting from lysis occurring between nucleosomes. On electrophoretic gels this results in the familiar "ladder" of DNA fragments giving multiples of 180 base pairs. Recently demonstrated in thymocytes has been nucleolysis into very large DNA pieces, specifically fragments around 50 and 300 kb. The large DNA fragments can be demonstrated by field inversion gel electrophoresis. It has been proposed that these may represent an important endonucleolytic event, since apoptosis from glucocorticoids in thymocytes occurred even when DNA breakdown into DNA ladders was blocked without blocking lysis into the very large fragments [15].

In CEM cells treated with oxysterols or glucocorticoids, there is DNA lysis into both large and small fragments, as well as DNA nicking. By TUNEL assay, DNA nicks begin to appear a few hours before the onset of the overt apoptotic changes that come in the second 24 h of culture following addition of steroid. Large DNA fragmentation begins approximately at 24 h and rapidly increases as the fraction of obviously apoptotic cells increases (Fig. 2). The appearance of DNA ladders occurs with or after the appearance of the large DNA fragments. The kinetics are quite similar for both oxysterols and glucocorticoids, although the exact pattern of fragments differs somewhat. In CEM cells, oxysterols appear to cause more marked DNA nicking, as shown by TUNEL assay, than do glucocorticoids. Also, shown in both Figs. 1 and 2 are data for clone M10R5, selected from CEM-C7 cells for resistance to high levels of 25-hydroxycholesterol. Fig. 1 shows that oxysterol resistance conferred no cross-resistance to glucocorticoid. Fig. 2 shows that M10R5 cells display DNA lysis when treated with dexamethasone; they showed none after exposure to oxysterol [1]. Conversely, clones selected for resistance to glucocorticoids remained sensitive to oxysterol (unpublished data, not shown).

Thus, both caspase activation and endonucleolysis occur concomitant with or just prior to morphologic apoptosis, beginning around 24 h after the addition of steroid. It is striking that the approximate time course of interruption of the cell cycle by glucocorticoids par-

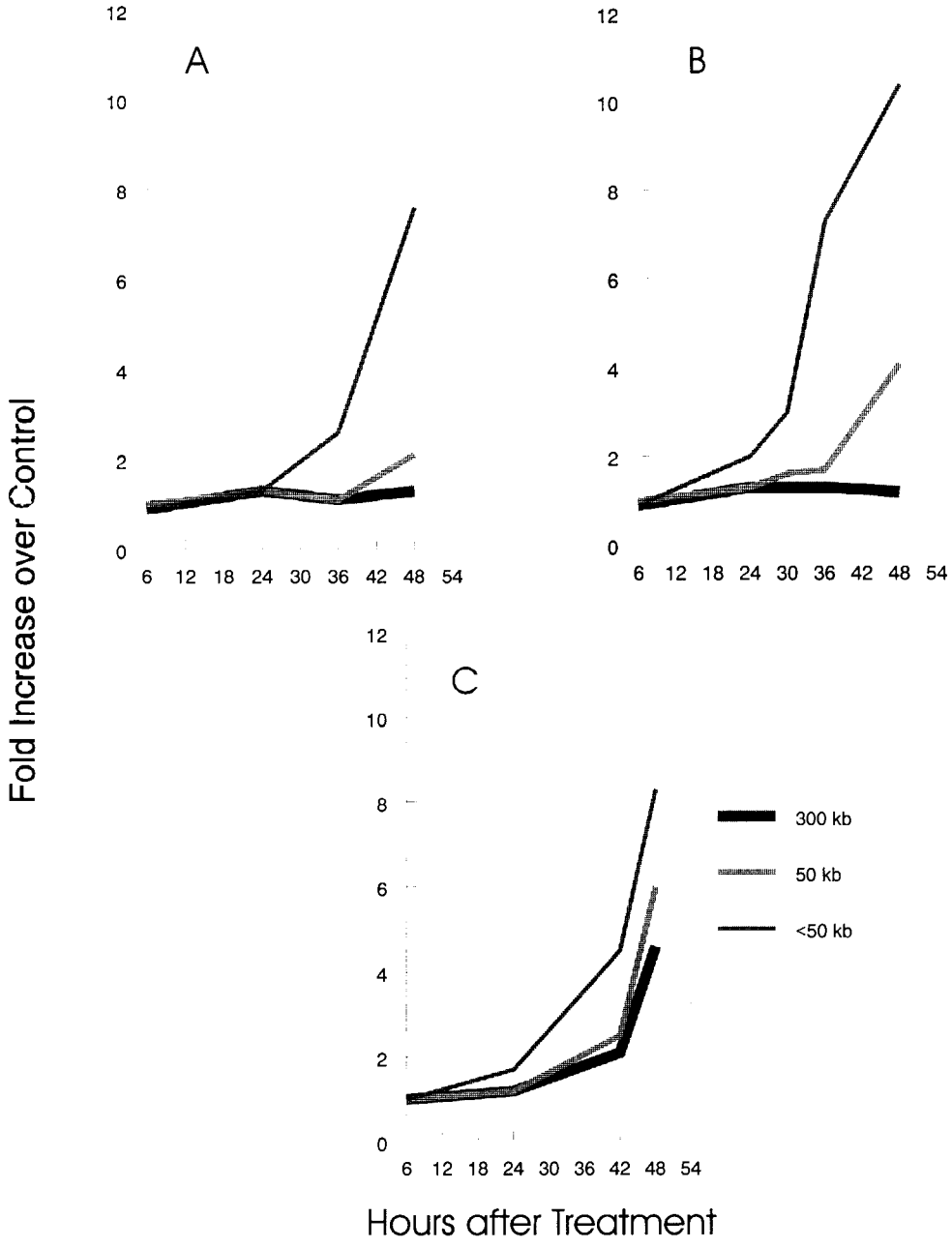

Fig. 2. Kinetics of appearance of large DNA fragments in cells sensitive to glucocorticoid and/or oxysterol. Three sizes of DNA fragments from CEM-C7 cells (a) and M10R5 cells (b) treated with 1 μM dexamethasone. The DNA fragmentation pattern of CEM-C7 cells treated with 1 μM 25-hydroxycholesterol is given in (c). Reprinted with permission from Ref. 1.

alleles closely the decrease of cell viability in the culture that occurs shortly after 24 h. We have shown previously that dexamethasone causes CEM-C7 cells to stop cycling in the G_1/G_0 phase of the cell cycle and that cells could not be rescued once they had reached that point [16]. Therefore, the timing of cell cycle block in apoptosis with activation of caspases and DNAses is essentially coincident. Several works in the literature indicate that oxysterols can also interrupt the cell cycle in G_1 [17,18]. However, our experiments with CEM cells have been inconclusive with regard to oxysterol-evoked G1 arrest.

3.5. Synergism of cyclic AMP pathways and glucocorticoids

A long history of experiments has indicated that the cAMP and glucocorticoid pathways may be interactive in some cell types, particularly in lymphoid cells [19,20]. Accordingly, we examined the effect of forsko-

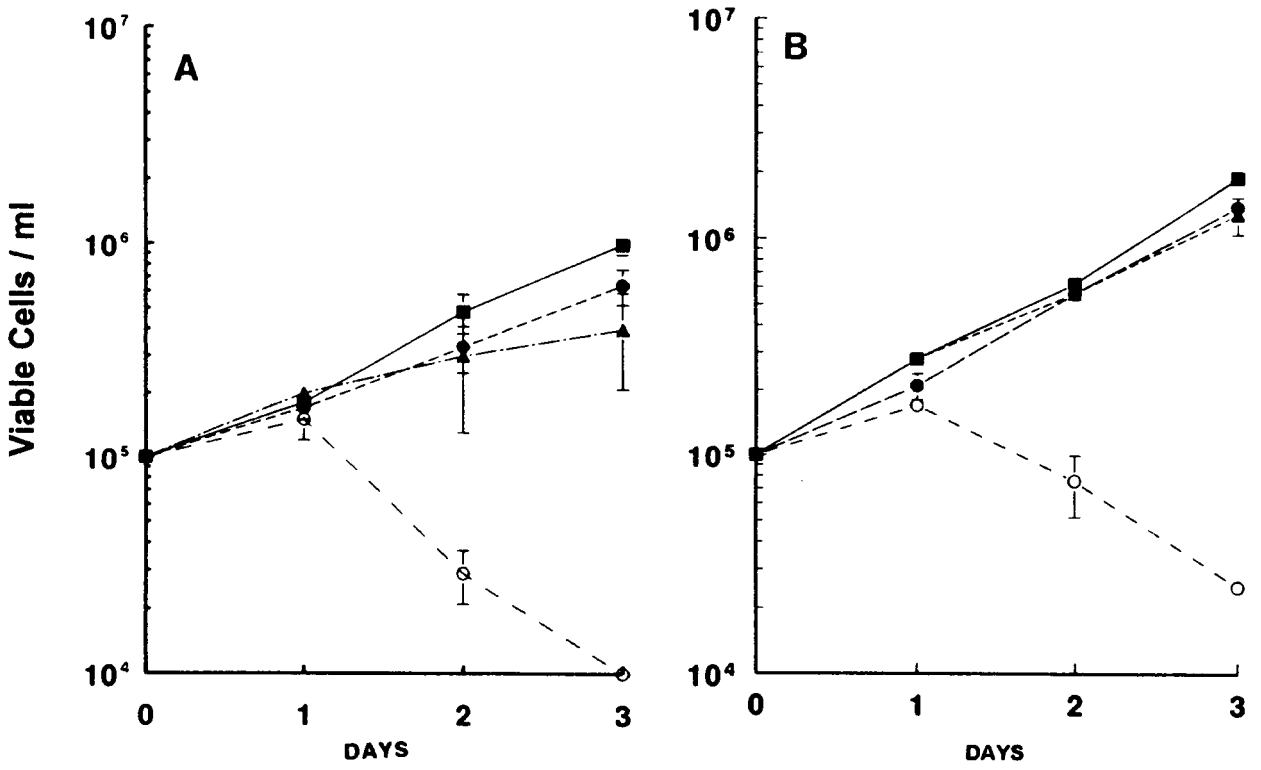

Fig. 3. Synergistic interaction between the glucocorticoid and protein kinase A pathways. CEM-C7 (A) and CEM-C1 (B) cells were incubated with 20 μM forskolin and/or dexamethasone [20 nM for C7 and 1 μM for C1]. Closed triangles = dexamethasone; closed circles = forskolin; open circles = dexamethasone + forskolin; closed squares = control cells with 0.3% ethanol as vehicle. Viable cells determined at times shown; $n = 3$ [no standard deviation bars indicated they fell within the size of the symbol]. Reprinted with permission from Ref. 21.

lin, an activator of the cAMP pathway, on dexamethasone-evoked responses in CEM cells [21,22]. The most dramatic effect was seen with clone CEM-C1 and its subclones. CEM-C1 cells were cloned originally from the CEM cell line without special selection and were subsequently observed to be resistant to glucocorticoid [23]. Unlike resistant cells deliberately selected from the sensitive clone CEM-C7, all of which showed abnormal glucocorticoid receptors, the CEM-C1 cells appeared to have a quantity of glucocorticoid binding sites equivalent to that of clone CEM-C7. Furthermore, these receptors appeared to be functional as judged by the ability to induce certain intracellular genes [24]. We hypothesized that some supporting mechanism, upon which action of the glucocorticoid receptor depended, was missing in CEM-C1 cells. To determine whether this interaction might involve the cAMP pathway, we treated CEM-C1 cells with forskolin in addition to dexamethasone. The results showed that while neither forskolin nor dexamethasone at high concentrations affected the growth of CEM-C1 cells, the combination caused dramatic apoptotic cell death. The time course for this death resembled that of the wild-type CEM-C7 cells in the presence of dexamethasone alone. That this death was apoptotic could be seen by the cell morphology and by tests of DNA lysis, e.g., TUNEL assay. Cyclic AMP analogs such as 8-bromo cAMP produced similar effects. As might be expected, the consequences of forskolin treatment included increased levels of cellular cAMP and also increased phosphorylation of CREB (cAMP response element binding protein) [21]. The precise molecular linkage between these changes and synergism with the glucocorticoid pathway remains to be determined. However, it is clear that an activated glucocorticoid receptor is required, since the apoptotic response evoked by the combined treatment could be blocked if the anti-glucocorticoid RU486 was added to displace dexamethasone from its receptor. We did not observe agonist activity for RU486 as an apoptotic agent under the circumstances of the combined glucocorticoid and forskolin treatment. This combination has been reported in other cell systems to convert RU486 to at least a partial agonist [25,26]. We have not, however, examined the induction of specific genes by RU486 in the CEM system. Dose response studies showed that the concentration of glucocorticoid required to kill forskolin-treated cells closely resembled that required to kill wild-type cells with glucocorticoid alone, i.e., concentrations consistent with those required to occupy receptor. We hypothesize that forskolin treatment "enables" the glucocorticoid receptor

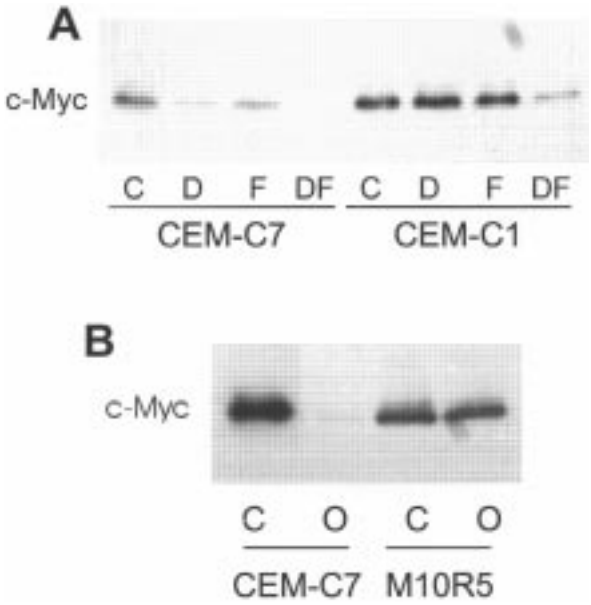

Fig. 4. Suppression of c-Myc protein by each of the three treatments by 24 h: dexamethasone, 25-hydroxycholesterol, and dexamethasone + forskolin. (A) Glucocorticoid treatment: To cells in logarithmic growth ethanol vehicle [C] or 20 nM dexamethasone for CEM-C7-14 cells and 1 μM for CEM-C1-15 cells [D] or 10 μM forskolin [F], or dexamethasone + forskolin [DF] was added and c-Myc protein was etermined by western blotting using the polyclonal antibody Mycl-9E-10.2. A representative experiment is shown here. (B) Oxysterol treatment: To cells in logarithmic growth ethanol vehicle [C] or 1 μM 25-hydroxycholesterol [O] was added to CEM-C7-14 or M10R5 cells and c-Myc protein was determined by Western blotting using the polyclonal antibody Mycl-9E-10.2.

to be responsive to its ligand and hence evoke apoptotic cell death. This interpretation is consistent with that for earlier results on a mouse lymphoid line [19]. We did not, however, consistently find increased GR levels in forskolin-treated cells, and therefore the data in CEM cells do not support GR induction as a general mechanism explaining the two pathways' interaction [19].

Potentiation between forskolin and dexamethasone was also observed in the wild-type CEM-C7 cells. This could be demonstrated by using a low concentration of glucocorticoid, one which in itself was only partially lethal. When this low (2×10^{-8}M) concentration of dexamethasone was given alone, only a portion of the cells died in 48 h. Addition of 20 μM forskolin alone slowed cell growth slightly, without loss of viability. A combination of forskolin and low dose dexamethasone, however, completely killed the culture (Fig. 3).

3.6. Suppression of c-myc by three forms of treatment that lead to apoptosis

After each of the three treatments: dexamethasone, 25-hydroxycholesterol or dexamethasone and forskolin, during the time preceding the irreversible changes

Table 2
1 μM Dexamethasone treatment of CEM-C7-14 cells regulates c-*myc* mRNA primarily at the transcriptional level. To logarithmically growing cultures of CEM-C7-14 cells, 1 μM dexamethasone in ethanol was added for 24 h or ethanol alone as control for 0 h. The cells were harvested for RNA or nuclei were isolated. The *c-myc* message levels were determined by Northern blot analysis. Nuclear run-on assay was employed to measure synthesized *c-myc* mRNA levels. Data are represented as 0 h levels = 100%

Time (h)	c-*myc* mRNA	Newly synthesized c-*myc* mRNA
0	100%	100%
24	30%	32%

of the second phase of events, there is a significant and deep suppression of the products of the c-*myc* proto-oncogene (Fig. 4). We have previously shown that glucocorticoid treatment of CEM-C7 cells and other clones of sensitive CEM cells leads to a rapid and dramatic down-regulation of c-*myc* [3,27]. This begins by an hour after addition of the steroid, and c-*myc* mRNA and protein fall rapidly thereafter, reaching a minimum at 12–18 h, at which low level they remain. Suppression of c-Myc protein levels can be seen after 3–6 h of glucocorticoid treatment. Later, the cells enter the apoptotic crisis. These changes do not occur in cells lacking a functional glucocorticoid receptor, nor do they occur in the CEM-C1 clone [21]. We therefore hypothesize that the c-Myc changes are essential early steps in the reversible phase, one that ultimately leads to apoptosis. Several tests of the hypothesis have been carried out. Transient transfection of cells with plasmids expressing c-Myc prevented subsequent cell death. Reducing c-Myc with an antisense oligonucleotide also caused complete cell death, akin to that seen after glucocorticoid addition [3]. The reversal of the apoptotic sequence by adding the anti-glucocorticoid RU486 in a timely fashion resulted in rapid restoration of c-*myc* levels and recovery of the culture [22]. The negative regulation of c-*myc* in CEM cells by dexamethasone occurs by a direct inhibition of transcription, as judged by nuclear run-on assays (Table 2). Our mRNA half-life studies indicate that there is essntially no change in the half-life of the stable c-*myc* message (data not shown).

Oxysterols also cause down regulation of c-*myc* mRNA and protein in CEM cells destined for apoptosis, but by mechanisms different from those utilized by glucocorticoids. Because of the prominent role that Myc appears to play in glucocorticoid-induced cell death, we examined the effects of 25-hydroxycholesterol on the c-*myc* message and protein pools. The results showed that there is a slow, but clear, diminution of c-*myc* mRNA beginning approximately 12 h after the addition of the steroid and continuing thereafter. The c-Myc protein decreases much more rapidly

after the administration of the oxysterol. As early as 6 h after the steroid, c-Myc protein is reduced significantly, and it falls very rapidly thereafter to a low level. Measurement of c-*myc* transcription by nuclear run-on assays showed that there was little or not effect of 25-hydroxycholesterol on transcription of the c-*myc* message. There was, however, a decrease in the half-life of *myc* mRNA following addition of the oxysterol. The differing kinetics of change of the protein and the message indicate that oxysterol has two effects on this system. One is a destabilization of c-*myc* message that accounts for the slow decrease in mRNA pool size. But much more rapidly, the oxysterol causes loss of c-Myc protein. Dissociation of regulation of the protein and its message has been reported in other systems [28–31]. CEM-C7 subclone M10R5, selected for resistance to 25-hydroxycholesterol, failed to show any change in c-*myc* message or protein in the face of lethal concentrations of oxysterols. The central point is that oxysterols, as well as glucocorticoids, cause a down-regulation of the c-*myc* gene product c-Myc in these cells, during the time preceding the overt increases in caspases, nucleases, and apoptotic death.

In CEM-C7 and CEM-C1 cells, dexamethasone and forskolin synergistically down-regulated c-Myc. In CEM-C1 cells, the negative regulation of c-*myc* expression correlated with the lytic potency of the agent(s). Dexamethasone alone failed to evoke a suppressive response, but combined treatment with dexamethasone and forskolin caused significant suppression. The exact level of control by which this occurs has not yet been demonstrated. A population of cells selected for resistance to the double drug treatment failed to show the negative regulation of c-*myc* expression.

4. Conclusions

Three different forms of treatment, glucocorticoid, oxysterol, or glucocorticoid plus forskolin, cause apoptotic cell death of CEM cell clones. These all show a similar time course: an initial period of approximately 24 h, in which growth is not grossly affected, followed by a period when increasing numbers of cells show apoptosis. Within thisl atter phase dexamethasone-treated cells are locked into G_1/G_0. Cell cycle events upon oxysteol treatment and upon combined treatment with forskolin and dexamethasone remain to be clarified. In each case, as the second phase of events unfolds, caspase and nuclease activation commence and increase dramatically, concomitant with increasing numbers of dying and dead cells.

Some critical level of the c-Myc protein seems to be essential for the viability of these lymphoid cells. First, giving antisense oligonucleotides to lower c-Myc kills the cells. Secondly, expressing c-Myc in a form that does not suppress, inhibits dexamethasone-evoked cell death. Analogous tests for the effect of expressing c-*myc* or antisense c-*myc* in cells treated with oxysterol and forskolin plus dexamethasone treated cells remain to be carried out. Mechanistically, the glucocorticoid and oxysterol treatments down-regulate c-Myc quite differently. It is impressive, therefore, that in both cases the final effect is a large reduction in c-Myc levels antecedent to biochemical and morphologic apoptosis.

We believe therefore that a significant step in the initial pathway leading to apoptosis in this cell system is the negative regulation of c-*myc* expression. The intervening steps between this and later stages remain to be clarified. For glucocorticoids, one intermediate step appears to be the prolonged induction of c-*jun*[32]. New experiments will be required to see whether this gene product is altered in circumstances following the other treatments. The steps between the initial events so far identified and the death of these lymphoid cells must be filled in by examining other candidate genes that may participate in the sequence of events leading to the apoptotic catastrophe.

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