Acta Biochim Biophys Sin (2008): 595-600 | © 2008 Institute of Biochemistry and Cell Biology, SIBS, CAS | All Rights Reserved 1672-9145
http://www.abbs.info; www.blackwellpublishing.com/abbs | DOI: 10.1111/j.1745-7270.2008.00433.x

Review

Stepping stones in the path of glucocorticoid-driven apoptosis of lymphoid cells

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Cumulative work on glucocorticoid (GC) regulation of genes in lymphoid cell cultures has revealed that apoptotic sensitivity to GCs depends on sufficient active GC receptors in the cells. The actions of the ligand-driven GC receptor that lead to apoptosis depend on interactions with other major cell-signaling systems, including the MAPK pathways, the cAMP/PKA pathway, the hedgehog pathway, the mTOR system and the c-*myc* system. The balance between these systems determines whether a given cell responds to GCs by undergoing apoptosis. A central core of networked genes may be found under GC control in many types of malignant, GC-sensitive cells. The partial core list identified should be tested in clinical cell samples from hematologic malignancies.

Keywords glucocorticoid; apoptosis; lymphoid cell

Early Background

How adrenal corticosteroids, specifically glucocorticoids (GCs), kill lymphoid cells is a longstanding problem in steroid hormone action. The mechanism of this effect is of great importance to both basic science and clinical applications. The lympholytic effect was discovered early in steroid research, when adrenal extracts were observed to obliterate over 90% of mouse thymic cells. Later, it was shown that the active principles in the extracts were GCs. The thymic cells killed by GCs were found to be immature lymphocytes in the T-cell lineage. It is not clear whether GCs acted to kill them by a direct effect on the lymphoid cells themselves or indirectly, by affecting other systems upon which the viability of the immature lymphocytes depended. In the mid-20th century, the general hypothesis of steroid hormone action, which stated that GCs could act by regulating gene expression, developed. Evidence obtained *in vivo* on the induction of certain liver enzymes, such as tyrosine aminotransferase, supported the hypothesis [1–3], but there was still considerable debate on the subject. The development of the hepatoma tissue culture (HTC) cell line and other hepatoma cell lines, which responded to GCs with increased production of the enzyme tyrosine aminotransferase [4–7], allowed more direct tests of the hypothesis. Use of HTC cells, for example, permitted experiments showing that GCs can act directly on their target cells to cause transcription-dependent increased synthesis of a specific protein [4,8,9]. In other words, no cellular systems other than the target cells themselves need be involved for a GC to induce an enzyme. Rodent and *in vitro* cellular experiments suggested that a component of control by GCs could also exist at the post-transcriptional level [8–10]. Although later work in HTC cells concluded that this was a secondary effect of the techniques used at the time [11], considerable data confirm that, at least in some systems, post-transcriptional regulation due to steroids does exist [12]. This is especially dramatic in the control of vitallogenin synthesis by estrogens [13]. But the most basic and general regulatory effect of GCs and other steroids is to change the level of expression of specific genes by altering their rate of transcription. These changes in expression can be increases or decreases. Both are important for the effects of GC's on lymphoid cells.

Given that GCs regulate lymphoid cell gene expression, the next question to answer was "How"? The key to this question was intracellular receptors for GCs in lymphoid cells [14]. These receptors bind steroids with high affinity and act as ligand-driven transcription factors. Use of tissue culture cell systems to study gene induction had already proven so valuable that cultured cell systems for studying the lympholytic effects of GCs were soon sought. The first reported system appears to have been S49 cells, a line derived from a mouse lymphoma [15]. With these cells, it was shown that GCs act directly on the target cell for liver gene induction. However, the measured response was

Received: May 5, 2008 Accepted: May 9, 2008
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cell death. This allowed strong selective pressure on the cultured cells, which in turn allowed selection of GC-resistant cells. Among these, several classes of resistant cells were identified. Though a few resistant clones appeared to have normal receptors (as could be best determined by the pre-gene cloning methods then available), the majority had either lost or mutated their GC receptor.

Because the mouse cell model could only partially mimic the behavior of human lymphoid cells, we sought a human lymphoid cell system that was sensitive to GC-dependent cell death. In the 1970s, we screened the few available lines of human leukemic lymphoblasts and found a useful tool in a human lymphoblastoid cell line (CEM), which came from a case of childhood lymphoblastic leukemia [16]. From the original line, without deliberate selective pressure, we cloned cells that were highly sensitive to GC-driven cell death (e.g., clone C7) and others that were highly resistant (e.g., clone C1). The use of clonal cell stocks has allowed many studies to be done without the confusion that often comes from using uncloned cell lines, which inevitably include phenotypically mixed populations. Particularly interesting was the fact that the GC-resistant C1 cells contained as many abundant high-affinity receptors for GCs as the sensitive C7 cells. The resistant C1 cells' receptors could mediate gene induction and, therefore, were functional [17]. This receptor-positive, GC-resistant cell phenotype was an exciting model to have because, clinically, many human hematologic malignancies that become GC resistant retain GC receptors [18–21].

The GC-sensitive clone C7 cells also were valuable. With them, we demonstrated again that the lethal effects of GCs are directly on the affected cells. The extent of kill corresponded to the concentration of GCs that occupied increasing proportions of the GC receptors, and maximal cell kill corresponded to maximal receptor occupancy. Selection for resistant subclones of C7 cells was done in two ways. First, in an experiment based on Luria-Delbrück fluctuation analysis, we used a maximal receptor-filling concentration of GCs and selected for resistant survivors. This method, developed for analysis of prokaryotic organisms, shows whether drug resistance results from spontaneous mutations or from non-genetic adaptations to the drug. GC resistance in the sensitive C7 clone occurred in a manner consistent with random, spontaneous mutations and at a surprisingly high rate of about 10^{-5}. This rate is more consistent with a haploid mutation rate than with a diploid, autosomal gene and the GC-receptor is a diploid gene. Resistance almost always appeared to be due to an alteration in the GC receptor, giving rise to a phenotype not previously described [22,23]. In clone after clone of these GC-resistant cells, the GC receptors behaved similarly: they could bind steroid *in vitro*, but under conditions that lead to receptor activation to its functional form as a transcription factor, they lost the ability to retain GCs. Thus we termed such receptors "activation-labile" (act^l).

Later, the reason for the high mutation rate and the high frequency of a single phenotype was found. Both CEM C7 and CEM C1 cells and, in fact, the cells of the patient from which the CEM line came are haploid for wild-type GC receptors [24–26]. The abnormal allele contains a point mutation in the receptor L753F that gives the act^l phenotype. The wild-type allele mutates at the expected haploid rate, allowing selection for clones that retain only the L753F mutant receptor, which does not mediate GC-dependent cell death.

In the second method of selection, C7 cells were first subjected to intensive chemical mutagenesis and then selected for GC resistance. A different phenotype predominated in the resulting GC-resistant clones [23]. The majority appeared to lack GC-binding (receptor) activity altogether. Later it was found that at least some of these still were expressing the act^l receptor allele [27]. These combined experiments showed that, for human lymphoid cells to die in response to GCs, GC receptors in sufficient numbers and with normal function are absolutely required. The CEM C1 clone, which displays GC resistance despite having sufficient GC receptors, lacks the essential mechanisms required for the cell death response to GC. It should be noted that, due to epigenetic instability over long-term culture periods, expression levels of the glucocorticoid receptor in C1 cells dwindle, often leading to populations with reduced receptors. Recloning of C1 cells allows recovery of resistant cells with receptor quantities akin to C7 cells, which leads to the conclusion that GC receptors are necessary but not sufficient for GC-dependent lymphoid cell death. Subsequent studies on these cells have provided deeper insights into the interrelated signaling pathways that support GC action.

Apoptosis is the Type of Cell Death Caused by GCs in Lymphoid Cells

Prior to the recognition of apoptosis as a form of cell death, GC-dependent death of lymphoid cells was simply referred to as "lysis" or "death". When histological studies defined apoptosis and the biochemical cytological correlates of cell shrinkage, DNA lysis, membrane eversion of phosphatidyl serine etc. were uncovered, it became apparent that GC-

driven lymphoid cell death is classic apoptosis [28,29]. As the complex caspase and Bcl_2 systems, which are so important for regulation of apoptosis, have become better understood, it is clear that they are the ultimate regulators of this apoptosis. The caspases have been shown to be involved in CEM cell apoptosis [30]. However, it is not clear exactly what the step-by-step pathway is for GC-dependent lymphoid cell apoptosis. The mitochondrial breakdown-dependent subpathway of caspase activation is involved, but the exact steps remain to be determined. Experiments testing for direct regulation of caspase and Bcl_2 family genes suggest that GC-driven lymphoid apoptosis involves more than direct induction of caspase or pro-apoptotic Bcl_2 family genes. The induction of such genes is involved, but how their expression and the activity of their protein products are controlled is complex.

The Time-course of GC-driven Apoptosis Implies a Gene Cascade

To date, results from cultured lymphoid cell lines have shown that considerable time must elapse after the addition of GCs before the first biochemical evidence of apoptosis can be detected. The exact interval between initial exposure to GCs and the early stages of biochemical apoptosis varies between cell lines, but the interval seems to exceed at least 12 h. In GC-sensitive CEM cell clones, this lag period for biochemical apoptosis ranges between 20 h and 48 h. Several points about the lag are noteworthy. First, GCs must be continually present during this period for apoptosis to ensue. Premature removal of GC or addition of a competitive antagonistic ligand to displace the GC agonist from its receptor results in failure to cause apoptosis. The cells then simply resume normal growth. Second, the onset of apoptosis does not occur in a synchronized pattern. Initially, a few cells die, and gradually more are recruited. Maximal cell death may require 1–2 d. Third, GCs stop the cell cycle in a G_1- or G_0-like state. This cycle inhibition may be inextricably linked to eventual apoptosis, as in CEM cells [31], or may be dissociated from cell death, as in P1798 cells [32]. Fourth, once the biochemical processes of apoptosis have begun, they cannot be reversed by removing the GCs. Thus, GCs do not act as an abrupt switch. Rather, they act as drivers of a time-dependent continuum that ultimately triggers overt apoptosis.

These facts can be evaluated in light of the known actions of GCs on gene regulation. GCs and other steroids can regulate genes by primary, delayed primary or secondary actions [33]. Primary gene control is relatively rapid. A ligand-activated receptor enters the nucleus in minutes to bind at specific, open regulatory sites on the DNA. Altered transcription and all the subsequent steps that end in altered protein expression are completed within minutes thereafter. From specific examples studied, new protein can be seen as early as 15 min after adding GCs to the system. As a rule of thumb, primary induction may require up to 2 h due to requirements for RNA processing, protein folding and the like. In some cases, primary induction may be delayed for several hours (delayed primary induction). The mechanisms behind the delay are not well understood, but they presumably relate to requirements for time-consuming chromatin modifications ultimately dependent on protein complexes formed in the nucleus between the GC receptor and a large and varied number of other proteins. No new protein synthesis is required for either form of primary gene regulation by GCs. In contrast, secondary regulation does require new protein synthesis. In this type of regulation, the primary events driven by the ligand-activated GC receptor result in production of proteins, which in turn (secondarily) regulate genes.

The behavior of cells exposed to GCs and destined for apoptosis strongly suggests that all the above mechanisms, particularly the slower ones, are involved. That GC exposure does not act as a brief switch tells us that the GC receptor must continually act to modulate gene expression. It also implies the involvement of a gene network. This network must involve interactions between the GC signaling system and other signal transduction pathways. Interactions between the GC and cAMP pathways have been known for many years [34]. More recently, a wealth of data has been presented, showing important connections between steroid-driven and other signal transduction networks [35–39]. Our work based on CEM cell clones and extended to other cell lines, as well as that of others, has begun to reveal this network.

Before microchip-based gene array methods were available, we found by surveying a small number of intuitively selected genes, that GCs rapidly and selectively repress transcription of c-*myc* [40]. Later, we showed that constitutive expression of c-*myc* partially protected against GC-dependent apoptosis [41]. Since the protein product of c-*myc* is a master regulator of transcription, our findings gave support to the hypothesis of interactive networks in GC-driven lymphoid cell apoptosis. Adding to the conviction that multi-gene, multi-signaling networks were working in conjunction to drive GC-dependent apoptosis were manipulations of the cAMP pathway. Remembering the known interdependence in lymphoid cells of GCs and cAMP actions [42,43], we tested the effects of activating the cAMP path. Forskolin activates adenyl

cyclase, leading to elevation of intercellular cAMP and activation of cAMP-dependent protein kinase A (PKA). We found that activation of PKA by forskolin synergized with GCs in a clone of GC-sensitive CEM cells, and even more impressive, PKA activation converted the C1 clone of completely resistant cells to GC-sensitive cells [44]. We since have discovered that a specific isoform of PKA is involved and that this cAMP/GC-pathway interaction links to the hedgehog signal transduction pathway [45].

Studies with Large Gene Arrays Reveal the Outlines of MAPK Pathway Interactions with GCs and their Receptors

Microchip-based gene arrays have allowed analysis of thousands of genes for altered expression in the presence of GCs. We have employed a closely related set of three CEM cell clones to determine which genes are best related to GC-driven lymphoid cell apoptosis. The clones employed are: CEM-C7-14, CEM-C1-6 and CEM-C1-15. These are subclones of the original C7 and C1 clones, derived without selective pressure. CEM-C7-14 is inherently sensitive to GC-driven apoptosis. CEM-C1-15 is inherently resistant and shows characteristics very similar to those of its clonal parent CEM C1. Such recloning periodically is necessary to preserve phenotype in long-term cultures. CEM-C1-6 is a sister clone to C1-15 and is a spontaneous revertant to GC sensitivity. At the outset of work with these three clones, we hypothesized that since clones C7-14 and C1-6 are sensitive to GC-driven apoptosis, they would share a set of GC-regulated genes distinct from those in resistant clone C1-15. This proved to be the case. Using microchips that could detect approximately 12,600 genes and arbitrarily setting allowable induction and repression limits high to minimize "false positives", we uncovered sets of 39 induced and 21 repressed genes that were unique to the two sensitive clones [46]. The clone resistant to GC-dependent apoptosis was not unresponsive to GCs, but for the most part showed regulation of genes differently than those in the sensitive cells. Among the genes uniquely repressed in the two sensitive clones was c-*myc*, confirming our earlier results. The repression of c-*myc* is rapid and consistent with a primary GC receptor effect. Of the genes repressed at later times, 40% appear to be c-*myc* dependent (our unpublished results).

Subsequent experiments revealed that an important action of GCs in the sensitive clones is to activate p38, a member of the mitogen-activated protein kinase (MAPK) system. We also discovered that p38 MAPK specifically phosphorylates the serine at position 211 in the human glucocorticoid receptor [47]. Phosphorylation of S^{211} enhances the ability of the receptor to regulate transcription and apoptosis [47,48]. By using chemical inhibitors on specific classes of MAPKs, we found that inhibition of p38 reduced GC-dependent apoptosis, whereas inhibition of the other two major MAPK classes, extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK), enhanced apoptosis. This balance was critical for determining the sensitivity of CEM cells. The resistant clone C1-15 displayed high basal JNK and ERK activity relative to that of p38 [49]. Inhibition of JNK and ERK converted C1-15 cells to GC sensitive. Both forskolin and rapamycin, treatments known to cause GC-resistant CEM cells to become GC-sensitive cells [44,50], affected the MAPKs appropriately by reducing activated ERK and JNK and increasing activated p38. Several pathways that modulate GC-dependent apoptotic sensitivity converge on the MAPK pathway [51]. Many of these GC-regulated events depend on transcription; others are post-transcriptional protein modifications that affect enzymic activities.

An Initial Gene Profile for Apoptotic GC Sensitivity

Focusing at first on transcription-level regulation, we examined the responses of genes in cell lines representative of several types of hematologic malignancies [52]. Clinically, T-lineage and B-lineage lymphoid cells tend to differ in response to GCs and other drug treatments. Adult and childhood leukemias also tend to differ in their responses. Malignant and normal myeloid lineage cells tend to resist GC-driven apoptosis, but an uncommon type of myeloid leukemia is GC sensitive. Therefore, we selected GC-sensitive cell lines representative of several types of leukemia: pediatric T-lineage (CEM), pediatric B-lineage (SUP-BIS), adult B-lineage (RS4; 11), and myeloid (Kasumi-1). We also included comparisons of the effects of GCs on the genes of normal mouse thymocytes. As a negative control, we employed the CEM-C1-15 clone. Since we knew of several treatments that could render these cells GC sensitive, we were able to compare the genes affected in C1-15 cells in their GC-resistant and GC-sensitive states.

The time-course of apoptotic response in the sensitive cells conformed to the pattern discussed above. Each line displayed a lag phase after the addition of GCs and before cell death manifested itself. For gene expression analysis, a nested set of comparisons was performed. To avoid missing any regulated genes of importance, no arbitrary fold-changes in expression were imposed, and any

consistent change ≥20% from background was initially accepted. After categorizing the changes in expression at this level, simple tests of statistical significance were carried out and the lists revised accordingly. When all consistent changes in expression of mRNAs common to all GC-sensitive CEM cells (including C1-15 cells converted to GC sensitive by treatment with two structurally different GCs) were compiled, 96 regulated genes were revealed. Of these, 35 were found to fit into a single network in which c-*myc* and NR3C1 (the glucocorticoid receptor gene) were hubs.

Additional data from the two B-lineage leukemias and the GC-sensitive acute myeloblastic leukemia resulted in a reduced list of 27 genes regulated (up or down) in all sensitive cell lines. The fact that these results were derived from quite different cell types suggests a core of GC-regulated genes common to all or many apoptotically sensitive hematologic malignant cells. Ten of these genes were regulated in normal mouse thymocytes. Considering the differences between the systems (growing, malignantly transformed versus non-growing, non-transformed), it is striking that a third of the genes were still found in common.

These lists are undoubtedly incomplete. The gene microchips employed were capable of showing only approximately 12,000 human genes and approximately 6000 mouse genes. Further studies with chips querying the entire genome will no doubt reveal additional GC-sensitive genes common to apoptotically sensitive hematological cells. Furthermore, other than CEM clones, only single examples of each malignant cell type were employed. Each of these gave rise to subsets of genes not common to all cell types. By querying other examples of each type, it will be possible to see whether specialized gene sets can be identified that will specifically profile each malignant cell type (e.g. adult B-cell leukemia). An early attempt at clinical correlations with a few patient samples has been made [53]. Even at this current level of identification, the data encourage examination of additional clinical specimens to see whether the *in vitro* results will prove applicable to prognostic evaluations in patients.

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