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Gene networks in glucocorticoid-evoked apoptosis of leukemic cells☆

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Abstract

To discover the genes responsible for the apoptosis evoked by glucocorticoids in leukemic lymphoid cells, we have begun gene array analysis on microchips. Three clones of CEM cells were compared: C7–14, C1–15 and C1–6. C7–14 and C1–15 are subclones from the original clones C7 (sensitive to apoptosis by glucocorticoids) and C1 (resistant). C1–6 is a spontaneous revertant to sensitivity from the C1 clone. Previously we presented data on the sets of genes whose expression is altered in these cell clones after 20 h exposure to dexamethasone (Dex). The two sensitive clones, which respond by undergoing apoptosis starting about 24 h after Dex is added, both showed >2.5-fold induction of 39 genes and 2-fold reduction of expressed levels from 21 genes. C1–15, the resistant clone, showed alterations in a separate set of genes.

In this paper, we present further analysis of the data on genes regulated in these cell clones after 20 h Dex and compare them with the genes regulated after 12 h Dex. Some, but not all the genes found altered at 20 h are altered at 12 h, consistent with our hypothesis that sequential gene regulation eventually provokes full apoptosis. We also compare the levels of basal gene expression in the three clones. At the basal level no single gene stands out, but small sets of genes differ >2-fold in basal expression between the two sensitive and the resistant clone. A number of the genes basally higher in the resistant clone are potentially anti-apoptotic. This is consistent with our hypothesis that the resistant cells have undergone a general shift in gene expression.

Keywords: Glucocorticoid; Apoptosis; Dexamethasone (Dex)

1. Introduction

Glucocorticoids provide one of the least toxic mainstay therapies for several types of lymphoid malignancies. This therapeutic usage originally was based on the observation that acute administration of high-dose corticosteroids to young mice leads to dramatic thymic involution. This effect is due to an apoptotic response of young thymocytes to the steroids, and it can be demonstrated to begin in isolated immature mouse thymocytes in a matter of a few hours [1–3].

Equivalent human experiments have been difficult to perform, due to the inaccessibility of the relevant thymic cells. However, glucocorticoids often appear to cause apoptosis of many lymphoid leukemia and lymphoma cells, and these often have been employed in vitro as model systems. The mechanisms by which glucocorticoids evoke apoptosis in cells capable of responding are still the subject of active research. While glucocorticoids can down-regulate the expression of lymphokines upon which normal lymphoid cells depend, and thereby reduce their viability, these steroids also seem to have powerful direct apoptotic effects on the cells. This seems particularly relevant to the responsive malignancies, since by their very nature malignant lymphoid cells often have lost dependence on the normal supporting lymphokines.

We have therefore employed clones of the childhood acute lymphoblastic leukemia cell line CEM to investigate the direct, apoptotic effects of the synthetic steroid dexamethasone (Dex), as a representative, therapeutically relevant, glucocorticoid. We have shown repeatedly over many years, that addition of Dex in concentrations sufficient to fully occupy the glucocorticoid receptor, leads to a highly reproducible time course of events, culminating in the apoptosis and eventual lysis of sensitive clones of CEM cells [4]. A striking property of the time course in the system is that the clearly apoptotic events only begin to appear after approximately

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24 h in the constant presence of steroid. Only after that do cells show increased caspase activity, DNA lysis, growth arrest, karyorrhexis, etc. Cell death is increasingly manifest between 24 and 96 h. Many molecular events occur during the 24 h preceding the onset of overt apoptotic events. It is our hypothesis that a network of Dex-initiated primary and secondary gene regulatory events occurs during the first 24 h, culminating in a set of circumstances that precipitates full apoptosis. During the prodromal and early apoptotic stages, Dex must remain present all the while for cell death to occur. Removal, or reversal by antagonist, of Dex action during the first day completely aborts the apoptotic program. This does not mean that all alterations in gene expression are directly caused by Dex. Some, even many, could be secondary to the changes caused and sustained in directly Dex-sensitive genes.

To test the hypothesis, we have compared the expression of genes in three closely related clones of CEM cells, two sensitive to Dex-evoked apoptosis, the third resistant. We predicted that in the two sensitive clones we would find a common set of genes regulated that would differ from those in the resistant gene. Initially, we chose to examine the genes affected by an exposure interval of 20 h in Dex. This time point was chosen to be just before the onset of major apoptotic events. The first analysis of the data identified 39 genes whose mRNA pools were increased significantly and 21 genes whose mRNAs were reduced in both sensitive clones but not the resistant clone [5]. A different set of genes was regulated in the resistant clone. In this communication we present further analysis of this data. We also examine in detail the differences in basal gene expression between the three clones, to see if they offer insights to their sensitive and resistant phenotypes. Finally, we present data on the changes in gene expression found after 12 h in Dex and compare these with those seen after 20 h.

2. Materials and methods

2.1. Reagents

Dex and other reagent grade chemicals were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO). The RNeasy total RNA isolation kit was from Qiagen (Santa Clara, CA), T7-oligo (dT) promoter primer from Affymetrix (Santa Clara, CA), and the BioArray high yield RNA transcription labeling kit from Enzo Biochem (New York, NY). The following reagents were purchased from Invitrogen (Carlsbad, CA): SuperScript II reverse transcriptase, dNTP mix, *E. coli* DNA ligase, *E. coli* DNA polymerase I, RNase H, and T4 DNA polymerase.

2.2. Cell culture

All CEM clones used in this study were subcloned in 1996 from original clones C1 and C7 in semisolid agarose medium in the absence of any selective pressure [6]. Thereafter, the cells were carefully maintained in logarithmic growth in Cellgro RPMI 1640 tissue culture medium (Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum from Atlanta Biologicals (Norcross, GA) at 37 °C in a humidified 5% CO_2/95% air incubator.

2.3. RNA extraction

When cells had grown to a density of 4×10^5 cells/ml, they were treated with either ethanol vehicle (≤1% final concentration) or 1 μM Dex in vehicle for 12 or 20 h. Approximately 1×10^7 cells were harvested, washed once with chilled phosphate buffered saline, pH 7.4 (Cellgro) and resuspended in lysis buffer (RNeasy kit). The cell lysate was passed through a QIAshredder column (Qiagen) and processed for total RNA isolation as per the protocol provided (RNeasy kit). As an internal control, a Northern blot analysis for c-*myc* would be performed on each sample to confirm that a standard response to Dex had occurred [7]. RNA samples were then stored at −70 °C in ethanol until used for either GeneChip® analysis or Northern hybridization. An aliquot fraction of the cells in each experiment was kept in culture for several days, to confirm the cells apoptotic or resistant behavior.

2.4. Target labeling and hybridization

First-strand cDNA synthesis was performed using 10–25 μg of total RNA, a T7-$(dT)_{24}$ oligomer (5′ GGCCAGTGAATTGTA ATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$ 3′) and SuperScript II reverse transcriptase (Life Technologies, Baltimore, MD). Second strand synthesis converted the cDNA into a double-stranded DNA template, which was subjected to an in vitro transcription reaction using bacteriophage T7 RNA polymerase. The "target" cRNA was labeled with biotin during the in vitro transcription reaction and then fragmented to a mean size of 200 bases to facilitate hybridization to probe sequences of the HG_U95Av2 Affymetrix GeneChip® Arrays. Each target RNA sample was initially hybridized to a test array that contained a set of probes representing genes commonly expressed in a majority of human cells, e.g. actin, alu, transferrin receptor, transcription factor ISGF-3, 18S RNA, and 28S RNA to confirm the successful labeling of the target RNAs and prevent the use of degraded or non-representative target RNA samples. Hybridization of GeneChip® Arrays was performed at 45 °C for 16 h in 0.1 M MES buffer, pH 6.6, 1 M sodium chloride (NaCl), 0.02 M EDTA and 0.01% Tween 20 detergent. Four prokaryotic genes (*bioB*, *bioC*, and *bioD* from the *E. coli* biotin synthesis pathway and *cre*, the recombinase gene from P bacteriophage) were added to the hybridization cocktail as internal controls. Arrays were washed using both non-stringent (SSPE/Tween 20 detergents, 25 °C) and stringent (1 M NaCl, 50 °C) conditions prior to staining with phycoerythrin streptavidin (10 μg/ml

final concentration). GeneChip® Arrays were scanned using a Gene Array Scanner (Hewlett Packard, Palo Alto, CA) and analyzed using the Affymetrix Microarray Suite 5.0 software.

2.5. Data analysis

Three independent experiments for each time point were performed. In each, the ethanol- and Dex-treated samples from every cell clone were assessed. The raw data were obtained using Affymetrix software. Details regarding Affymetrix GeneChip® design and the Affymetrix Microarray Suite (MAS) 4.0 and 5.0 algorithms can be obtained from the Affymetrix expression analysis technical manual or their website at http://www.affymetrix.com. Initial data processing was done using the Affymetrix MAS 5.0 that uses statistical algorithms, as opposed to the empirical algorithms in MAS 4.0, to yield a signal intensity value for each of the 12,626 genes on the HG_U95Av2 array. Affymetrix 5.0 Pivotdata text files for control and treated chips were imported into GeneSpring™ (Silicon Genetics, Redwood City, CA), version 5.0.1 and combined into single experiments for each replicate. For each individual experiment, control and treated chip were normalized together using the 50th percentile distribution of all genes. All subsequent analyses were performed with the GeneSpring™ software package.

2.6. Creating significant induced and repressed gene lists

In order to determine the genes regulated by Dex, the signals on individual chips receiving the products derived from Dex-treated cells were filtered to find the 5000–6000 genes called "present". All other genes were dismissed for that particular experiment. All present genes were then compared to their counterparts on the control chips to find those that were induced at least 2.5-fold. To be included on the suppressed list, that particular gene had to be "present" on the control chip and repressed at least two-fold in the Dex-treated samples. These lists were then compared to elucidate those genes induced or suppressed in at least two out of the three experiments. Genes regulated by Dex in each of the three clones were compared with the results expressed by Venn diagrams and lists of specific genes.

2.7. Evaluating variability among 12 and 20 h control samples

Merge replicates into a single experiment

→ Normalize data → Filter out "absent" genes

→ Run Welch t-test (P-value < 0.05)

→ Filter for genes expressed $>$ two-fold

Following the schematic, replicate samples (three controls at each time point) were merged into single experiments. To normalize data, any raw measurements less than zero were set to zero, then replicate chips were normalized to the 50th percentile, and finally, individual genes were normalized to their respective medians. For an individual gene to be considered further in data analysis, it must have been flagged "present" in at least two of the three replicate experiments at a given time point. A Welch t-test was run to search for genes statistically different (P-value < 0.05) between the 12 and 20 h data. Finally, reciprocal lists (12 h $>$ 20 h and 20 h $>$ 12 h) were obtained and pooled together to determine how many total genes varied more than two-fold from one time point compared to the other.

2.8. Basal level expression differences between clones

Each cell line had six control chips analyzed. These replicate chips were merged and normalized as mentioned above to create a baseline value for each individual gene. For an individual gene to be considered further in data analysis, it must have been flagged "present" in at least five of the six replicate experiments. This produced lists varying in length from 4500–4700 genes for each of the three clones. These genes were then compared to a second cell line using a Welch t-test. A P-value of 0.05 was used to screen initially for genes that were graded significantly different in one cell line compared to another (500–1000 genes at this level of stringency). We have shown previously [5], by randomizing such data sets and remaking comparisons, that there is a high probability when such small numbers of replicate experiments are used, of chance occurrences resulting in false "significant" differences between genes showing <2-fold data values. Therefore, the 500–1000 genes identified by the first level stringency filter were compared to find those genes expressed at >2-fold difference between the three pairs of clones (usually less than 200 genes).

3. Results

3.1. Identification of a unique set of genes relevant to glucocorticoid-evoked apoptosis

In a previous report [5], we identified a finite set of genes whose mRNA was induced or repressed above chosen limits after 20 h of Dex treatment. These changes in gene expression were seen in two apoptosis-sensitive clones (C1–6 and C7–14) but not in the apoptosis-resistant sister clone C1–15. The data analysis that identified these genes was based on levels of expression defined by the Affymetrix 4.0 software. It has been necessary to analyze the data from subsequent experiments based on initial chip fluorescence analysis by Affymetrix 5.0 software. Consequently, we have reanalyzed all our experiments with this newer algorithm so as to be able to make further comparisons as new data accrued. The reanalysis has resulted in some regrouping of genes, though the majority of genes originally identified have

Table 1
Glucocorticoid-repressed genes distinctive to CEM cells destined for apoptosis

Affymetrix 5.0			Affymetrix 4.0		
Probe ID	Average fold change in C1–6	Average fold change in C7–14	Probe ID	GenBank accession	Name
1077_at[a]	7.2	4.6	1077_at	M29474	RAG1
37393_at[a]	12.3	8.7	37393_at	L19314	HRY
1973_s_at[b]	2.9	3.7	1973_s_at	V00568	c-*myc*
			37724_at		
40455_at[a]	5.9	2.9	40455_at	AB020637	KIAA0830
41259_at[b]	5.3	3.9	41259_at	Al553745	HSPC111
41415_at[b]	2.9	2.4		L36720	BYSL
36203_at[b]	3.0	2.4		X16277	ODC1
35731_at[b]	2.8	2.2		X16983	ITGA4
33570_at[b]	3.1	2.2		U34962	CSX
			40692_at	M99439	TLE4
			34805_at	AA195301	MGC2574
			34517_at	X66435	HMGCS1
			38277_at	M29550	PPP3CA
			38505_at	AL050151	DKFZp586J0720
			39285_at	AC003038	DNA from cosmid R30923
			32118_at	AF076838	RAD17
			34341_at	U00238	GPAT
			41861_at	AL050019	DKFZp564C186
			2061_at	L12002	ITGA4
			31838_at	U79274	HSU79274
			34998_at	AF059531	PRMT3
			40729_s_at	Y14768	LTB
			40020_at	AB011536	CELSR3
			35246_at	U18934	TYRO3
			40982_at	AA926957	FLJ10534
9	Total		21		

The lists of genes are those identified by GeneSpring™ software analyses of the same raw data, after initial evaluation by Affymetrix version 4.0 or 5.0 (shown in appropriate columns). The average fold change is given for all three experiments using Affymetrix 5.0 data only. Genes that surpass the chosen criteria in three of three experiments for both C1–6 and C7–14 have Probe IDs indicated with 'a' (in superscript), while those with 'b' (in superscript) exceeded the criteria in two of three experiments for either apoptosis-sensitive clone. The absence of a Probe ID in a column indicates that it did not meet our criteria during the analysis.

also been reidentified from the Affymetrix 5.0 database. Previously, a unique set of 39 genes were found to be induced >2.5-fold, compared to their time-matched controls, in the two apoptosis-sensitive clones. Based on initial evaluation of the same data by Affymetrix 5.0, the number of genes in this induced-in-sensitive group declined, as 10 genes no longer met the criteria, three new genes appeared, and one gene (GRAP2) that had not done so now was revealed to be regulated in the apoptosis-resistant clone. With these genes reassigned, a total of 31 genes now were found to be induced >2.5-fold in the sensitive clones exclusively. As to gene repression, our earlier report had identified 21 genes whose mRNA pools were reduced by Dex >2-fold in the two sensitive clones as compared to the resistant clone. After the reanalysis, four new down-regulated genes were identified, but 16 genes identified initially no longer qualified. Thus, the new result gave a total of nine deinduced genes (Table 1) unique to the two apoptosis-sensitive clones.

Our chosen cut-off criteria for inclusion as a relevant regulated gene were that its mRNA level be increased ≥2.5-fold in at least two of the three experiments in each sensitive clone. In fact, 11 of the 31 induced genes met the criteria in all three experiments and three of the repressed genes met the criteria for all experiments. We examined the genes that did not the meet the criteria in one of the three experiments and found in almost all cases, that they were changed relative to controls, only not sufficiently to meet the cut-offs. When all data were averaged, most of the averaged values were ≥2.5-fold compared to the controls (Table 2). Because the low sample numbers made the standard deviations from these means very large in some cases, these are not shown. We have chosen to include these genes as candidates for future analysis, for reasons given in Section 4.

3.2. Comparisons of basal levels of gene expression within each clone at 12 and 20 h

In all experiments, non-steroid, treated time-matched controls are run simultaneously with Dex-treated samples. One question of importance in these studies is whether basal gene expression varies significantly over time in the logarithmically growing population. We evaluated the constancy of

Table 2
Glucocorticoid-induced genes distinctive to CEM cells destined for apoptosis

Affymetrix 5.0			Affymetrix 4.0		
Probe ID	Average fold change in C1–6	Average fold change in C7–14	Probe ID	GenBank accession	Name
37294_at[a]	4.2	7.2	37294_at	X61123	BTG1
41592_at[a]	27.5	11.8	41592_at	AB000734	SOCS-1
36227_at[a]	6.4	8.0	36227_at	AF043129	IL7R
1370_at[a]	4.1	4.2	1370_at	M29696	IL7R
36591_at[b]	3.0	8.9	36591_at	X06956	TUBA1
330_s_at[b]	2.5	6.0	330_s_at	HG2259-HT2348	TUBA1
36231_at[a]	57	6.5	36231_at	AC002073	clone DJ515N1
37544_at[b]	3.3	5.3	37544_at	X64318	E4BP4
41872_at[b]	4.3	4.3	41872_at	AF073308	DFNA5
35985_at[a]	3.6	3.1	35985_at	AB023137	Paralemmin 2
38717_at[b]	6.7	4.0	38717_at	AL050159	DKFZp586A0522
32112_s_at[a]	16.9	3.2	32112_s_at	A1800499	AIM1
32113_at[b]	12.8	3.4	32113_at	U83115	AIM1
32168_s_at[a]	4.6	4.5	32168_s_at	U85267	DSCR1
995_g_at[b]	12.7	3.1	995_g_at	X58288	PTPRM
41524_at[a]	6.4	3.3	41524_at	L08488	INPP1P
656_at[b]	14.5	2.9	656_at	L08488	INPP1P
1102_s_at[b]	2.2	4.2	1102_s_at	M10901	GR alpha
			36690_at	M10901	GR alpha
38378_at[b]	2.9	2.8	38378_at	M37033	MOX44
1427_g_at[b]	2.4	2.3	1427_g_at	D89077	SLAP
1426_at[b]	2.9	2.5		D89077	SLAP
32542_at[a]	18.0	3.1	32542_at	AF063002	SLIM1
735_s_at[a]	3.3	3.5	735_s_at	HG2167-HT2237	PK Ht31
1461_at[b]	2.3	2.5	1461_at	M69043	MAD-3, NFKBl
31508_at[a]	3.8	2.5	31508_at	S73591	VDUP1
32215_i_at[b]	2.1	2.6	32215_i_at	AB020685	K1AA0878
			32216_r_at	AB020685	K1AA0878
31611_s_at[b]	9.2	6.8	31611_s_at	AF032457	BIMEL
35854_at[b]	4.2	5.4	35854_at	L14269	SLC18A2
35164_at[b]	2.3	2.5	35164_at	AF084481	DFNA6
37112_at[b]	2.5	3.2	37112_at	AB002384	DIFF40
32526_at[b]	3.5	2.1	32526_at	AA149644	JAM3
1717_s_at[b]	6.4	4.5	1717_s_at	U45878	BIRC3
36634_at[b]	2.5	2.4	36634_at	U72649	BTG2
706_at[b]	1.7	3.7		HG4582-HT4987	GR beta
40589_at[b]	2.4	3.0		U40572	SNTB2
40046_r_at[b]	3.1	2.2		AF009426	C18orf1
			38799_at	AF068706	AP1G2
			37645_at	Z22576	CD69
			1814_at	D50683	TGFBR2
			1815_g_at	D50683	TGFBR2
			390_at	X85740	CMKBR4
			38671_at	AB014520	KIAA0620
			38661_at	X75315	RNPC1
			35917_at	W26631	MAP1A
			33804_at	U43522	PTK2B
			35763_at	AB011112	KIAA0540
			32227_at	X17042	PRG1
31	Total		38		

The lists of genes are those identified by GeneSpring™ software analyses of the same raw data, after initial evaluation by Affymetrix version 4.0 or 5.0 (shown in appropriate columns). The average fold change is given for all three experiments using Affymetrix 5.0 data only. Genes that surpass the chosen criteria in three of three experiments for both C1–6 and C7–14 have Probe IDs indicated with 'a' (in superscript), while those with 'b' (in superscript) exceeded the criteria in two of three experiments for either apoptosis-sensitive clone. The absence of a Probe ID in a column indicates that it did not meet our criteria during the analysis.

basal gene expression at 12 and 20 h for each clone. Comparison of the results from triplicate experiments at 12 and 20 h showed that, in general, basal gene expression was stable in the ∼4500 genes identified as expressed by the chips employed. The apoptosis-resistant clone, C1–15, showed the greatest variability, with 30 genes (about 0.67%) that varied in expression levels >2-fold (P-value < 0.05) between the 12 and 20 h controls. The two apoptosis-sensitive clones, C1–6 and C7–14, showed less variability, as they only had two genes each that met these criteria. None of these 34 genes varied more than two-fold, comparing the two time point baselines, in more than one clone.

3.3. Sample variability within clones

Taking advantage of this constancy in basal gene expression, data from all replicates at both 12 and 20 h for a given clone were pooled to give a stronger statistical base. The data from each independent cell sample, analyzed on a single chip, was then compared to this pooled data to see how many genes varied (>2-fold) on two or more chips compared to the baselines. Generally, individual samples closely resembled the pooled data. No clone had a gene that was >2-fold variable in four or more of the replicate experiments. The sensitive C7–14 clone was the most stable from sample to sample, with two genes that varied in three independent samples more than two-fold compared to the pooled baseline. For example, the six values for Probe ID *1870_at*, corresponding to a gene encoding a protein–tyrosine phosphatase, were 0.70, 0.95, 1.05, 0.39, 2.38, and 2.34-fold different, compared to the normalized control mean value. C7–14 showed an additional 10 genes that varied >2-fold in two of the six replicates. In the sensitive C1–6 clone, no genes varied >2-fold from the averaged baseline in three or more replicates, and only 18 strayed from the baseline on two chips. Again, the apoptosis-resistant clone C1–15 showed the highest degree of variability, with two genes >2-fold outside the average in three replicates and another 29 in two of the six experiments. These results indicate a low level of variation in basal gene expression due to uncontrolled biological factors or technical inconsistencies. None of the outlier genes found during this process was identified in comparisons of the three different clones as being induced or repressed by Dex.

3.4. Comparison of basal level gene expression in sensitive versus resistant clones

Our initial survey did not reveal any one gene whose differential expression at the basal level was an obvious explanation of the sensitivity of clones C1–6 and C7–14 or the resistance of clone C1–15 [5]. To evaluate the possible influence of basal gene expression further, we have now compared the clones with the pooled 12 and 20 h data. Since C1–6 is hypotetraploid, whereas C1–15 and C7–14 are identically pseudodiploid, we compared basal expression of each individual apoptosis-sensitive clone to the other. Since all data are scaled automatically by the primary software, and comparison of the scaling factors used allows an estimate of the overall level of gene expression. This comparison did not show a universal increase in basal gene expression in C1–6 relative to C7–14. Pairwise comparison between those two clones revealed 126 genes expressed >2-fold higher in C1–6 than in C7–14 and 161 genes expressed >2-fold higher in C7–14 than in C1–6.

The apoptosis-resistant clone C1–15 expressed higher mRNA levels for 33 genes when matched pairwise to the pooled data of each of the two apoptosis-sensitive clones (Table 3). Most of these genes are expressed more highly in the resistant C1–15 clone, as opposed to being expressed at average levels in this clone and under-expressed in the sensitive clones. Several of the genes identified in this comparison have been shown previously to be anti-apoptotic agents in various cell types (Table 3, [8–14]). The two apoptosis-sensitive clones shared a smaller group of 24 genes seen expressed higher than the chosen limit, when compared to the average expression level in the single resistant clone (Table 4). The mRNA for both alpha and beta glucocorticoid receptor isoforms are present in this select list, as is a member of the caspase family. A few of the identifiable genes are known pro-apoptotic agents (Table 4, [15–17]). In contrast to the genes identified as more highly expressed in the resistant clone, for the most part the basal levels of the mRNAs of the genes identified in the sensitive clones are close to the average gene expression levels. Their relatively high levels in the sensitive clone are due to the fact that in most cases the resistant clone under-expresses the mRNA levels for this set of genes, hence by comparison they score >2-fold higher in the sensitive clones. The combined data comparisons revealed what had not been apparent before, that the mRNA levels for GRα and GRβ in C1–15 cells were about half that in C7–14 cells. We confirmed that there was also a similar reduction at the level of steroid binding by the GR (not shown). Each sensitive clone also uniquely expressed a limited number of genes at elevated levels when compared to the pooled, averaged data from the other sensitive clone plus the resistant clone. Thus, with regard to these non-overlapping sets, the apoptosis-sensitive C1–6 clone uniquely expressed higher mRNA levels of 36 genes (>2-fold), while the other sensitive clone C7–14 constitutively expressed more mRNA from 71 genes, including caspase family member caspase-2 (data not shown).

In sum, this analysis of basal gene expression, based on Affymetrix 5.0 software “calls” of gene expression, confirms our previous impression that no single gene stands out in these clones as likely to be causative of sensitivity on one hand or resistance, on the other, to Dex-provoked apoptosis. On the other hand, the somewhat elevated expression of a whole collection of potentially anti-apoptotic genes in the resistant clone, coupled with the under-expression of a group of pro-apoptotic genes in the same clone, may have relevance to the resistant phenotype seen in the cells.

Table 3
Genes expressed basally higher in C1–15 as compared to both apoptosis-sensitive clones C1–6 and C7–14

Probe ID	Name	GenBank	C1–15 vs. C1–6	C1–15 vs. C7–14	Reference
1077_at	RAG1	M29474	3.0	4.0	
1147_at	EAR-3	HG3510-HT3704	8.8	6.2	
1182_at	PLCLI	D42108	14.4	3.8	
2047_s_at	JUP	M23410	3.3	8.7	[8]
2086_s_at	TYRO3	D17517	2.5	2.5	
35246_at	TYRO3	U18934	2.9	2.2	
31874_at	GAS2LI	Y07846	3.1	2.1	
32570_at	HPGD	L76465	83.9	4.1	
37322_s_at	HPGD	X82460	55.0	4.4	
32614_at	SV2B	AB018278	17.3	12.3	
32745_at	NLVCF	AF034091	2.4	3.3	
32747_at	ALDH1	X05409	2.6	6.0	[9]
33442_at	KIAA0367	AB002365	8.2	10.4	
34183_at	DKFZp434C171	AL080169	5.4	4.7	
34319_at	S100P	AA131149	2.5	2.6	[10]
34460_at	PRAX-1	AB014512	3.5	3.9	
35626_at	SGSH	U30894	10.1	2.9	
36192_at	KIAA0193	D83777	21.7	7.1	
36491_at	TMSNB	D82345	2.8	2.1	[11]
36591_at	TUBA1	X06956	2.0	5.6	
36617_at_	ID1	X77956	9.9	3.4	
36618_g_at	ID1	X77956	5.5	2.5	[12]
36816_s_at	CFTR	M28668	8.9	10.3	
36821_at	DKFZp564A026	AL050367	5.3	10.9	
38037_at	DTR	M60278	9.1	7.6	
38317_at	TCEAL1	M99701	5.1	5.3	
38526_at	PDE4D	U02882	4.6	5.8	
38750_at	NOTCH3	U97669	5.9	5.5	[13]
38824_at	HTATIP2	AF039103	12.7	10.0	
40201_at	DDC	M761802	6.3	14.2	
40297_at	STEAP	AC005053	9.0	7.7	
40512_at	CHN1	X51408	3.3	3.0	
40699_at	CD8A	M12824	3.5	2.8	
41191_at	KIAA0992	ABO23209	2.8	3.6	
41503_at	KIAA0854	AB020661	3.2	3.1	
576_at	NOS3	M93718	2.7	3.7	[14]
33	Total				

3.5. Comparison of regulated genes that correlate with glucocorticoid-evoked apoptosis after 12 and 20 h exposure to Dex

Under our general hypothesis, the culminating set of genes whose altered expression evokes apoptosis may not be entirely the same as those expressed at earlier times. We propose that an interactive gene expression network builds the circumstances that set off full apoptosis. To begin to examine this possibility, we examined the expression data after 12 h of exposure to Dex. For comparative purposes we kept the same criteria as used for the 20 h set. Briefly, to be included, an induced or deinduced gene had to meet the cut-off criteria in at least two out of the three data sets and not appear as regulated in the resistant clone more than once. Thus, to be included, a gene had to be "called" present by the Affymetrix 5.0 software in the Dex-treated sample and to show a fold change increase >2.5 in at least two out of the three experiments. For a gene to be included on a repressed gene list, it had to be called present in the non-steroid treated control and to decrease >2-fold in at least two out of the three experiments. With these restrictions, small lists of genes were found to be regulated uniquely after 12 h of Dex in the two apoptosis-sensitive clones (Table 5), while a different, even smaller list of genes was elucidated for the apoptosis-resistant clone C1–15. Fifteen genes were identified as being regulated when meeting our criteria. All but one (RTP801) were found induced beyond the chosen limits at both 12 and 20 h. In the C1–6 cells, RTP801 was induced >2.5-fold at 12 h in each of the three experiments; however though it showed increase in all three at the 20 h time point, it exceeded the 2.5-fold limit only once (average of three experiments, 2.8-fold). Therefore, this gene is not presented in Table 2. So, because of its induction at 12 h, we include it in Table 5(A).

The number of genes repressed after 12 h exposure to Dex also was less than that after 20 h. Table 5(B) shows the three genes that were repressed more than two-fold

Table 4
Genes expressed basally higher in C1–6 and C7–14 (>2-fold) as compared to C1–15

Probe ID	Name	GenBank	Average fold in C1–6[a]	Average fold in C7–14[a]	Reference
1102_s_at	GR alpha	M10901	3.6	2.0	
1445_at	CCRL2	AF014958	3.1	5.4	
1741_s_at	IGFBP2	S37730	3.0	10.0	
40422_at	IGFBP2	X16302	2.3	5.9	
195_s_at	CASP4	U28014	3.2	2.2	[15]
32588_s_at	ERF2	X78992	3.6	4.3	
32636_f_at	SMG1	AB007881	2.8	3.0	
33273_f_at	IGL	X57809	2.3	2.7	
33646_g_at	GM2A	X61094	2.5	3.2	
33647_s_at	GM2A	AA224768	2.1	2.5	[16]
35820_at	GM2A	X62078	2.2	3.6	
34818_at	ETV5	X96381	4.5	2.5	
35985_at	AKAP2	AB023137	2.5	2.6	
36480_at	PHKA2	X80497	2.1	2.2	
36873_at	VLDLR	D16532	4.2	3.6	
37265_at	KIAA0237	D87074	3.0	2.2	
37398_at	PECAM1	AA100961	3.2	4.6	
37641_at	IFI44	D28915	3.4	2.0	
38017_at	CD79A	U05259	2.1	3.1	
38091_at	LGALS9	Z49107	2.8	2.4	[17]
766_at	LGALS9	AB006782	3.1	2.5	
38287_at	PSMB9	AA808961	3.9	4.4	
38514_at	IGLL1	M27749	4.4	5.1	
40522_at	GLUL	X59834	3.7	37.0	
41184_s_at	PSMB8	X87344	2.5	2.5	
41356_at	BCL11A	W27619	5.2	2.8	
41827_f_at	unknown	Al932613	6.0	7.0	
706_at	GR beta	HG4582-HT4987	3.2	2.1	
24	Total				

[a] Basal level fold difference as compared to C1–15 basal expression.

Table 5
Glucocorticoid-regulated genes distinctive to CEM cells destined for apoptosis

Probe ID	Common name	C7–14		C1–6	
		Average fold change at 12 h	Average fold change at 20 h	Average fold change at 12 h	Average fold change at 20 h
(A) Induced genes					
1717_s_at	BIRC3	2.8	4.5	4.7	6.4
31508_at	VDUP1	3.0	2.5	2.7	3.8
32168_s_at	DSCR1	4.7	4.5	7.9	4.6
32542_at	SLIM1	2.8	3.1	7.6	18.0
36227_at	IL7R	3.3	8.0	5.5	6.4
36231_at	clone DJ515N1	3.5	6.5	3.0	5.7
330_s_at	TUBA1	4.5	7.5	3.9	2.8
37294_at	BTG1	8.7	7.2	5.2	4.2
37544_at	E4BP4	4.4	5.3	3.7	3.3
37645_at	CD69	3.9	5.3	1.8	2.3
38717_at	DKFZp586A0522	2.8	4.0	6.7	6.7
39827_at	RTP801	3.8	3.7	4.5	2.8
41592_at	SOCS-1	9.6	11.8	28.0	27.5
41872_at	DFNA5	3.6	4.3	3.0	4.3
706_at	GR beta	4.2	3.7	3.5	1.7
(B) Repressed genes					
1077_at	RAG1	2.2	4.6	1.7	7.2
1973_s_at	MYC	2.5	3.7	2.0	2.9
37393_at	HRY	3.2	8.7	3.0	12.3

The lists of genes are those uniquely regulated by the two apoptosis-sensitive clones C7–14 and C1–6 after both 12 and 20 h exposure to Dex. The average fold change is given for all three experiments at each time point as compared to the control averages. In a few cases, one outlier value caused the average to fall below the chosen limits.

in both apoptosis-sensitive clones and not in the resistant clone. The resistant clone uniquely induced one gene (zinc finger protein 202) after 12 h exposure to Dex and repressed an additional five genes (CD43, WWP1, PTEN, NR1D2, and CAML). One gene, DSIPI also known as GILZ (glucocorticoid-induced leucine zipper), was greatly induced (>10-fold) in all three clones at both time points. No repressed genes were seen in common to the resistant and sensitive clones.

4. Discussion

The new technology of gene expression analysis by microchip allows a much wider and less biased inspection of complex cellular control processes then heretofore possible. Results from microchip gene arrays are not without their own bias, of course, but they allow more complete data than earlier methods, such as mRNA subtraction techniques. Some preliminary gene array approaches have been used to study steroid effects on lymphoid cells already [18–26]. We have employed microchip analysis of gene expression in a nested set of three cell clones, to obtain fresh insights on the long-standing problem of how glucocorticoids kill lymphoid leukemic cells. The results are promising and allow several general and specific conclusions. These in turn give the basis for constructive new experiments.

Our approach is to study intensively a system of closely related clones of CEM cells, as a model for glucocorticoid-induced leukemic lymphoid cell apoptosis. By doing so, we intend to provide a database against which other lymphoid leukemic cells and perhaps even normal lymphoid cells can be compared. The first three clones to be studied are C7–14 and C1–6 (both of which undergo apoptosis when exposed to glucocorticoids) and C1–15, which is totally resistant. C1–6 is a spontaneous revertant to sensitivity from resistant clone C1, whereas C1–15 is a sister subclone of C1 that retained resistance. All three clones express the GR. When the subclones were first isolated they had equal numbers of binding sites in repeated assays, averaging 10,000 in both clones [27]. In fact, C1–15 had been cloned out of the C1 population expressly to re-establish the original C1 phenotype with GR levels equal to those in C7 [28], since over a period of years GR levels in C1 had fallen [27]. Now it appears that they have done so also in subclone C1–15. The GR of C1–15 cells does seem sufficient to cause regulation of certain genes, however [5]. The reason for the reduction in GR in C1 and C1–15 cells over long periods of time will bear further investigation. It was noted that transfection of C1 cells with the GR gene, to restore higher GR levels, had in several clones restored apoptotic sensitivity to glucocorticoid [29]. On the other hand, treatment of C1 cells with forskolin to activate the protein kinase A pathway also rendered the cells apoptotically sensitive to glucocorticoid, as it did in C1–15 cells [27]. The two results are not necessarily contradictory, since it is possible that greater protein kinase A activity could enhance the activity of an otherwise limiting quantity of GR.

Fundamental to our clonal cell system is the fact that in the "sensitive" clones, agonist ligands (such as Dex) for the GR must be present for a considerable time before irreversible apoptotic events occur. This is true of many, if not all, malignant lymphoid cell systems. The standard timing of events in sensitive CEM clones in log growth is that though Dex is added, the cells continue to grow logarithmically for about 24 h and only then do they begin to show irreversible effects. Over the ensuing 72 h, in a stochastic fashion, increasing numbers of cells become irreversibly committed to apoptosis. Eventually, all but a very small, newly selected resistant subpopulation die. We hypothesize that during the prodromal, reversible phase, a network of genes change expression as a direct or indirect effect of the steroid on its receptor. Obviously, the initial direct effects of Dex could alter expression of genes that encode regulatory factors. Changes in these could then affect other genes, and so on. To what extent this is so is unknown, although we have shown in this system that the acute transcriptional down-regulation of c-*myc* leads to reduction of ornithine decarboxylase (ODC) expression [30]. The ODC promoter is know to contain a c-*myc* binding site and to depend strongly on c-*myc* for transcription. Regardless of whether its effects are direct or indirect, Dex must continue to be present during the prodromal period in order for the apoptotic response to occur. This suggests that if secondary effects occur, they depend on changes in rapidly turning over pools of factors whose altered expression is Dex-dependent. Ultimately, as a result of the cumulative changes, conditions are reached which provoke the full apoptotic machinery. A corollary of this hypothesis is that the necessary changes in gene expression will be seen in the sensitive clones but not the resistant clone. (An interesting side issue is whether gene expression in the resistant clone responds at all to the steroid.)

We have begun to test the hypothesis by examining the gene expression changes in the three clones at relatively long times after addition of Dex but before irreversible apoptosis starts. Preliminary reports of gene changes in other clones of CEM cells, after short exposures to Dex have been presented [25,26]. Our first set of data was taken after 20 h Dex exposure. This gave results consistent with our hypothesis. A set of genes was identified that was altered in expression only in the two sensitive clones [5]. We have now added to this data from cells exposed to the steroid for 12 h. Again, the two sensitive clones showed a set of genes whose regulation differed from that of the resistant clone. Most of these had also been identified in the 20 h data set. In the genes whose products increased, only one newly appeared at 12 h, and reanalysis of the 20 h data for that gene suggest that it may also be induced at both times. Among the repressed genes, however, many more were found at 20 h than at 12 h. Thus, the picture begins to emerge that fewer genes are altered in expression—at least to as great an extent—after 12 than after 20 h in Dex. The genes induced after 12 h continue to

be induced at the later time and are joined by additional induced genes. The same general trends are true for repressed genes, though the proportion of repressed genes increases more greatly by 20 h.

The resistant clone C1–15 appears to respond to steroids in a similar pattern, but with different genes affected. As yet no single Dex-dependent gene alteration provides an explanation for the resistance of C1–15 cells to steroid-evoked apoptosis.

Examination of basal levels of gene expression in each of the three clones showed that the great majority of the ∼4500 genes detected on the microchips employed remained constant in expression at 12 and 20 h. This is reassuring, since we take pains to keep the cells in log growth and constant conditions. We did not find a strong gene–dose effect on overall basal levels of gene expression when we compared the hypotetraploid, sensitive clone C1–6 with the nearly diploid clone C7–14. When apoptosis-sensitive/resistant clones were compared, finite numbers of genes grouped to the two categories were shown to be differentially expressed. For the most part, further experiments will be required to see whether these differences in basal gene expression fully or partially account for the sensitive and resistant phenotypes.

The data analysis we present underscores the dependence of results obtained by such methods on the microchips employed, on technology for resolving primary data from the chips, and on the software used to analyze that data. As we have continued to study the system, the Affymetrix software for analyzing the basic fluorescence data was "upgraded" from version 4.0 to 5.0. The newer version assigns a *P*-value to the values from each gene site on the chip. Since the new data required use of version 5.0, we reran our earlier data, based on version 4.0, on the new system, in order to allow 12 and 20 h data comparisons. All subsequent comparative analyses were carried out by a single software system. The effect was to alter somewhat the composition of the gene sets identified. Though most 20 h genes initially identified were reidentified, others dropped out. This does not necessarily mean that they were incorrectly identified as regulated in the first analysis. The result points to the need for caution in accepting as absolute the data output from any given analysis.

Our choice of criteria, allowing each "accepted" gene to fall below certain cut-offs fold for change in one of the three experiments, was based on the argument that in such small data sets, one error might occur. The high cost of these microchips make larger *n*s impractible. One might argue that if the gene truly was important for apoptosis, it should respond in every experiment. However, it should be remembered that our limits were chosen to improve our chances of identifying genes that clearly were affected by the treatment, apart from random or ambiguous changes. This does not mean that the genes identified are all those whose regulation has importance. When we calculated averages for all three experiments for the "two out of three" genes, as might be done in a standard set of biochemical assays, in most cases, the average for each regulated gene met our criteria for fold change, adding strength to the argument that they were legitimate members of the sets. Such genes obviously will require further analysis before the consequence of their regulation becomes clear. Also, other genes of great importance may never be altered >2.5-fold up or >2-fold down. Only when sufficient replicate experiments have been done will it be possible to identify these with a high degree of certainty.

Several conclusions may be drawn from the results with this system to date.

- The data support our hypothesis that the sensitive clones should show distinctive sets of Dex-regulated genes. This is definitely not to say the genes identified so far are all of those important in the process. The chips employed only represent ∼12,600 genes; so using our present criteria, and extrapolating to the estimated 30–40,000 genes believed to be expressed in human cells, one would estimate roughly three times as many genes would be found to change. But smaller or larger gene sets can be identified by employing different criteria. Only more experiments and further analysis will identify the full set. This truth does not, however, invalidate the basic conclusions that a distinctive group of genes is regulated in both sensitive clones and that those identified thus far are likely to be relevant.
- The resistant clone shows gene regulation by Dex, but of a different gene set than seen in the sensitive clones.
- There are a few genes regulated common to both sensitive and resistant cells.
- The regulation results in both increased and decreased levels of the mRNAs of particular genes.
- Smaller numbers of genes appear to be regulated (to have changed as greatly) at 12 h into the prodromal period than at 20 h. Those that are regulated at 12 h continue to be regulated in the same direction (increased or decreased) after 20 h. These results suggest an amplifying "cascade" of events.
- Among the upregulated genes in the sensitive clones are several that in various systems have been shown to be pro-apoptotic or growth inhibitory-apoptotic and a few the literature describes as anti-apoptotic.
- The "core" group of three down-regulated genes (c-*myc*, RAG1 and HRY) identified at both 12 and 20 h all have been associated with growth promotion and anti-apoptotic activities.
- Changing ratios of GRα and GRβ do not account for sensitivity versus resistance.
- Basal gene expression is generally rather constant in the three clones while in log growth. Resistant clone C1–15 tends to show more variability, as it does in its regulated genes.
- Among basally expressed genes, the resistant clone expresses higher levels of a set of genes among which are found a number associated with resistance to apoptosis in

some systems. Conversely, the sensitive cells tend to underexpress a set of genes among which are found several thought to be anti-apoptotic. Therefore, in comparison these show up as relatively overexpressed in the resistant clone. The sensitive clones also basally express relatively higher levels of a few pro-apoptotic genes. (However in log growth, cell viability in all three clones is ≫95%.)

In conclusion, this system is providing a strong basis for analysis of the genes involved in glucocorticoid-evoked apoptosis of lymphoid leukemic cells. The data allow for comparisons with other related hematopoietic malignancies, and may have pertenance to normal lymphoid cells as well. Single genes and groups of genes are being identified that bear closer scrutiny for their roles in the process.

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