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Phenobarbital responsiveness as a uniquely sensitive indicator of hepatocyte differentiation status: requirement of dexamethasone and extracellular matrix in establishing the functional integrity of cultured primary rat hepatocytes

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Abstract

We used a serum-free, highly defined primary hepatocyte culture model to investigate the mechanisms whereby dexamethasone (Dex) and extracellular matrix (ECM) coordinate cell differentiation and transcriptional responsiveness to the inducer, phenobarbital (PB). Low nanomolar levels of Dex and dilute concentrations of ECM overlay were essential in the maintenance of normal hepatocyte physiology, as assessed by cell morphology, LDH release, expression of the hepatic nuclear factors C/EBPα, -β, -γ, HNF-1α, -1β, -4α, and RXRα, expression of prototypical hepatic marker genes, including albumin and transferrin, and ultimately, cellular capacity to respond to PB. The loss of hepatocyte integrity produced by deficiency of these components correlated with the activation of several stress signaling pathways including the MAPK, SAPK/JNK, and c-Jun signaling pathways, with resulting nuclear recruitment of the activated protein-1 (AP-1) complex. In Dex-deficient cultures, normal cellular function, including the PB induction response, was largely restored in a dose-dependent manner by reintroduction of nanomolar additions of the hormone, in the presence of ECM. Our results demonstrate critical and cooperative roles for Dex and ECM in establishing hepatocyte integrity and in the coordination of an array of liver-specific functions. These studies further establish the PB gene induction response as an exceptionally sensitive indicator of hepatocyte differentiation status.

Keywords: Dexamethasone; Extracellular matrix; Primary hepatocytes; Phenobarbital; Cell culture

Introduction

The balance between liver differentiation and growth is determined by the coordinated interaction of a myriad of extracellular signals in the microenvironment of the liver cell [1,2]. This interplay, including cell–extracellular matrix (ECM) [3] and cell–cell interactions [4], function in the context of a diversity of growth factors and other hormonal stimuli to determine the course of hepatocyte proliferation, differentiation, and survival [5]. Although the molecular mechanisms integrating these diverse signals have not been entirely elucidated, cell–ECM interactions likely function to enhance the fidelity of receptor-activated signal transduction pathways associated with growth factors [6].

Glucocorticoids (GCs) play pivotal roles in determining the functional integrity of a number of cell types. The associated biological effects are mediated by the intracellular glucocorticoid receptor (GR), which upon ligand activation and nuclear translocation, regulates a diverse collection of genes through both transcriptional and post-transcriptional mechanisms [7–9]. GR regulatory mechanisms include direct cross-talk between the GR and other transcription factors, notably the activated protein-1 (AP-1) and NF-κB complexes [10,11]. The ability of GCs to transrepress both of these factors is likely of fundamental physiological significance, resulting, for example, in their profound anti-inflammatory and antineoplastic properties [12,13]. Growth factor and cytokine-mediated stimulation of AP-1 activity may proceed

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through activation of several members of the mitogen-activated protein kinase family, including extracellular-regulated kinase (Erk1/2) MAPK, p38 MAPK, and c-Jun NH_2-terminal kinase (JNK) [14,15]. Other steroids and retinoids also mitigate cellular stress as evidenced by their pharmacological actions as anti-inflammatory, immunosuppressive, and anti-neoplastic agents [16,17]. Recent evidence demonstrates that GCs inhibit apoptotic events in a number of cell types through induction of specific survival pathways, including the activation of serine/threonine survival kinase (*sgk*-1) [16,17].

Induction of pharmaceutical and carcinogen-metabolizing enzymes, such as the cytochrome *P*450s, is a hallmark feature of cellular defense, with broad toxicological significance [18,19]. Phenobarbital (PB) is a prototypical hepatic enzyme inducer, exhibiting pleiotropic effects on liver physiology, including hypertrophy and altered cell–cell communication [20]. The PB gene induction response is liver-selective and appears uniquely sensitive to the cellular microenvironment. The molecular mechanisms of the response have been characterized in part, with key features including the nuclear translocation of the constitutive androstane receptor (CAR) [21], its subsequent partnering with RXRα and interaction of the nuclear receptor dimer complex with a PB response-enhancer module (PBREM), a DNA enhancer element present upstream of PB inducible genes [22,23].

Previous studies have demonstrated a critical requirement for both ECM and nanomolar concentrations of Dex in directing the robustness of in vivo-like xenobiotic responsiveness in primary rodent [24–26] and human hepatocytes [27]. Although it has been reported that Dex exposures themselves enhance CAR expression levels [27], an alternative hypothesis for the potentiative effects of Dex on the PB induction process is that Dex impinges on a cluster of additional signaling pathways that act in concert with CAR to direct the overall expression of the hepatic phenotype.

In the present investigation, we used a well-defined and serum-free primary rat hepatocyte culture system [24,28] to investigate the interplay between Dex and ECM as modulators of cell survival, cellular differentiation, and PB responsiveness. The influence of ECM and Dex on the hepatic phenotype was profound. Our results demonstrate critical and cooperative roles for Dex and ECM in the promotion of hepatocyte integrity, in the coordination of an array of liver-specific functions, and establish the PB gene induction response as an exceptionally sensitive indicator of hepatocyte differentiation status.

Materials and methods

Cell culture materials and chemicals

All cell culture media and Trizol™ were purchased from Life Technologies (Grand Island, NY). Matrigel™, ITS+ [insulin, transferrin, selenium, bovine serum albumin [BSA] and linoleic acid], and Nu-Serum™ were obtained from Becton Dickinson (Bedford, MA). Transferrin, selenium, and BSA–linoleic acid were obtained from Sigma (St. Louis, MO). Collagenase (Type 1) was purchased from Worthington Biochemical Corporation (Lakewood, NJ).

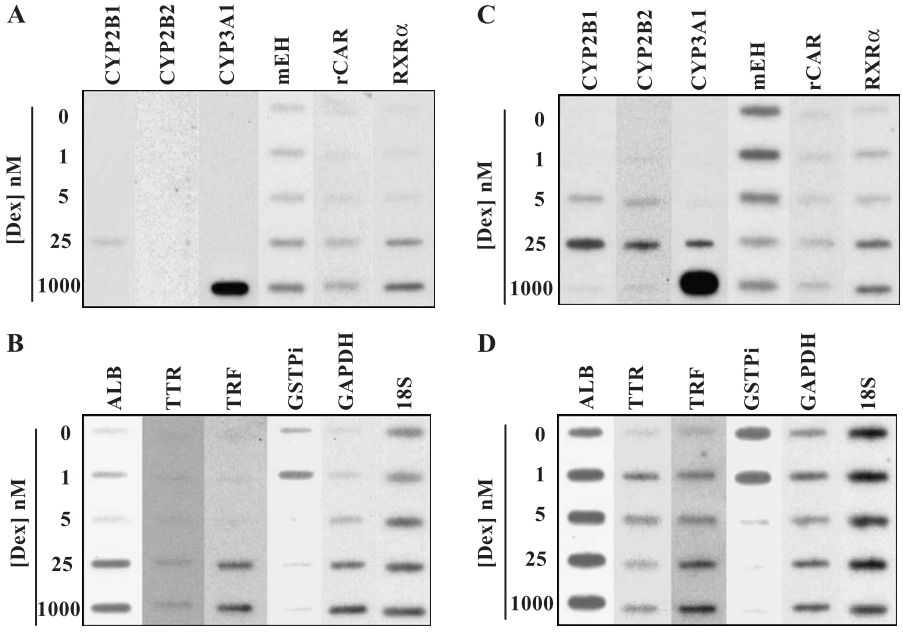

Fig. 1. Effect of ECM overlay and Dex concentration on PB-inducible and liver-specific gene expression. Primary rat hepatocytes were cultured for a total of 96 h under the stated Dex concentrations (nM) and in the absence (Panels A, B) or presence (Panels C, D) of an ECM overlay. In the PB-treated dishes, PB (500 μM) was added after 72 h in culture and maintained for an additional 24 h. Total RNA was isolated and evaluated by slot-blot analysis for the expression levels of PB-inducible and liver-specific genes as described under Materials and methods. Ribosomal 18S and GAPDH RNA hybridization levels were used as normalization standards.

Tissue culture-treated plastic (100 mm) and collagen-coated Biocoat dishes were obtained from Becton Dickinson (Franklin Lakes, NJ). Dexamethasone (Dex; 9αfluoro-16αmethyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione), and bovine insulin were obtained from Sigma. The lactate dehydrogenase (LDH)-based cell cytotoxicity kit was obtained from Promega (Madison, WI).

Isolation and culture of hepatocytes

Rat hepatocytes were isolated from adult Sprague–Dawley male rats (Simenson Labs, Gilroy, CA) by a two-step collagenase perfusion in situ and cultured with modification [25,28] of a protocol described previously [24]. Briefly, cells were plated on tissue culture-treated plastic dishes in William's E medium in the presence of 10% Nu-Serum™ (no serum was added to cells cultured on collagen-coated dishes) and 100 nM dexamethasone (Dex). Cells were allowed to attach for 3 h before switching to a serum-free medium (William's E, 10 nM insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenium, 1.25 mg/ml BSA, 5.35 μg/ml linoleic acid, and variable concentrations of Dex-1, 5, 25, or 1000 nM). Subsequently, cells were cultured in the latter formulation with daily medium changes. In other experiments, cells were cultured in the serum-free formulation with Dex additions occurring at varying time points (4, 24, 48, or 72 h after plating).

Matrigel™ overlay

A dilute concentration (233 μg/ml, final concentration) of ECM (Matrigel™) was added to respective plates as an overlay [24] following the first medium change, 4 h after plating.

Phenobarbital treatments

Typically, cells were cultured for 72 h before the addition of PB (500 μM). Cells were then maintained in PB for 24 h at which point cells were harvested for endpoint analyses. Representative data are shown from multiple experiments performed independently with several different hepatocyte preparations.

RNA analysis

Total RNA was isolated using Trizol™ as described previously [28] and analyzed by slot-blot hybridization [25]. The antisense exon 3 DNA oligonucleotide probe, MR/CAR-rp [5′-GGCCTTGCTGACCTCACA-3′], was used as a hybridization probe for rat CAR RNAs.

cDNA hybridizations

A cDNA probe specific for rat RXRα was an *Eco*RI–*Xho*I fragment of a rat RXRα cDNA obtained from a clone derived from a Sprague–Dawley rat liver Matchmaker cDNA library (Clontech, Palo Alto, CA). cDNA probes specific for rat GST-Pi and mEH were used as previously reported [25,29], while a cDNA probe for rat GAPDH was obtained from Sigma. PCR products were radiolabeled using the DECAprime™ DNA labeling kit (>3000 Ci $mmol^{-1}$, New England Nuclear/Dupont, Boston, MA). Hybridization was performed essentially as described [25], except conducted at 60 °C in the absence of formamide.

Determination of LDH release

Levels of LDH were determined in triplicate in the extracellular medium, spectrophotometrically, as per manufacturer's instructions (Promega), as reported [30].

Analysis of PKB, SAPK/JNK, p42/44 MAPK, and c-Jun phosphorylation status

Primary hepatocytes were cultured in the presence or absence of varying concentrations of Dex and ECM overlay for 96 h before cell harvest and analysis of extracts by SDS-PAGE [31]. Following manufacturer's blotting instructions,

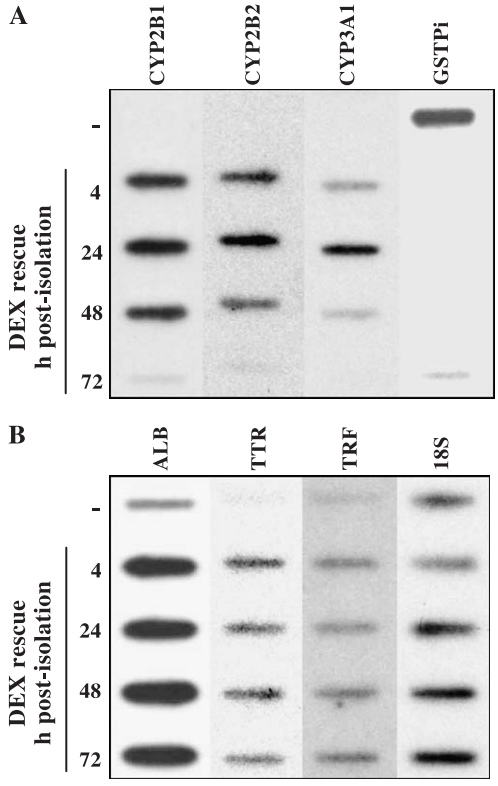

Fig. 2. Time course of Dex rescue on the expression levels of PB-inducible and liver-specific gene expression in primary rat hepatocytes. Primary rat hepatocytes were cultured for a total of 96 h in the presence of an ECM overlay. At the indicated time points (4, 24, 48, or 72 h post-plating), Dex (25 nM) was added to the medium and the cells were continued in culture until the 96 h time point. The " – " designation indicates that no Dex was added to the cultures. Panel A: PB-induction responses; Panel B: Live-specific marker genes.

the membranes were probed with phosphospecific antibodies that recognize phosphorylated PKB (Akt), SAPK/JNK and p42/44 MAPK, and c-Jun, respectively (New England Biolabs, Beverly, MA). The membranes were then incubated with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:2000 dilution). Treatments were normalized in parallel by assessment of protein loading using phosphorylation-independent antibodies against Akt (α-Akt), SAPK/JNK, and p42/44 MAPK. The ECL chemiluminescence system (Amersham, Piscataway, NJ) was used to visualize specific immunoreactive proteins.

Electromobility shift assays

Electromobility shift assays (EMSA) were conducted essentially as previously reported [30], using nuclear extracts isolated from hepatocytes treated with the various concentrations of Dex and in the presence or absence of ECM overlay. Double-stranded consensus oligonucleotides for activated protein-1 (AP-1) (5′-CGCTTGATGACTCAGCCGGAA-3′, Santa Cruz, Santa Cruz, CA), C/EBPα (5′-TGCAGATTGCGCAATCTGCA-3′, Santa Cruz), HNF-1α (5′-CCAGTTAATGATTAACCACTGGC-3′, Geneka Biotechnology, Montreal, Quebec, Canada), HNF-3α (5′-GCCCATTGTTTGTTTTAAGCC-3′, Geneka), HNF-4 (5′-GGAAAGGTCCAAAGGGCGCCTTG-3′, Geneka), and RXRα (5′-AGCTTCAGGTCAGAGGTCAGAGAGCT-3′, Santa Cruz) were end-labeled with γ^{32}P ATP using T4 polynucleotide kinase (Amersham). Wild type and mutant oligonucleotides were used in 80-fold excess to the radiolabeled probe in competition experiments. Hepatocyte nuclear protein was isolated using the NuPer kit (Pierce,

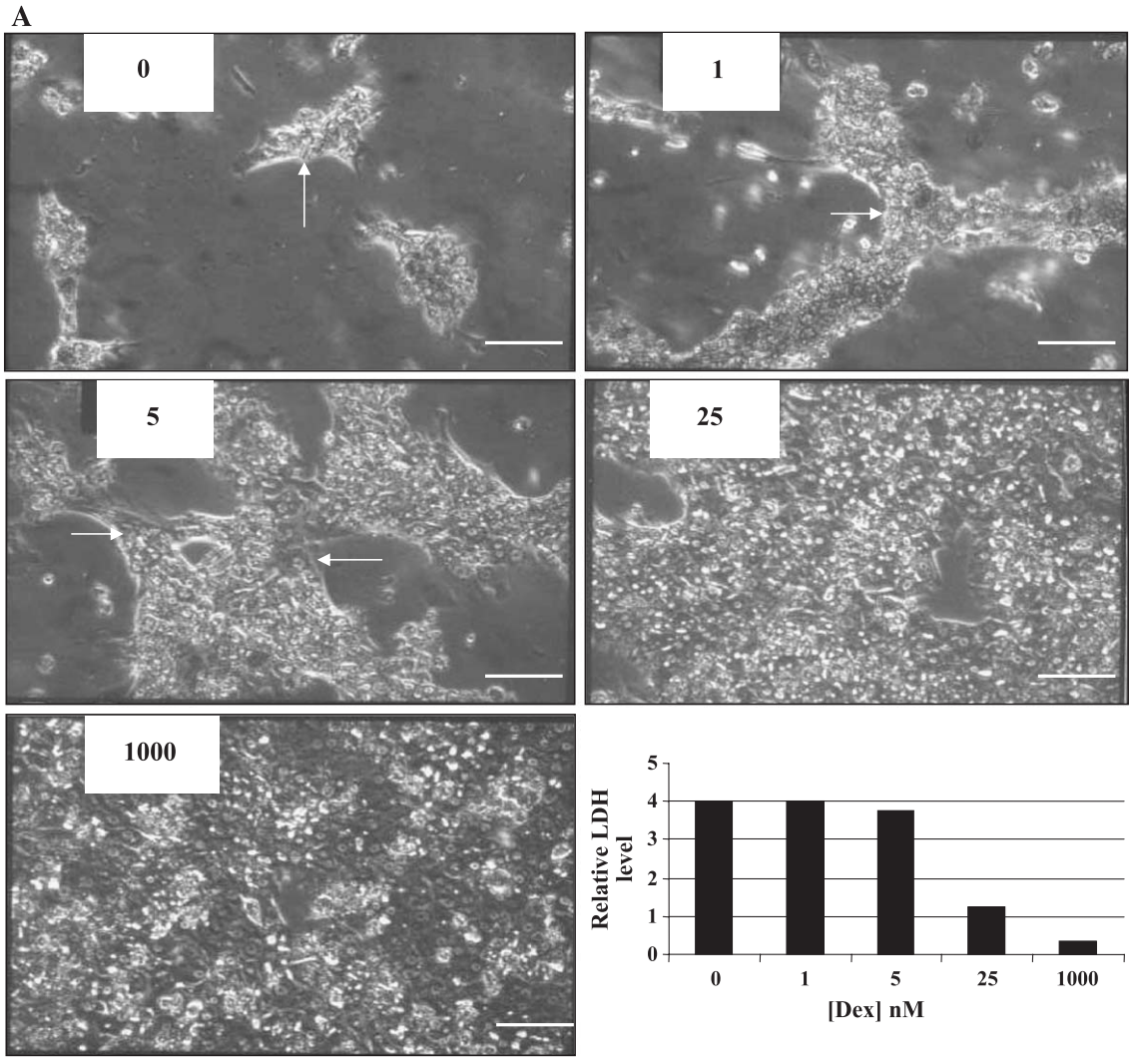

Fig. 3. Effect of ECM overlay and dexamethasone concentration dependency on hepatocyte morphology and viability. Primary rat hepatocytes were cultured for a total of 96 h under the stated dexamethasone concentrations (nM), in the absence (Panel A) or presence (Panel B) of an ECM overlay. The effect of both agents on the representative morphology (20× magnification) of primary rat hepatocytes is shown. Arrows identify evidence of perturbed morphology: condensed cytoplasm and rounded-up cells, attributed to cytotoxicity. The lower right panel shows the relative level of LDH leakage associated with each Dex concentration. Scale bar = 50 μm.

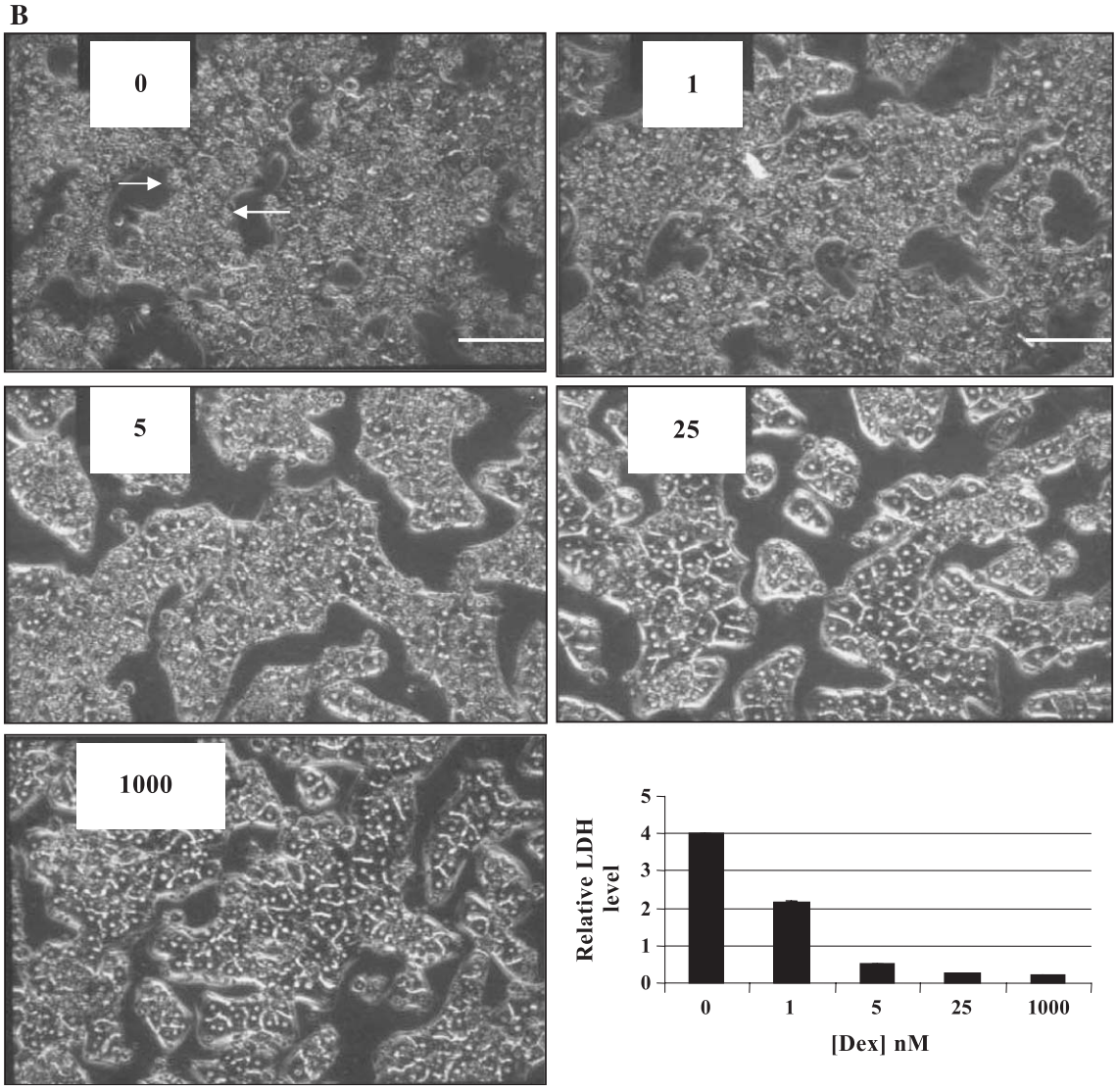

Fig. 3 (*continued*).

Rockford, IL). Five to ten micrograms of nuclear protein was incubated in binding buffer (50 mM Tris–Cl, pH 7.5, 2.5 mM DTT, 2.5 mM EDTA, 250 mM NaCl, 5 mM $MgCl_2$, and 20% glycerol) for 15 min at RT. The labeled probe (0.5 ng) (approximately 100,000–200,000 cpm in a 20-μl reaction volume) was added and incubated for an additional 15 min at RT. From this reaction mixture, nuclear protein complexes were resolved on 5% TBE gels (BioRad, Hercules, CA), dried, and subjected to autoradiography. Protein concentration was determined with bovine serum albumin as standard using a commercial kit (BCA protein assay reagent, Pierce).

In separate analyses, the expression pattern of the respective transcription factors was determined by SDS-PAGE in the same nuclear extracts as used for EMSA analysis. The primary antibodies for each family member were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Results

Previous studies have indicated that Dex was an important modulator of xenobiotic responsiveness in cultured primary hepatocytes [25]. To better determine the mechanistic basis of this response, we performed a series of experiments manipulating Dex concentrations and ECM exposures in a highly defined hepatocyte culture system and evaluated the biological impact of these manipulations on selected cellular endpoints.

The dose dependency of Dex on PB-inducible and liver-specific gene responses

Typically, cells were cultured for 72 h before challenge with PB. During the initial 72-h culture period, cells were maintained in the presence or absence of ECM overlay, and

under varying concentrations of Dex. To examine gene induction endpoints, cells were then treated with 500 μM PB and continued in the presence of the inducer for an additional 24 h. Total RNA was then isolated and subjected to expression profiling of various PB-inducible genes and other liver-selective markers (Fig. 1A). Levels of 18S and GAPDH RNA were assessed as standard comparators. In the absence of ECM overlay, PB treatment resulted in a modest induction of CYP2B1, but only in the presence of 25 nM Dex. No induction of either CYP2B2 or 3A1 mRNAs was evident under the latter conditions, although CYP3A1 induction was evident with 1000 nM Dex. The expression level of mEH was not altered appreciably by Dex, although PB appeared to modestly induce mEH in the presence of higher Dex concentrations. In contrast, the levels of both CAR and RXRα mRNAs increased in a dose-dependent manner with Dex addition.

The mRNA levels of the liver-specific markers, albumin, transthyretin, and transferrin were affected markedly by Dex additions (Fig. 1B). The expression level of GST-Pi, a gene typically quiescent in differentiated adult hepatocytes, was clearly elevated in the presence of ≤ 1 nM Dex. Elevating the Dex concentration resulted in suppression of the aberrant GST-Pi response. The mRNA level of the housekeeping gene, GAPDH, was also reduced in the absence of ECM overlay, and with limiting Dex conditions.

Interdependency of ECM overlay and Dex on hepatic gene expression

We further examined the coordination between ECM and Dex on cellular responses in cultured hepatocytes. Even low concentrations (e.g., 5 nM) of Dex, in the presence of an ECM overlay, markedly intensified the PB-induction response for CYP2B1, 2B2, and 3A1 (Fig. 1C). Optimal PB induction of CYP2B and 3A1 was obtained in the presence of 25 nM Dex plus ECM overlay. The highest concentration of Dex tested (1000 nM) in-

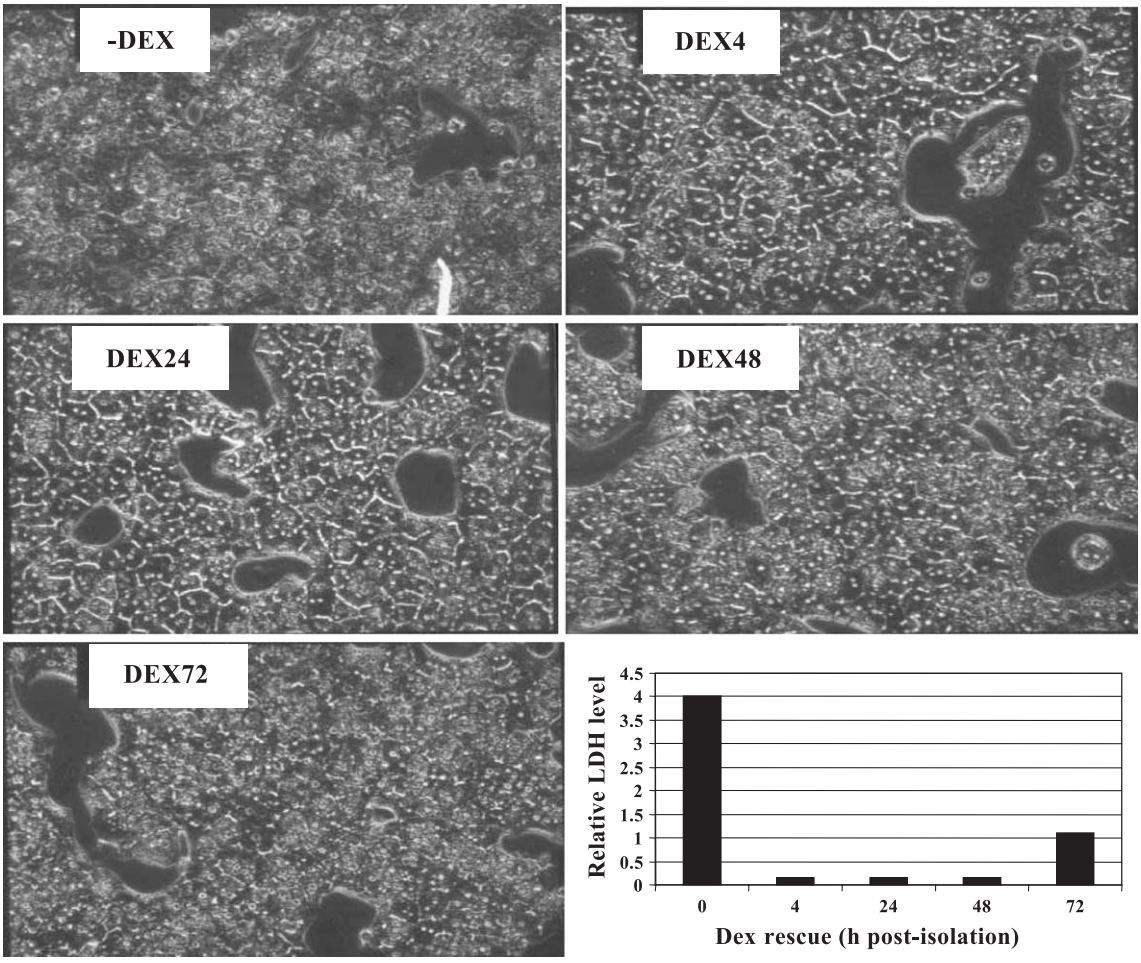

Fig. 4. Effect of time course of Dex rescue on hepatocyte morphology and viability. Primary rat hepatocytes were cultured in total for 96 h in the presence of an ECM overlay. At the indicated time points (4, 24, 48, or 72 h post-plating), Dex (25 nM) was added to the medium and the cells were continued in culture until the 96-h time point. The delayed effect of Dex addition on the representative morphology (20× magnification) of primary rat hepatocytes is shown. Arrows identify evidence of perturbed morphology indicated by condensed cytoplasm and rounded cells attributed to cytotoxicity. The lower right panel shows the relative level of LDH leakage associated with the time course of Dex rescue. Scale bar = 50 μm.

creased the expression of CYP3A1 mRNA, although simultaneously inhibiting the CYP2B PB-induction response. The latter result is consistent with previously published observations demonstrating a biphasic effect of Dex on PB induction [25]. mEH levels appeared only marginally impacted by the additions of Dex.

Since the nuclear receptors CAR and RXRα are integral to the PB induction response, we also examined their respective mRNA levels under the specified culture conditions. Altering Dex concentration was without substantive effect on CAR mRNA expression level; however, RXRα mRNA levels were clearly increased in the presence of ≥ 25 nM Dex.

While examining the expression character of several liver-specific genes (Fig. 1D), we observed that albumin mRNA levels were not adversely impacted by the absence of Dex. However, transthyretin and transferrin levels were clearly reduced under the same conditions, with transferrin exhibiting the most sensitivity to Dex manipulation. Dex omission resulted in substantial up-regulation of GST-Pi mRNA expression, a response that was reverted to the normal quiescent level in the presence of ≥ 5 nM Dex.

Dex rescue of hepatic gene expression

We tested whether the aberrant hepatic responses characterized under Dex deficiency were reversible. The results of these 'Dex-rescue' experiments are presented in Fig. 2. In most cases, additions of Dex to cells that had been deprived of the hormone for 4, 24, or 48 h were effective in restoring robust levels of hepatic responsiveness to PB (Fig. 2A) as well as albumin, transthyretin, and transferrin expression (Fig. 2B). Interestingly, PB induction responses could no longer be restored in cells that were deprived of Dex for 72 h, despite restoration of the other more classical markers of hepatic expression. These results imply that PB responsiveness is a uniquely sensitive indicator of hepatocyte differentiation status.

Effects of Dex and ECM overlay on hepatocyte morphology and cell viability

We assessed the impact of Dex modulation and ECM addition on cultured hepatocytes using cell morphology and viability endpoints. Either Dex omission or the absence of ECM overlay resulted in a marked perturbation of cell morphology from that typically associated with differentiated hepatocytes in primary culture. The representative photomicrographs, shown in Figs. 3A and B, demonstrate that limiting conditions of Dex and/or ECM omission disrupted typical cuboidal networks and bile canalicular structures. Morphological deterioration was clearly evident at Dex concentrations ≤ 5 nM, with cells exhibiting condensed cytoplasm, abnormal rounding of cell structure, and formation of fibroblast-like protrusions (Fig. 3). Less LDH leakage (Fig. 3 insets) was apparent at higher Dex concentrations (25–1000 nM). The presence of ECM protected the cells, as morphological disruption and LDH leakage was evident in ECM-treated cells only when Dex was entirely omitted (Fig. 3A versus 3B). In

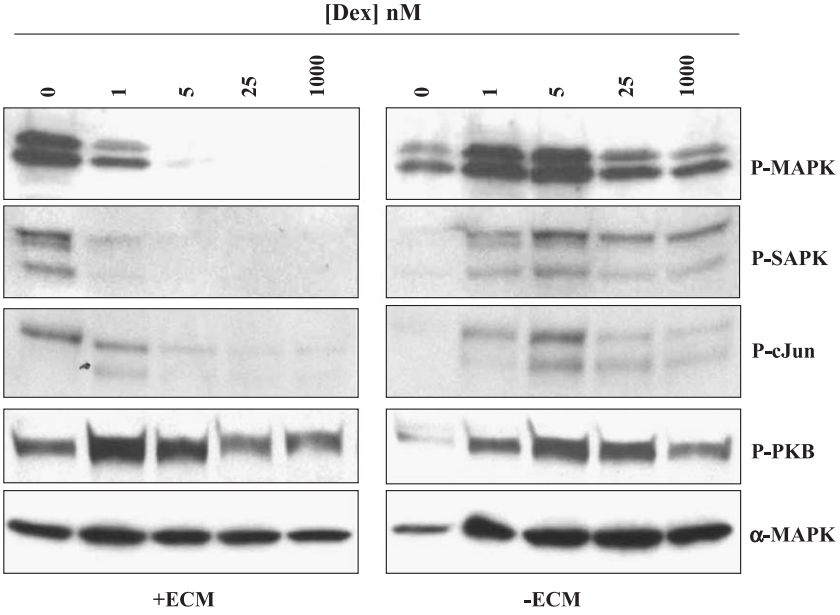

Fig. 5. Effect of ECM overlay and dexamethasone concentration on the activation of stress signaling pathways in primary rat hepatocytes. Primary rat hepatocytes were cultured for 96 h under variable concentrations of Dex and in the presence (+ECM) or absence (−ECM) of an ECM overlay. Total cell extracts were prepared as stated in Materials and methods and analyzed by Western blot analysis. Phosphospecific antibodies were used to discern the phosphorylation status of p42/44 MAPK (Thr202/Tyr204), SAPK/JNK (Thr183/Tyr185), c-Jun (Ser63), and Akt (Ser473). The levels of immunoreactive proteins also were assessed with phosphorylation-independent antibodies; α-MAPK is shown.

fact, in the presence of ECM overlay, Dex concentrations of 1–5 nM were sufficient to maintain the formation of intact networks with tight junctions and bile canalicular structures (Fig. 3B).

Dex rescues hepatocyte morphology and cell fidelity

To determine if the perturbations in cell morphology produced by Dex omission were reversible, we conducted a time course experiment. In a similar scheme to that described for Fig. 2, 25 nM Dex was added back to cells that had been deprived of the hormone for varying periods. As shown in Fig. 4, the complete omission of Dex resulted in markedly compromised hepatocyte morphology and enhanced cytotoxicity, as evidenced by LDH leakage. The morphological deterioration was not triggered until ≥ 72 h post-isolation, as noted by a decline in the fidelity of cellular structure. These results paralleled the gene expression indices examined in the studies described in Fig. 2.

Dex and ECM modulate the auto-phosphorylation of mitogenic and stress-inducible signaling pathways

In a previous study [30], we demonstrated that primary hepatocytes and their inherent signaling pathways are uniquely sensitive to chemical stress. Here, we hypothesized that the compromised differentiation status of hepatocytes produced by the absence of Dex, denoted by perturbation of hepatocyte morphology, reduction in cellular viability, and altered responsiveness marker genes, was associated with a stress-related cytotoxic response. Cell extracts were prepared from hepatocytes cultured for a total of 96 h under varying Dex concentrations and ECM environments. Western immunoblot analysis and phospho-specific antibodies were used to assess the phosphorylation status of several key signaling proteins involved in transducing the stress response.

Cells cultured in the absence of Dex exhibited a marked stimulation of p42/44 MAPK, SAPK/JNK, and c-Jun phosphorylation (Fig. 5). The presence of ECM attenuated the activation of these pathways, even at the 1 nM Dex dose. The stress activation responses were blunted completely with 5 nM Dex. In contrast, cells cultured in the absence of an ECM overlay exhibited stress pathway activation responses that could only be attenuated modestly by Dex, even at the highest concentrations of Dex tested. Thus, there is an apparent synergy between the effects of ECM and Dex in providing attenuation of the stress cascades. No alterations of the p38 MAPK pathway were observed under these same conditions (data not shown). As depicted in Fig. 5 for immunoreactive MAPK, the non-phosphorylated state of SAPK/JNK, MAPK, and Akt were also determined with control antibodies and the respective levels of these proteins were unchanged across the Dex treatment conditions (data not shown). The results presented in Fig. 5 using phosphospecific antibodies were confirmed in separate assays by measuring functional activities associated with each respective pathway (data not shown). It is interesting to note that omission of Dex or ECM only had minimal impact on the phosphorylation status of PKB, a critical and positive effecter of cell survival and death (Fig. 5). This latter result suggests that the cell survival stimulus associated with Dex is independent of a PI3-kinase pathway.

Dex stabilizes the DNA binding activity of liver-specific transcription factors and suppresses the AP-1 associated stress pathway

Since cell-specific transcription factors are clearly important determinants of cellular differentiation, we asked whether the activated stress responses occurring in the absence of Dex were associated with altered DNA binding activities of specific nuclear transcription factors. As in our previous experiments, cells were cultured for 96 h either in the

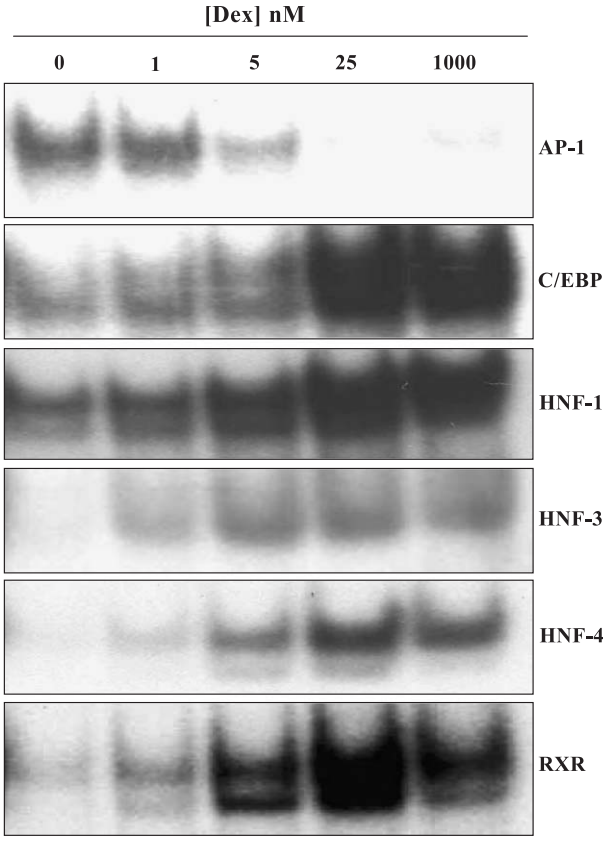

Fig. 6. EMSA analysis examining Dex-mediated stimulation of DNA binding of liver-specific transcription factors and suppression of AP-1 complex in primary rat hepatocytes. Primary rat hepatocytes were cultured for 96 h in the presence of an ECM overlay and under varying concentrations of Dex (nM). Total nuclear protein was extracted as stated in Materials and methods and 10 μg of nuclear protein was incubated with double-stranded oligonucleotides to AP-1, C/EBPα, HNF-1α, -3α, -4α, and RXRα, as described in Materials and methods.

presence or absence of ECM overlay and specific concentrations of Dex (0, 1, 5, 25, 1000 nM). Nuclear extracts were subsequently prepared and subjected to EMSA analysis using double-stranded oligonucleotide probes encompassing consensus-binding sites for AP-1, C/EBPα, HNF-1α, -3α, -4α, and RXRα. The results of these studies are presented in Fig. 6. Dex omissions resulted in a potent stimulation of AP-1 binding. The magnitude of this aberrant response was attenuated by gradually increasing the concentration of Dex such that the complex was inhibited at Dex concentrations ≥ 5 nM. These results correlated well with the marked attenuation of the SAPK/JNK and c-Jun signaling pathways observed under the same culture conditions (Fig. 5).

Concomitant with the increase in binding of the stress-associated AP-1 complex, Dex omission markedly reduced the DNA binding activities associated with several other liver transcription factors. In absence of Dex, the cells exhibited a marked reduction in the binding of C/EBPα, HNF-1α, and RXRα, while the interaction of HNF-3α and -4α to their respective DNA targets was completely extinguished (Fig. 6). Competition studies with excess unlabelled oligonucleotides and with mutated probes were performed to verify the specificity of the interactions (data not shown).

Dex rescues hepatocytes from intracellular stress

The capacity of Dex to rescue the respective transcription factor DNA binding activities and stress-activation responses was evaluated. Dex omission resulted in the marked stimulation of the phosphorylation status of the MAPK, SAPK/JNK, and c-Jun signaling pathways (Fig. 7A). Delayed additions of Dex (4, 24, 48, or 72 h post-isolation) successfully attenuated the activation of these responses. Consistent with the activation of these stress pathways, limiting Dex conditions also triggered enhanced nuclear accumulation of the AP-1 complex (Fig. 7B). Reintroduction of Dex back to the culture environment, even at 48–72 h post-plating, largely restored the levels of AP-1, C/EBPα, HNF-1α, -3α, -4α, and RXRα, as assessed with EMSA experiments. Cells that were maintained in the absence of an ECM overlay required substantially higher concentrations of Dex (25–1000 nM) to restore levels of transcription factor binding (data not shown).

Effect of Dex dose and time course on the rescue of liver-specific transcription factor expression

Finally, we sought to determine whether the reduction in DNA binding activities, previously noted for the hepatic transcription factors under conditions of reduced Dex, was attributed directly to lowered levels of their respective proteins. Nuclear extracts were prepared at several time points in the absence or presence of 25 nM Dex and assessed by Western immunoblot analysis using specific antibodies. Dex omissions resulted in the marked reduction

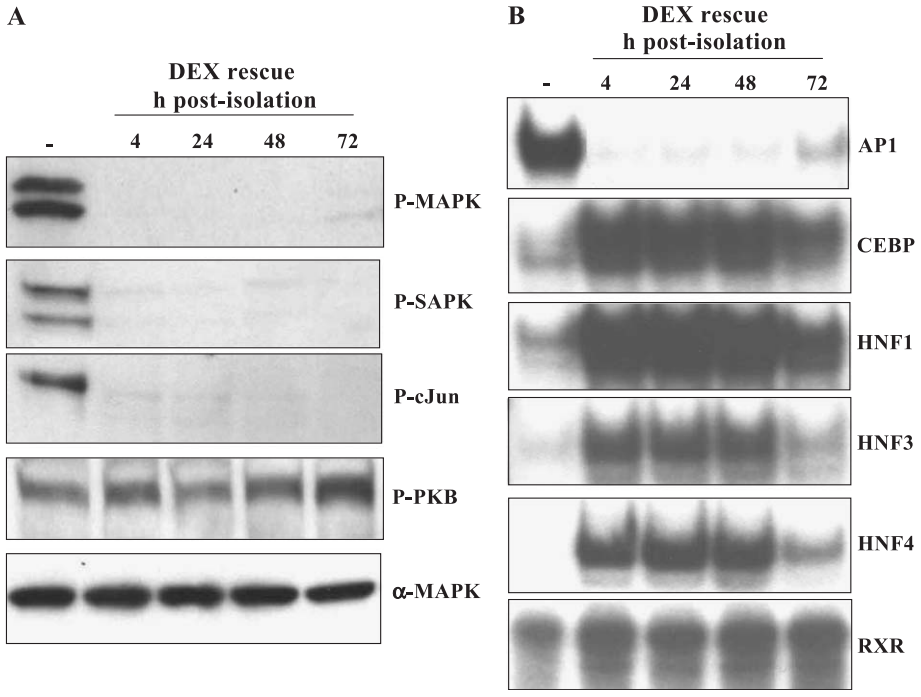

Fig. 7. Dex rescues hepatocytes from stress-associated signaling pathways. Primary rat hepatocytes were cultured for 96 h in the presence of an ECM overlay. At the indicated times (4, 24, 48, or 72 h post-plating), Dex (25 nM) was added to the medium and continued in the cultures until the 96-h time point. The delayed effect of Dex addition on stress-associated signaling pathways (Panel A, phosphorylation signals) and EMSA analysis (Panel B) of DNA binding activities associated with AP-1, AP-1, C/EBPα, HNF-1α, -3α, -4α and RXRα are shown.

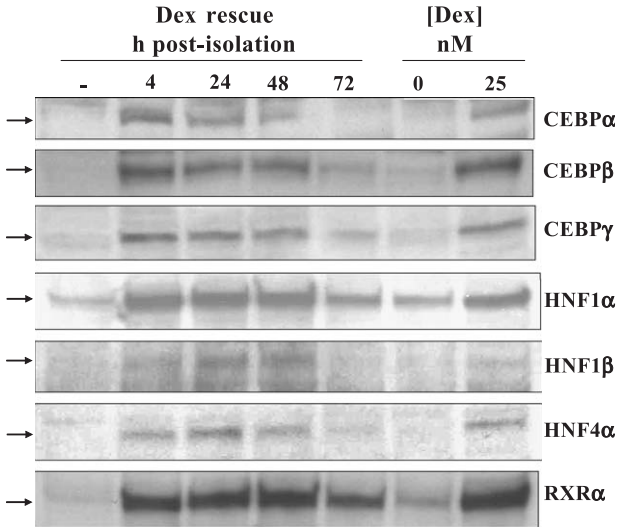

Fig. 8. Immunoblot determination of time course and dose effects of Dex rescue on the expression levels of liver-specific transcription factors in primary rat hepatocytes. See legend to Fig. 7 for details of experimental treatments.

of protein levels associated with all of the family members assessed (Fig. 8). As was the case in the previous experiments, subsequent Dex additions were capable of largely restoring the respective binding activities to levels consistent with those observed in freshly isolated hepatocytes (data not shown).

Discussion

Defined culture systems of primary hepatocytes provide a unique resource for study of the underlying biology of the liver parenchyma. Recent progress in the maintenance of cultures of primary rodent and human hepatocytes has enabled more detailed analysis of extracellular influences involved in liver differentiation, growth, and survival [32–34].

Cell–ECM interactions are critical in maintaining hepatocyte differentiation via the establishment of cell polarity, polygonal shape, and formation of bile canaliculi and junctional complexes. These effects are mediated through integrins [32,35,36], a family of transmembrane receptors, which in turn cross-talk with components of the cytoskeleton. ECM-associated modulation of cell shape appears to be inversely correlated with the control of gene expression. Additionally, it has been proposed that tissue phenotype depends on reciprocal interactions between the ECM and structural organization of the nucleus [37]. A number of studies have reported a correlation between ECM-induced hepatocyte differentiation and the expression or function of various liver-enriched transcription factors [38,39]. For example, the expression of HNF-3α and -4α appear to be induced and stabilized by Dex treatment [40,41].

Using a well-defined rat hepatocyte system, we previously established that robust, in vivo-like responsiveness to the prototypical hepatic inducer, PB, required the presence of dilute concentrations of ECM [24]. Subsequently, we also demonstrated that nanomolar additions of the synthetic glucocorticoid, Dex, were essential to sustain maximal levels of these induction responses [25,26], effects that have been corroborated by other investigators [27].

To better identify mechanisms associated with these responses that impact hepatocyte integrity, differentiation status, and xenobiotic responsiveness, in the current work we targeted specific cellular pathways for further analysis, pathways we hypothesized as potential mediators of the effects exerted by ECM and Dex. We examined the involvement of several stress-associated pathways, including the MAPK, SAPK/JNK, and c-Jun signaling pathways, and resulting nuclear recruitment of the AP-1 complex. In addition, we asked whether Dex and ECM might facilitate hepatocyte differentiation and homeostasis by altering functional expression of key liver-specific transcription factors.

Our results demonstrate the critical requirement for defined additions of both nanomolar concentrations of Dex and dilute additions of ECM in facilitating the maintenance of the differentiated liver phenotype in vitro. The results also confirm a paradoxical effect exhibited with highly elevated concentrations (≥ 1 μM) of Dex whereby PB-inducible CYP2B1/2 expression responses are attenuated. The latter responses were not associated with diminished DNA binding or altered expression levels of any of the transcription factors examined, nor did they result from any visible alteration of hepatocyte morphology or differentiation state. The mechanism through which high doses of Dex suppress the PB induction may reside in cross-talk with other nuclear receptors, such as PXR and remain to be further elucidated.

Our data demonstrate the requirement of Dex in the maintenance of the expression and respective DNA binding activities for the liver-enriched transcription factors, C/EBPα, -β, -γ, HNF-1α, -1β, -3α, -4α, and RXRα. Recently, other investigators reported that reintroduction of the liver-specific transcription factor, HNF-4α, reinstated a differentiated phenotype [42] and classical parenchymal morphology [43] in de-differentiated hepatoma cells via restoration of E-cadherin expression and concomitant responsiveness to glucocorticoids, such as Dex. Using gene disruption techniques, Li et al. [44] have demonstrated that HNF-4α represents a master regulator of hepatic differentiation; without it, hepatocytes lose a broad spectrum of liver-specific functions. Thus, as reported here, in the absence of Dex, the down-regulation of HNF-4α function, together with other liver-specific transcription factor interactions, likely contributes to the loss of hepatocyte differentiation features assessed with our experimental endpoints.

Results of our investigations demonstrated that culture medium additions of an ECM overlay, and particularly of Dex, to deficient cultures produced rather spectacular res-

toration of the polygonal and cuboidal morphology, characteristics associated normally with the adult liver [25,35,45]. Nanomolar concentrations of Dex similarly sustained hepatocyte viability, as manifested by a marked reduction in the extracellular leakage of LDH. Combined, these observations provide compelling evidence for the importance of Dex and ECM in the maintenance of hepatocyte physiology.

Recent reports have proposed that both GCs [46,47] and ECM [48,49] induce G1 growth arrest and subsequent blockage of S phase progression, through suppression of cyclins A and D1 and concomitant induction of cyclin-dependent kinase (CDK) inhibitors $p21^{Waf1/Cip1}$ and $p27^{kip1}$ [50]. Similarly, GC-mediated cell cycle arrest triggered by p21 induction in rat hepatoma cells requires the concomitant steroid induction of C/EBPα [48,51], and ECM-mediated induction of $p21^{Waf1/Cip1}$ results in increased p53 phosphorylation [50,52]. Hepatocytes in vivo are typically in a G1 cell cycle-arrested state, consistent then with predicted effects of Dex and ECM influence. The dramatic activation of auto-phosphorylation of the Erk1/2 MAPK pathway in primary hepatocytes seen in our experiments in the absence of either Dex or ECM would be anticipated to result in a loss of control of the signaling machinery regulating cell cycle progression and mitogen-activated growth. Thus, our findings suggest that Dex and ECM function to block proliferative signals both at the level of AP-1 activation and cell cycle progression, thereby facilitating the expression of a differentiated cellular phenotype.

The differentiated phenotype of the hepatocyte clearly includes robust expression of albumin, transferrin, transthyretin, and the PB induction response, as seen in the Dex- and ECM-treated panels in Fig. 1. The contributions of Dex and ECM on hepatocyte differentiation status are further demonstrated by the remarkable dose-dependent suppression that Dex exerts on GST Pi expression, a gene that is normally repressed in adult liver but activated by AP-1 in preneoplastic liver foci. It is noteworthy that neither albumin, transthyretin, or transferrin mRNA expression—prototypical markers of hepatocyte function—are not as sensitive to Dex or ECM deficiency as the PB induction response is itself.

A recent study indicated that Dex treatments augment the levels of CAR mRNA [27] in human hepatocytes. In our experiments, the potentiative effects of Dex, on the PB induction process, on transcription factor expression, and in attenuating the stress pathway endpoints examined, were typically manifested with 1–5 nM Dex concentrations. Only Dex concentrations ≥ 25 nM led to a modest elevation in the level of detectable RXRα mRNA and protein, however, CAR levels were not noticeably affected by this or any of the Dex levels tested. These results support a broader role for Dex (and ECM) in the facilitation of the PB induction response, indicating that Dex and ECM coordinately orchestrate a cluster of additional signaling networks that act independently of the nuclear receptors CAR and RXRα to modulate xenobiotic responsiveness in the hepatocyte.

In the absence of Dex and any exogenous mitogenic stimuli, we observed a remarkable auto-phosphorylation of the SAPK/JNK and MAPK (Erk1/2) signaling pathways. Resultant downstream nuclear effects of these perturbed signaling pathways included the nuclear recruitment of both AP-1 and NF-κB (NF-κB data not shown). These responses were mitigated by the dose-dependent readditions of Dex and, to a lesser extent, by reintroduction of an ECM overlay. It is interesting that neither the omission of Dex or ECM impacted the phosphorylation status of p38 MAPK (data not shown).

The aberrant intracellular stress response mounted by hepatocytes in the face of either Dex or ECM omission is likely of fundamental physiological relevance. By providing the cells with a physiologically stable and structured environment, ECM appears to facilitate the response of cells to soluble ligands via stabilization of receptor-mediated signaling pathways [5,6] and effects a balance between resulting phosphorylation–dephosphorylation events. The ECM responses appear closely coupled with those facilitated by the presence of physiological concentrations of GCs. Disruption at any tier of this cascade appears to result in the activation of aberrant and sustained auto-phosphorylation signals, such as those demonstrated in the stress pathway analysis conducted in the present investigation.

Taken together, we have demonstrated that low concentrations of Dex and ECM provide critical cues to the hepatocyte in culture, functioning to preserve expression character of a variety of hepatic nuclear factors, including C/EBPα,-β,-γ, HNF-1α, -1β, -4α, and RXRα; facilitate the expression of prototypical hepatic marker genes, including albumin and transferrin; preserve hepatic morphology; provide attenuation of several stress signaling pathways including that of MAPK, SAPK/JNK, c-Jun, and AP-1 activation; attenuate the expression of GST-Pi; and enhance hepatocyte viability as monitored through LDH release. We propose that the resultant phenotypic stability that Dex and ECM confer to the hepatocyte is critical for establishing the fidelity of cellular response to xenobiotics, particularly to the class of PB inducing agents.

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