HGF-, EGF-, and Dexamethasone-Induced Gene Expression Patterns During Formation of Tissue in Hepatic Organoid Cultures

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Corticosteroids, hepatocyte growth factor (HGF), and epidermal growth factor (EGF) play important roles in hepatic biology. We have previously shown that these molecules are required for formation of tissue with specific histology in complex organoid cultures. Dexamethasone suppresses growth and induces hepatocyte maturation; HGF and EGF are needed for formation of the nonepithelial elements. All three are needed for formation of the biliary epithelium. The gene expression patterns by which corticosteroids, HGF, and EGF mediate their effects in hepatic tissue formation are distinct. These patterns affect many gene families and are described in detail. In terms of main findings, dexamethasone induces expression of both HNF4 and C/EBPα, essential transcription factors for hepatocyte differentiation. It suppresses hepatocyte growth by suppressing many molecules associated with growth in liver and other tissues, including IL-6, CXC-chemokine receptor, amphiregulin, COX-2, HIF, etc. HGF and EGF induce all members of the TGF-β family. They also induced multiple CNS-related genes, probably associated with stellate cells. Dexamethasone, as well as HGF and EGF, induces expression of HNF6-β, associated with biliary epithelium formation. Combined addition of all three molecules is associated with mature histology in which hepatocyte and biliary lineages are separate and HNF4 is expressed only in hepatocyte nuclei. In conclusion, the results provide new and surprising information on the gene expression alterations by which corticosteroids, HGF, and EGF exert their effects on formation of hepatic tissue. The results underscore the usefulness of the organoid cultures for generating information on histogenesis, which cannot be obtained by other culture or whole animal models.

Gene arrays   Three-dimensional cultures   Hepatocytes   Biliary cells   Growth regulation

HEPATOCYTES isolated by perfusion of rat liver with collagenase contain a small contaminant population of other hepatic cells, such as stellate cells, biliary epithelium, endothelial cells, etc. The primary isolates reorganize to form tissue with recognizable, albeit altered, hepatic microarchitecture in roller bottle cultures under the influence of insulin, nicotinamide, corticosteroids (dexamethasone), hepatocyte growth factor (HGF), and epidermal growth factor (EGF). Histology in these organoid cultures consists of a surface layer of mature biliary epithelium, an intermediate layer of hepatocytes and stellate cells, and a layer of endothelial cells attached to the substratum (46). Biliary epi-thelium in the surface of these cultures is in large part derived from hepatocytes undergoing *trans*-differentiation (45). HGF and EGF are required for the formation of the biliary epithelium and the connective tissue. The effects on connective tissue are probably mediated by regulation of expression of TGF-β family members (46). Dexamethasone, in the absence of HGF and EGF, induces maturation of cells exclusively towards the hepatocytic lineage. Biliary epithelium is absent without addition of HGF or EGF. When dexamethasone, HGF, and EGF are all removed, immature hepatocytes present in the cultures undergo intensive proliferation. The reproducibility of histo-

logic alterations induced by addition of specific cytokines provides a useful model for analysis of the mechanisms by which these and other cytokines contribute to the morphogenesis of hepatic tissue. In the current studies, we analyzed the underlying gene expression patterns associated with the morphogenetic changes related to dexamethasone, HGF, and EGF. The results obtained provide unexpected insights as to the pathways involved and the nature of the signaling mediators related to proliferation and maturation of hepatic cellular elements during tissue formation.

MATERIALS AND METHODS

Animals

Fisher 344 male rats were obtained from Charles River Laboratories (Frederick, MD, USA). All procedures performed on the rats employed for these studies were approved under the IACUC protocol # 0699068A-1 and conducted according to National Institute of Health guidelines.

Materials

EGF was obtained from Collaborative Biomedical (Waltham, MA). Collagenase for hepatocyte isolation was obtained from Boehringer Mannheim (Mannheim, Germany). Vitrogen (from Celtrix Labs., Palo Alto, CA) was used for collagen coating of roller bottles. General reagents and retrorsine were obtained from Sigma (St. Louis, MO). EGF was purchased from Collaborative Biomedical. HGF used for these studies was the $\Delta 5$ variant and was kindly donated by Snow Brand Co. (Toshigi, Japan). Antibodies were obtained from the following sources: proliferating cell nuclear antigen (PCNA) (Signet Laboratories), amphiregulin (NeoMarkers, Inc, Fremont, CA), CXC chemokine receptor (Research Diagnostic, Inc., Flanders, NJ), vascular endothelial growth factor (VEGF), VEGF receptor Flk-1, IGF1, and HNF4 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Generation and Maintenance of Organoid Cultures

The techniques used to generate the organoid cultures from the livers of the rats were exactly as we recently described (46). Rat hepatocytes were isolated by an adaptation of Seglen's calcium two-step collagenase perfusion technique (63) as previously described from our laboratory (7). Freshly isolated hepatocytes were added to roller bottles (1450 cm^2 pleated surface) obtained from Falcon (Franklin Lakes, NJ). Each bottle contained 210 million freshly isolated hepatocytes in 250 ml of hepatocyte growth medium (HGM) supplemented with HGF (40 ng/ml)

and EGF (20 ng/m). The bottles were rotated at a rate of 2.5 rotations per minute and kept in an incubator maintained at 37°C, saturated humidity, and 5% CO_2. The HGM was used to maintain the cultures. The composition of the medium was also as recently described. DMEM liquid medium, HEPES, glutamine, and antibiotics were purchased from GIBCO/BRL (Grand Island, NY). ITS mixture (insulin, transferrin, selenium) was purchased from Boehringer Mannheim. All other additives were cell culture grade (Sigma). Unless otherwise indicated for specific experiments, the basal HGM consisted of DMEM supplemented with purified bovine albumin (2.0 g/L), glucose (2.25 g/L), galactose (2.0 g/L), ornithine (0.1 g/L), proline (0.030 g/L), nicotinamide (0.305 g/L), $ZnCl_2$ (0.544 mg/L), $ZnSO_4:7H_2O$ (0.750 mg/L), $CuSO_4:5H_2O$ (0.20 mg/L), $MnSO_4$ (0.025 mg/L), glutamine (5.0 mmol/L), and dexamethasone (10^{-7} mol/L). Gentamicin (50 µg/ml) was added to the basal HGM. The mixed basal HGM was sterilized by filtration through a 0.22-µm low-protein-binding filter system, stored at 4°C, and used within 4 weeks. ITS 1.0 g/L [rh-insulin 5.0 mg/L, human transferrin 5.0 mg/L (30% diferric iron saturated), selenium 5.0 µg/L] was added after filtration immediately before use. The growth factors, as required, were added to HGM fresh at the specified concentrations every time the medium was changed. Tissue samples were taken at different times during the cultures to assess histology.

Analysis of Gene Expression: Data Generation and Statistics

Affymetrix Chip Analysis. The Affymetrix oligonucleotide chips specific for the rat (U34 A chip) containing 8000 expressed sequences specific for the rat genome.

cRNA Preparation. RNA was isolated from organoid cultures kept in different conditions. The RNA was isolated at day 20 of the cultures. Three separate culture sets (each from a separate rat) were used to prepare RNA for analysis of gene expression. Total RNA was extracted and purified with Qiagen RNeasy kit (Qiagen, San Diego, CA). Five micrograms of total RNA was used in the first-strand cDNA synthesis with T7-d(T)$_{24}$ primer [GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)$_{24}$] by Superscript^{TM} II (GIBCO-BRL, Rockville, MD). The second-strand cDNA synthesis was carried out at 16°C by adding *E. coli* DNA ligase, *E. coli* DNA polymerase I, and RnaseH in the reaction. This was followed by the addition of T4 DNA polymerase to blunt the ends of newly synthesized cDNA. The cDNA was purified through phenol/chloroform and ethanol pre-

cipitation. The purified cDNA were then incubated at 37°C for 4 h in an in vitro transcription reaction to produce cRNA labeled with biotin using MEGA-script™ system (Ambion, Inc., Austin, TX).

Affymetrix Chip Hybridization. cRNA (15–20 μg) was fragmented by incubating in a buffer containing 200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc at 95°C for 35 min. The fragmented cRNA was then hybridized with a preequilibrated Affymetrix chip at 45°C for 14–16 h. After the hybridization cocktails were removed, the chips were then washed in a fluidic station with low-stringency buffer (6× SSPE, 0.01% Tween 20, 0.005% antifoam) for 10 cycles (2 mixes/cycle) and stringent buffer (100 mM MES, 0.1 M NaCl, and 0.01% Tween 20) for 4 cycles (15 mixes/cycle), and stained with SAPE (strepto-avidin phycoerythrin). This was followed by incubation with biotinylated mouse anti-avidin antibody, and restaining with SAPE. The chips were scanned in a HP ChipScanner (Affymetrix Inc., Santa Clara, CA) to detect hybridization signals.

Analysis. The analysis was conducted through an Affymetrix microarray suite 5.0 software. Data were exported and tabulated on Excel spreadsheet (Microsoft Office). The mean and standard error of the logarithm (base 2) of the data were expressed. Three totally separate preparations, each from a separate rat, and each used for the four experimental categories analyzed, were used for the studies. Means and standard error from each category of the three data sets (control, dexamethasone, HGF + EGF, and combined (dexamethasone + HGF + EGF) were derived. In addition, the *p*-value of the difference between the mean of the control cultures and the mean of the specially treated cultures was determined for each gene. Only genes in which the difference between the control and the treated category was less than 0.05 were used for the generation of the data presented.

RESULTS

Effects of Dexamethasone, HGF, and EGF on Histology of the Organoid Cultures

These results were as previously described in work from our laboratory (46). Cultures maintained in HGM alone (containing insulin and nicotinamide) exhibited a high rate of spontaneous proliferation (Fig. 1A). Mitoses were readily observed. The PCNA labeling index was 94% and the percent of nuclei labeled by Ki-67 was 24%. The cells had the appearance of small hepatocytes. No biliary epithelial cells were seen. Staining for HNF4 shows that only 27%

percent of nuclei stained strongly positive for this transcription factor (Fig. 1B). There was very little connective tissue. Addition of dexamethasone induced an increase in the size of the cells, which were recognized as mature hepatocytes (Fig. 1C). Several apoptotic hepatocytes were also noted. There was a dramatic decrease in cell proliferation (PCNA 39.7% and Ki-67 1%). Mitotic index was 0, whereas in the control cultures it was very high, at 2.3%. More than 95% of the nuclei stained strongly positive for HNF4 (Fig. 1D). No connective tissue or biliary epithelium were seen. Addition of HGF and EGF in the medium altered the histology of the organoid cultures (Fig. 1E). The cells had an appearance most resembling "oval cells" as described in livers of rats subjected to a variety of protocols causing liver regeneration and suppression of hepatocyte proliferation (2,17). There was no clear distinction between surface biliary epithelium and hepatocytes. Staining for HNF4 showed that 18% of the nuclei contained this transcription factor (Fig. 1F). Percent of nuclei labeled with PCNA was more than 90% and the percent labeled with Ki-67 was 16%. Combined addition of dexamethasone, HGF, and EGF resulted in separation of hepatocytic and biliary lineages, with biliary epithelium appearing on the surface of the organoid cultures (Fig. 1G). Cytokeratin 19 was seen only on the surface biliary epithelium (46). HNF4 was only observed in nuclei of hepatocytes, most of which (more than 80%) contained the transcription factor (Fig. 1H). PCNA labeling index was more than 70%. Ki-67 labeling for hepatocytes was less than 5%, whereas more than 60% of the biliary epithelium stained positive for Ki-67, as previously described (46).

Gene Expression Patterns Induced by Dexamethasone

Expression of genes following addition of dexamethasone was examined in three separate organoid cultures prepared from three different liver perfusions. The results show that dexamethasone suppressed growth of hepatic cells and promoted hepatocyte differentiation by affecting expression of many signaling molecules and signal transduction pathways. Table 1 shows a list of the genes affected and the fold increase or suppression induced by dexamethasone. Most of the genes affected were associated with previous literature as growth or differentiation regulators in liver or other related tissues. Of the group of genes whose expression change was statistically significant (*p* < 0.05), only the most dramatically affected genes are discussed below. The values in parentheses after each gene indicate the degree or gene expression in dexamethasone-treated cultures as percent of gene
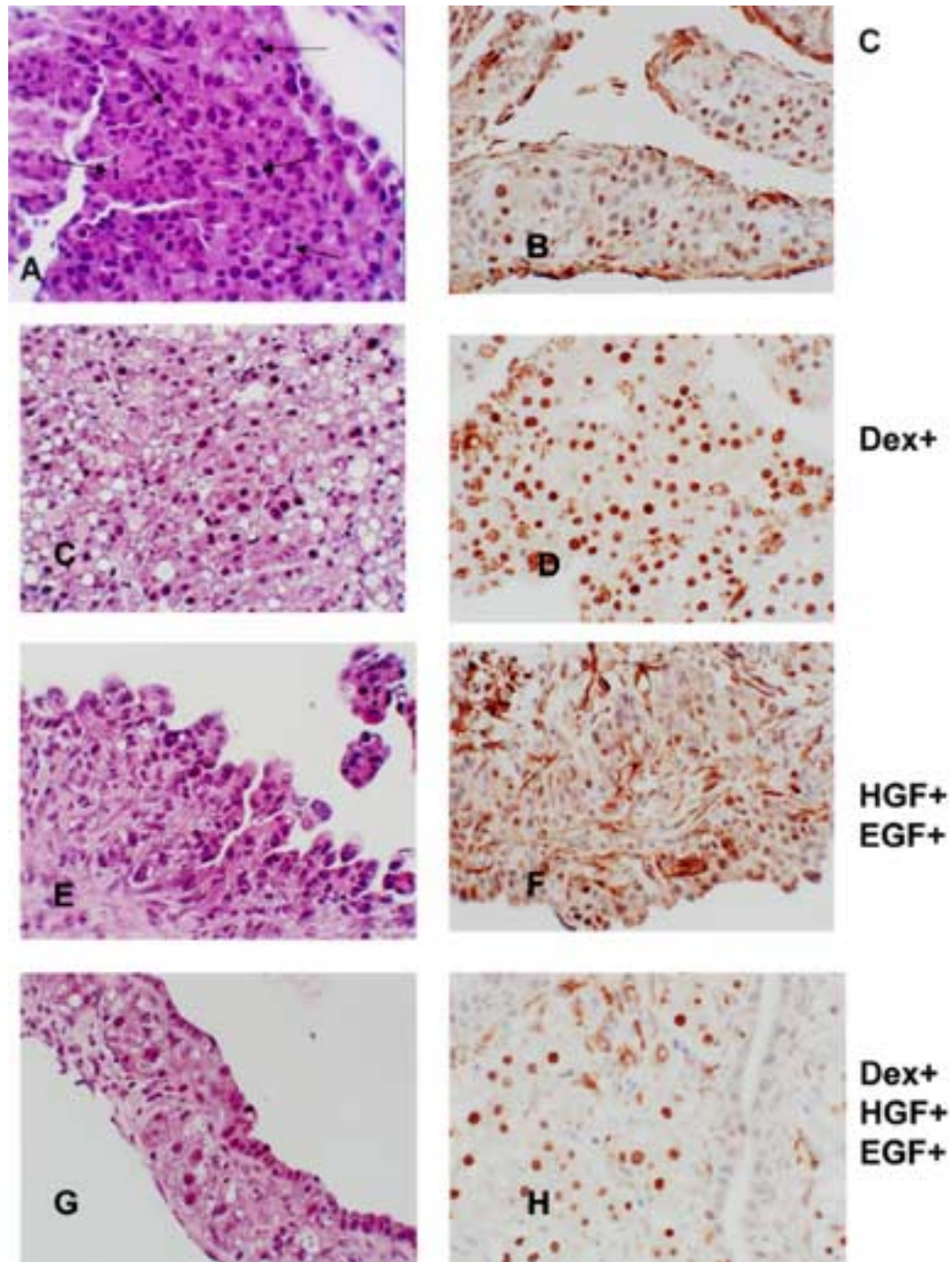
Figure 1. Histology of the organoid cultures under the influence of dexamethasone, HGF, and EGF. Medium supplements are shown to the right of the photomicrographs. (A, C, E, G) Sections of tissue from the organoid cultures stained with hematoxylin and eosin. (A) Arrows point to mitoses seen in the control cultures. (B, D, F, H) Immunohistochemical staining for HNF4. Notice the partition of the nuclear stain between hepatocytes and biliary epithelium in (H). Original magnifications: 400×.

TABLE 1
DEXAMETHASONE EFFECTS ON HEPATIC ORGANOID GENE EXPRESSION PATTERNS

Gene Name	Gene Number	Gene Expression (% of Control)
Growth Factors and Signaling Cytokines		
Suppressed genes		
Interleukin 6 (IL-6) gene	M26745	2.48%
Angiopoietin-2, partial	AF030378	2.60%
Pancreatitis-associated protein	M98049	3.05%
Nonselective-type endothelin receptor	S65355	3.20%
Amphiregulin (schwannoma-derived growth factor)	X55183	6.70%
Insulin-like growth factor-binding protein 3 (IGF-BP3)	M31837	7.35%
ET-B endothelin receptor	X57764	8.64%
CXC chemokine receptor (CXCR4)	U90610	8.84%
Drosophila polarity gene (frizzled)	L02529	10.39%
Plasminogen activator inhibitor 2 type A (PAI2A)	X64563	11.14%
Cyclooxygenase isoform COX-2	S67722	11.40%
Adrenomedullin precursor	D15069	11.40%
Neuropilin	AF016296	11.94%
IGF-1	X06107	12.50%
TGF-β1	X52498	16.49%
Gro	D11445	16.49%
CXC chemokine LIX	U90448	19.39%
Nonselective-type endothelin receptor	S65355	19.84%
Urokinase receptor uPAR-1	X71898	20.31%
Glioma-derived endothelial cell growth factor (ECGF)	M32167	21.27%
Vascular endothelial growth factor (VEGF)	L20913	22.79%
Putative pheromone receptor (Go-VN2)	AF016179	22.79%
Fibroblast growth factor receptor 1	AF000144	23.33%
Interleukin 2, (IL-2)	M22899	29.39%
Very low density lipoprotein receptor (VLDLR)	L35767	30.08%
Interleukin-1 beta-converting enzyme	S79676	30.08%
Corticotropin releasing hormone (CRH) gene	M54987	30.78%
Interleukin 10 (IL-10)	L02926	33.76%
Interleukin-15	U69272	38.78%
SCGF Stem cell growth factor	AB009246	39.69%
VEGF receptor-2/FLK-1	U93306	37.89%
Angiotensin/vasopressin receptor (AII/AVP)	M85183	41.56%
Cyclooxygenase 1	U03388	45.59%
Fas antigen ligand	U03470	46.65%
Increased genes		
IGF binding protein-1 (rIGFBP-1)	M58634	2785.76%
Growth hormone receptor	Z83757	1329.98%
Glucagon receptor	M96674	606.29%
Transforming growth factor-beta 3 (TGF-β3)	U03491	481.21%
Transferrin	D38380	438.73%
Epidermal growth factor receptor (EGFR)	M37394	428.71%
Asialoglycoprotein receptor (hepatic lectin)	K02817	364.69%
GABA(A) receptor beta-3 subunit	X15468	356.36%
Acetylcholine receptor beta-subunit	X74833	276.38%
Fos-related antigen	U34932	276.38%
Transforming growth factor-alpha (TGF-α)	M31076	151.57%
Transcription Factors		
Suppressed genes		
Hypoxia-inducible factor 1 (HIF-1)	Y09507	11.94%
Proliferating cell nuclear antigen (PCNA/cyclin)	M24604	39.69%
Increased genes		
Hepatocyte nuclear factor 4 (HNF4)	X57133	527.80%
Hepatocyte nuclear factor 3a (HNF3-β)	L09647	400.00%
Cyclin D2	D16308	390.86%
C/EBPα	X12752	356.36%
Hepatocyte nuclear factor 6 beta (HNF6-β)	Y14933	356.36%
c-Myc oncogene and flanking regions	Y00396	332.50%
Retinoid X receptor gamma (RXRγ)	AF016387	296.22%

TABLE 1
CONTINUED

Gene Name	Gene Number	Gene Expression (% of Control)
HNF3-γ	AB017044	235.11%
Stat3 protein	X91810	229.74%
HNF3/forkhead homolog-1 (HFH-1)	L13201	125.99%
AlF-C1	AB016536	257.87%
Matrix-Related Proteins and Proteases		
Suppressed genes		
Osteopontin	M14656	1.13%
Macrophage metalloelastase (MME)	X98517	2.16%
Dermatan sulfate proteoglycan-II (decorin)	Z12298	2.42%
Versican V3 isoform precursor	AF072892	4.42%
Decorin	X59859	8.06%
Embigin protein	AJ009698	9.47%
Lumican	X84039	11.94%
Platelet-endothelial cell adhesion molecule-1/CD31	U77697	11.96%
Collagen alpha1 type I	Z78279	14.69%
Osteonectin	Y13714	16.88%
Collagen type III alpha-1	M21354	18.51%
Tissue inhibitor of metalloproteinase type 2 (TIMP-2)	S72594	26.18%
Tenascin	U09361	30.78%
Fibronectin (fn-1) gene, partial	U82612	32.99%
Plasminogen activator inhibitor-1 (PAI-1)	M24067	37.89%
Increased genes		
Beta-2 glycoprotein I	X15551	37916.00%
Polyprotein 1-microglobulin/bikunin	S87544	1970.00%
Vitronectin	U44845	649.80%
Hepsin	X70900	324.90%
Fibronectin gene, exons 2b and 3a	M28259	317.48%
Brevican core protein	U37142	251.98%
Hepatocyte Differentiation Genes		
Suppressed genes		
Cytochrome P-450 isozyme 5 (P450 IVB2)	M29853	14.69%
Glutamine synthetase	M91652	23.87%
Increased genes		
Argininosuccinate synthetase (EC 6.3.4.5)	X12459	11404.00%
Cytochrome P450 monooxygenase (CYP2J3)	U39943	5079.68%
Apolipoprotein A-IV	M00002	3055.49%
Glycogen synthase	J05446	2785.76%
Fatty acid transporter	AB005743	2599.21%
Cytochrome P-450(M-1) gene, exon 9	M18363	2369.76%
Plasminogen	M62832	2315.63%
Plasma proteinase inhibitor alpha-1-inhibitor III	M22360	2211.06%
Alpha-2-macroglobulin gene, exons 5 and 6	M22670	2160.56%
UDP-glucuronosyltransferase, phenobarbital-inducible form	M13506	1637.40%
Apolipoprotein C-III gene	J02596	1492.85%
CYP2D2	AB008423	962.42%
Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase	M33648	918.96%
Canalicular multispecific organic anion transporter(cMOAT)	D86086	918.96%
Protein C	X64336	918.96%
P-glycoprotein	L15079	877.46%
Alpha-fetoprotein (AFP)	X02361	712.72%
Cathechol-O-methyltransferase	M93257	565.69%
Vitronectin	U44845	649.80%
Thrombin	M81397	515.75%
CYP2D3	AB008424	438.73%
Glutathione S-transferase (GST) Y(b) subunit	X04229	438.73%
Ceruloplasmin	L33869	409.35%
Ornithine carbamoyltransferase	K03041	381.94%
Fibrinogen B beta chain	U05675	364.69%
Tyrosine aminotransferase	X02741	340.27%
Alpha-fibrinogen	X86561	340.27%

TABLE 1
CONTINUED

Gene Name	Gene Number	Gene Expression (% of Control)
Fibronectin gene, exons 2b and 3a	M28259	317.48%
Sodium/bile acid cotransporter	M77479	317.48%
Fetuin	X63446	317.48%
Apolipoprotein B (apoB) gene	U53873	296.22%
Cytochrome P450 4F4 (CYP4F4)	U39206	289.45%
Glucokinase, alternatively spliced GK2 (EC 2.7.1.1)	X53588	289.45%
Argininosuccinate lyase, complete	D13978	289.45%
Acyl-CoA synthetase	AB012933	270.07%
Hereditary hemochromatosis protein homolog (RT1-CAFe)	AF008587	257.87%
Albumin gene	X76456	257.87%
Cytochrome P-450 IV A1 (CYP4A1)	M57718	257.87%
Major acute phase alpha-1 protein (MAP)	K02814	251.98%
Hemopexin	M62642	251.98%
Fibrinogen gamma chain	J00735	240.61%
HMG-CoA reductase	X55286	240.61%
Signaling Pathways		
Suppressed genes		
Phospholipase A2	X51529	5.97%
Type II cAMP-dependent protein kinase regulatory subunit	M12492	7.69%
Protein kinase C-eta	X68400	8.25%
Cyclooxygenase isoform COX-2	S67722	11.40%
cAMP phosphodiesterase, 3′ end	J04563	11.40%
Phospholipase C-III	M20637	13.09%
Activin type I receptor-like kinase ALK-5	S81584	13.40%
Dual-specificity protein tyrosine phosphatase (rVH6)	U42627	15.04%
Phospholipase C-1	M20636	32.99%
Interleukin-1 beta converting enzyme (IL1BCE)	U14647	41.56%
Cyclooxygenase 1	U03388	45.59%
Increased genes		
LIMK-2a	D31874	356.36%
Beta subunit of AMP-activated protein kinase	X95577	289.45%

N/A, not available.

The genes affected are grouped into function categories. The gene names are as specified in the Affymetrix annotation. Modifications were made if the gene is known in the literature or from the Unigene database under a more commonly used name. The second column indicates the gene identifier code in the Unigene database. The last column shows the percentile change in gene expression in the treated cultures over that in the control cultures. All data represent the mean of three separate measurements from three separate culture sets. All genes shown were chosen from the list in which the difference of the means between the control and the treated cultures was statistically significant at $p < 0.05$.

expression in control cultures not treated with dexamethasone, HGF, or EGF.

Genes Suppressed by Dexamethasone.

Growth Factors, and Related Cytokines and Receptors. Interleukin 6 (IL-6) (2.48% of control) was the most suppressed of the signaling molecules. Many recent studies have shown that IL-6 is a mitogen for bile duct epithelial cells and that it regulates expression of STAT3 at the early stages of liver regeneration. Mice with homozygous deletion of IL-6 gene have altered liver regeneration response (11,61). Angiopoietin-2 (2.60% of control) is involved with angiogenesis in liver and other tissues (58). Neuropilin 1, VEGF, and its receptor Flk-1 are also molecules involved in angiogenesis and all are suppressed by dexamethasone (39). This may be due to the fact that dexamethasone also suppresses the hypoxia-inducible factor (HIF) (11.94% of control), a transcription factor known to be expressed in many cells including hepatocytes (34) involved in coordinate induction of gene expression of many angiogenesis-related growth factors and their receptors. Pancreatitis-associated protein (PAP) (3.05 %) is a member of a family of proteins comprising a group of small secretory proteins, which can function as acute phase reactants, lectins, antiapoptotic factors, or growth factors (8).

It is expressed highly in hepatocellular carcinomas. Schwannoma-derived growth factor is also known as amphiregulin (6.7% of control). It is a ligand for the EGF receptor (52). There is no literature for any role of amphiregulin in liver development or regeneration. Of interest, dexamethasone, while suppressing amphiregulin, increases gene expression of another EGF receptor ligand, TGF-α, though only by 57% (Table 1). The chemokine stroma-derived factor (SDF)-1, and its receptor, CXCR-4 (8.84%), have been shown to be essential for the translocation of hemopoietic stem cells from the fetal liver to the bone marrow (64). The receptor *frizzled* (10.39% of control) is associated with signal transduction via β-catenin, which is involved in growth signaling during liver regeneration (48,49). Previous studies from this laboratory have shown the importance of uPAR (urokinase receptor) (20.31% of control) in generating growth signals at the earliest stages of liver regeneration (44). Work from several laboratories has implicated cyclo-oxygenase-2 (COX-2) (11.40%) as a growth regulator for neoplasms from many endodermal tissues including colon and pancreas (67).

Matrix-Related Proteins and Proteases.   Addition of dexamethasone was associated with lack of connective tissue formation in the cultures. The gene expression data correlate very well with this histologic finding. TGF-β1, a cytokine associated with fibrosis and induction of many matrix-related proteins in several tissues (30), was suppressed by dexamethasone (16.49% of control). Suppressed also were the genes for osteopontin (1.13%), macrophage metalloelastase (2.16%), dermatan sulfate proteoglycan (decorin) (2.42%), versican (4.42%), lumican (11.94%), collagen type I (14.69%) and collagen type III (18.51%), and tenascin (30.78%).

Genes Induced by Dexamethasone: Correlation With Hepatocyte Differentiation.   Addition of dexamethasone caused increase in size and appearance of histologically mature hepatocytes in the organoid cultures (Fig. 1C). These findings correlate well with the data from gene expression analysis in the dexamethasone-treated cultures. A wide range of enzymes and secretory proteins characteristic of hepatocyte differentiation become induced (Table 1). These include metabolic enzymes characteristic of hepatocytes, such as argininosuccinate synthetase, different species of cytochrome P450 family of proteins, tyrosine aminotransferase, glucokinase, and HMG-CoA reductase. Many secretory proteins made by hepatocytes are also included, such as vitronectin, thrombin, ceruloplasmin, fibrinogen, apolipoprotein C-III, fetuin, hemopexin, α-fetoprotein, etc. The changes seen are consistent with

the effects of dexamethasone on hepatocyte-associated transcription factors. HNF4 gene expression increases by 527.8%. All (100%) of hepatocyte nuclei stain positive for HNF4 after addition of dexamethasone (control: 27%). Increases are also seen in expression of HNF3a (400%), C/EBPα (356%), HNF6-β (350%), HNF3-γ (235%), and retinoid X receptor (RXR) (296%). Most of the genes associated with hepatocyte differentiation that are induced by dexamethasone are also controlled by HNF4 and/or C/EBPα (28).

Effects of HGF and EGF

Histologic Effects.   Addition of HGF and EGF together to control cultures caused a slight decrease in the rate of cell proliferation (KI-67: control, 24%; HGF + EGF: 16%) and altered the morphology of the cells (Fig. 1C). There was no clear distinction between hepatocytes and biliary epithelium, with most cells having mixed hepatobiliary features, best resembling the "oval" hepatocytes described in rat liver protocols (18). The epithelial cells are interlaced with other cell types with mesenchymal features. HNF4 staining was seen in only 18% of the nuclei.

Gene Expression Patterns Induced by Addition of HGF and EGF Combined.   Whereas the gene expression patterns induced by dexamethasone can be easily categorized, the patterns induced by addition of the two tyrosine kinase receptor ligands are more subtle, yet not less dramatic for some of the affected genes. Meaningful changes were seen in expression of growth regulatory molecules, matrix-related proteins, and transcription factors.

Expression of Genes Related to Growth Factors and Signaling Cytokines.   Results in this category are shown in Table 2. A variety of genes in this category were suppressed by adding HGF and EGF. Most suppressed was the expression of interleukin 2 and angiopoietin 2. Previous studies have shown expression of angiopoietin 2 in hepatocellular carcinomas and during liver regeneration (13). IL-2, however, is considered to be expressed primarily in lymphoid cells, though a recent article suggested production of IL-2 by hepatocytes (38). Members of the Gro family of proinflammatory cytokines are expressed in many cell types including hepatocytes (59). Profoundly suppressed also was the expression of IGF-1 as well as receptors for FGF-1, parathyroid hormone-like peptide, oxytocin, endothelin, CXC chemokines, VEGF and hyaluronan (motility receptor), and the α-2 adrenergic receptor. Amphiregulin was also suppressed, though not as much as with dexamethasone. TGF-α expression, to the contrary, was increased, more so

TABLE 2
HGF + EGF EFFECTS ON HEPATIC ORGANOID GENE EXPRESSION PATTERNS

Gene Name	Gene Number	Gene Expression (% of Control)
Growth Factors, Receptors, and Signaling Cytokines		
Suppressed genes		
Interleukin 2	M22899	15.04%
Angiopoietin-2	AF030378	16.49%
Gro	D11445	21.27%
Fibroblast growth factor receptor 1	AF000144	22.79%
IGF-I (insulin-like growth factor I)	X06107	22.79%
CXC chemokine receptor (CXCR4)	U90610	25.00%
Fit-1S (Fit-1)	U04319	25.58%
CINC-2 beta CXC chemokine	D21095	26.79%
CXC chemokine receptor (CXCR2) gene	U70988	28.06%
Endothelin	E02223	30.08%
VEGF receptor-2/FLK-1	U93306	32.23%
Prodynorphin (Preproenkephalin B) gene	M10088	32.99%
Follicle stimulating hormone beta-subunit	M36804	34.55%
Alpha2-C4 adrenergic receptor	X57659	34.55%
Placenta growth factor (PlGF)	L40030	37.89%
PPAR-\GKg	AB011365	39.69%
Receptor hyaluronan-mediated motility	U87983	40.61%
Parathyroid hormone-like peptide (PLP) gene	M34112	41.56%
Oxytocin receptor (OTR)	U15280	41.56%
Nonselective-type endothelin receptor	S65355	43.53%
Amphiregulin (Schwannoma-derived growth factor)	X55183	43.53%
Interleukin 10 (IL-10)	L02926	44.54%
Epidermal growth factor precursor	X12748	45.59%
OX40 ligand (Ox40l)	AF037067	42.53%
Increased genes		
IGF binding protein-1 (rIGFBP-1)	M58634	918.96%
Beta-type calcitonin gene-related peptide	M11596	680.53%
Angiotensinogen, 3′ flank	M12112#3	634.96%
Secretin receptor	X59132	552.77%
Alpha-type calcitonin gene-related peptide	M11597	552.77%
QIL-LD1 olfactory receptor	AF091563	540.14%
Transforming growth factor-beta 3 (TGF-β3)	U03491	540.14%
Transforming growth factor-beta 1 (TGF-β1)	X52498	123.11%
Transforming growth factor-beta 2 (TGF-β2)	M96643	135.03%
CB1 cannabinoid receptor	X55812	515.75%
Notch 2	M93661	459.48%
Secretin receptor	E04128	400.00%
LDL-receptor	X13722	390.86%
Class I MHC molecule	X67504	381.94%
Interleukin-1 receptor-related protein	U49066	373.21%
Transforming growth factor-alpha (TGF-α)	M31076	348.22%
Growth hormone receptor	S49003	340.27%
Tenascin	U09401	332.50%
Chemokine CX3C	AF030358	332.50%
Interleukin 1 receptor antagonist gene	M63101	332.50%
Interleukin 5	X54419	332.50%
Neuronal nicotinic acetylcholine receptor-related protein	J05231	324.90%
QLL-TN1 olfactory receptor	AF091562	324.90%
Kallikrein	M11566	324.90%
P2Y purinoceptor	U22830	310.23%
Delta-preprotachykinin	X56306	303.14%
Interleukin-12	S82489	296.22%
Myelin-associated glycoprotein (S-MAG)	X06554	289.45%
Beta 3-adrenergic receptor	S56481	282.84%
Hepatocyte growth factor (HGF)	D90102	282.84%
Transferrin	D38380	282.84%
Preprorelaxin	A16585	263.90%
Neu differentiation factor	U02316	251.98%

TABLE 2
CONTINUED

Gene Name	Gene Number	Gene Expression (% of Control)
Thyroid stimulating hormone receptor	M34842	246.23%
Retinoic acid receptor alpha 2 isoform (RAR)	U15211	240.61%
Putative pheromone receptor (Go-VN4)	AF016181	229.74%
HER-2 neu oncogene	X03362	229.74%
Vasopressin	M64785	224.49%
Luteinizing hormone receptor	M61211	219.36%
Thrombopoietin	E12181	219.36%
Transforming growth factor-beta 3	U03491	219.36%
Purinoreceptor (P2X2)	AF020757	214.35%
Pancreatitis-associated protein	M98049	214.35%
Transcription Factors		
Suppressed genes		
APEG-1	U57097	15.39%
CCAAT binding transcription factor-B subunit (CBF-A11)	M60617	33.76%
PPAR-γ	AB011365	39.69%
Distal-less 3 (Dlx-3) homeobox protein	D31734	41.56%
fos-related antigen (Fra-1)	M19651	41.56%
c-Fos	X06769	43.53%
Gas-5 growth arrest homologue	U77829	43.53%
Increased genes		
Hepatocyte nuclear factor 6 beta	Y14933	620.46%
Cyclin D1	X75207	552.77%
Hepatocyte nuclear factor 4	D10554	448.98%
Zinc finger protein AT-BP1	X54249	282.84%
c-Jun	X17163	282.84%
DEAF-1 related transcriptional regulator (NUDR)	AF055884	251.98%
Retinoic acid receptor alpha 2 isoform (RAR)	U15211	240.61%
bHLH protein	D82868	224.49%
Variant hepatic nuclear factor 1 (vHNF1)	X56546	224.49%
Zinc finger transcription factor REST	AF037199	317.48%
Zinc finger protein AT-BP1	X54249	282.84%
Zinc finger protein AT-BP2	X54250	276.38%
Matrix-Related Proteins and Proteases		
Suppressed genes		
Osteopontin	M14656	26.18%
Neutrophil collagenase	AJ007288	30.08%
Embigin protein	AJ009698	35.36%
Neuroglycan C precursor	U33553	37.89%
Platelet-endothelial cell adhesion molecule-1/CD31	U77697	38.78%
Cell-binding bone sialoprotein	J04215	38.78%
190 kDa ankyrin isoform	AF069525	39.69%
Receptor hyaluronan-mediated motility	U87983	40.61%
Vascular cell adhesion molecule-1	M84488	40.61%
Neuroglycan C precursor	U33553	37.89%
Increased genes		
SHPS-1	D85183	857.42%
Integrin beta 4 subunit	U60096	857.42%
N-cadherin	AF097593	540.14%
Phosphacan	U04998	503.97%
Plasminogen	M62832	400.00%
Tenascin	U09401	332.50%
K-cadherin	D25290	317.48%
Connexin 40 (GJA5)	AF022136	263.90%
Neurexin III-alpha gene	L14851	257.87%
140-kDa NCAM polypeptide	X06564	204.67%
Pax-6	S74393	310.23%
Hepatocyte Differentiation		
Suppressed genes		
Liver glutathione S-transferase Ya subunit	K00136	7.69%
Lipophilin	M25888	17.27%

TABLE 2
CONTINUED

Gene Name	Gene Number	Gene Expression (% of Control)
Cytochrome P-450 MC gene	E00778	21.76%
von Willebrand factor	U50044	25.58%
Cytochrome P450 (CYP1B1)	U09540	27.42%
Cytochrome P-450-11β	D11354	27.42%
Low molecular weight fatty acid binding protein	J02773	31.50%
Liver fatty acid binding protein	V01235	32.23%
Lactate dehydrogenase-C (LDH-C)	U07177	32.23%
Zn-alpha-2-glycoprotein	X75309	32.99%
Cytochrome P450 (CYP1B1)	U09540	35.36%
Hydroxysteroid sulfotransferase	X63410	36.18%
Intestinal fatty acid binding protein (FABP)	K01180	36.18%
Beta-fibrinogen	M35602	37.03%
Beta-alanine oxoglutarate aminotransferase	D7839	39.69%
Cationic amino acid transporter-1 (CAT-1)	U70476	39.69%
Glutathione S-transferase Yc1 subunit	S72505	40.61%
Salivary proline-rich protein (RP4) gene	M64791	40.61%
Delta 4-3-ketosteroid 5 beta-reductase	S80431	41.56%
Fructose 6-phosphate,2-kinase	S67900	43.53%
Increased genes		
Alpha-fetoprotein (AFP)	X02361	1269.92%
Cytokeratin 21	M63665	818.70%
SH3-containing protein p4015	AF026505	781.73%
Cytokeratin 19	X81449	712.72%
Beta-2 glycoprotein I	X15551	592.44%
Dipeptidyl peptidase IV (DPP IV)	J04591	481.21%
Inducible nitric oxide synthase (iNOS)	D44591	481.21%
Hepatic squalene synthetase	M95591	428.71%
Cytochrome P450 arachidonic acid epoxygenase	U04733	418.92%
Plasminogen	M62832	400.00%
Liver glycogen phosphorylase	M59460#2	381.94%
L-type pyruvate kinase	X05684	381.94%
UDP-glucuronosyltransferase	S70364	348.22%
Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase	M33648	340.27%
Cytokeratin-8	S76054	332.50%
Serine pyruvate aminotransferase	X6357	317.48%
Pyruvate carboxylase	U32314	317.48%
Apha-2-macroglobulin gene	M22670	317.48%
Organic cation transporter OCT1A	U76379	303.14%
Phosphorylase kinase gamma-subunit	X07320	276.38%
Peroxisome assembly factor-2	D63673	276.38%
Intestinal lactose-binding lectin (L-36)	M73553	270.07%
17-beta hydroxysteroid dehydrogenase type 2	X91234	263.90%
Type I 5 alpha-reductase {longer transcript}	S81448	263.90%
Apolipoprotein A-IV	M00002	257.87%
Cytochrome P-450 IID5	J02869	257.87%
Apolipoprotein B (apoB)	M14952	246.23%
Annexin-V	AF051895	246.23%
Tissue factor protein	U07619	240.61%
Serine protease inhibitor 2.4	X69834	240.61%
Protein C	X64336	235.11%
High-affinity carnitine transporter	AB017260	235.11%
Keratin 14	D63774	229.74%
Beta-4N-acetylgalactosaminyltransferase	D17809	229.74%
Hepatic squalene synthetase	M95591	224.49%
Alpha-1 inhibitor III	J03524	219.36%
Squalene epoxidase	D37920	209.46%
Signaling Pathways		
Suppressed genes		
Platelet phospholipase A2	X51529	7.18%
G protein gamma-5 subunit	M95780	14.36%

TABLE 2
CONTINUED

Gene Name	Gene Number	Gene Expression (% of Control)
Type II cAMP-dependent protein kinase regulatory subunit	M12492#1	21.27%
12-Lipoxygenase	L06040	25.58%
Intracellular calcium-binding protein (MRP14)	L18948	25.58%
Putative G protein-coupled receptor (CNL3)	AF064706	26.18%
Phospholipase C-1	M20636	30.08%
12-Lipoxygenase	S69383	30.78%
JUN-Kinase (stress activated protein kinase) gamma	L27129	30.78%
Clathrin light chain (LCA1)	M15882	32.99%
Phospholipase C-III	M20637	33.76%
Inositol polyphosphate 4-phosphatase	U26397	36.18%
Guanyl cyclase (GC-S-beta-2)	M57507	37.89%
Endothelial nitric oxide synthase (NOSIII)	AF085195	43.53%
Calpain isoform Lp85	AF052540	43.53%
Mitogenic regulation SSeCKS (322) gene	U23146	38.78%
Increased genes		
SHPS-1	D85183	857.42%
Kinesin-related protein KRP4 (KRP4)	AF035953	680.53%
Rabphilin-3a related protein	AF022774	649.80%
Protein kinase C delta-bindig protein	D85435	515.75%
Inducible nitric oxide synthase (iNOS)	D44591	481.21%
Serine/threonine kinase (gamma-PAK)	U35345	470.22%
cAMP-dependent protein kinase inhibitor protein	M64092	470.22%
Bta subunit of AMP-activated protein kinase	X95577	459.48%
Ca^{2+}-dependent activator protein (CAPS)	U16802	390.86%
G-protein coupled receptor pH218	U10699	364.69%
ROK-alpha	U38481	348.22%
LIMK-2a	D31874	340.27%
Stanniocalcin (rSTC)	U62667	310.23%
Regucalcin	D67071	303.14%
Dynein-like protein 4	D26495	282.84%
Beta 3-adrenergic receptor	S56481	282.84%
Microtubule associated protein (MAP2c)	X17682	276.38%
Na,K-ATPase alpha-2 subunit	M28648	270.07%
Cyclin dependent kinase 2-alpha	D28753	263.90%
MAP kinase kinase kinase 1 (MEKK1)	U48596	257.87%
Ezrin p81	X67788	257.87%
Agrin	M64780	246.23%
MAP kinase kinase kinase 1 (MEKK1)	U48596	246.23%
Retinoblastoma protein (Rb)	D25233	240.61%
EH domain binding protein epsin	AF018261	229.74%
Guanine nucleotide-binding protein (Gz-alpha)	U77483	219.36%
SNAP-25A	AB003991	214.35%
Regulator of G-protein signaling 12 (RGS12)	AF035151	214.35%
Guanylate cyclase	M26896	209.46%
Protein tyrosine phosphatase	L19933	204.67%
Nuclear serine/threonine protein kinase	AF036959	204.67%
CNS Related Genes		
Suppressed genes		
Glial fibrillary acidic protein alpha (GFAP) gene	AF028784	27.42%
Transcriptional repressor of myelin-specific genes (SCIP)	M72711	29.39%
Prodynorphin (Preproenkephalin B) gene	M10088	32.99%
Neuroglycan C precursor	U33553	37.89%
Neuritin	U88958	38.78%
Increased genes		
Synuclein	X86789	664.99%
CB1 cannabinoid receptor	X55812	515.75%
Neurofibromatosis protein type I	M82824	390.86%
Myelin protein MVP17	U31367	381.94%
QLL-TN1 olfactory receptor	AF091562	324.90%
Pax-6 [rats, small eye rSey, Partial Mutant, 1292 nt]	S74393	310.23%

TABLE 2
CONTINUED

Gene Name	Gene Number	Gene Expression (% of Control)
Brain serine protease bsp2	AJ005642	303.14%
Myelin-associated glycoprotein (S-MAG)	X06554	289.45%
Neuroendocrine-specific protein (RESP18)	L25633	289.45%
BMAL1b	AB012600	289.45%
Homer-1c	AF093268	282.84%
Blue cone opsin-like pigment	U63972	257.87%
Synaptotagmin XI	AF000423	257.87%
Neurexin III-alpha gene	L14851	257.87%
Neuron-specific protein PEP-19	M24852	240.61%
Mint2	AF029107	229.74%
Putative pheromone receptor (Go-VN4)	AF016181	229.74%
PIT-1-beta	L01507	229.74%
Neurexin I-beta	M96375	229.74%
Synapse associated protein 25A	AB003991	214.35%
Synuclein SYN2 (alternatively spliced)	S73008	209.46%
Synapse-associated protein 102	U50147	204.67%
NCAM polypeptide	X06564	204.67%

N/A, not available.
The genes affected are grouped into function categories. The gene names are as specified in the Affymetrix annotation. Modifications were made if the gene is known in the literature or from the Unigene database under a more commonly used name. The second column indicates the gene identifier code in the Unigene database. The last column shows the percentile change in gene expression in the treated cultures over that in the control cultures. All data represent the mean of three separate measurements from three separate culture sets. All genes shown were chosen from the list in which the difference of the means between the control and the treated cultures was statistically significant at $p < 0.05$.

than with dexamethasone. Prominent increases in gene expression were seen for several genes in this category. IGF BP1, a known immediate early gene whose expression dramatically increases after partial hepatectomy (42), was most prominently induced. A large group of genes composed of ligands and receptors associated with G proteins was also prominently increased. This category included secretin and its receptor, angiotensinogen, olfactory receptors, cannabinoid receptors, etc. There was prominent induction of TGF-β3 and a mild induction of TGF-β1 and 2. There was also marked increase in the expression of *Notch*. The latter is generally considered to be an inhibitor of terminal differentiation in multiple systems (21,55) and was increased (459.48%). This is meaningful in view of the histologic evidence that addition of HGF and EGF is associated with cells with intermediate hepatobiliary morphologic characteristics analogous to oval cells. Expression of HGF itself was also induced (282%), as wells as expression of HER-2 neu (229%).

Expression of Genes Associated With Hepatic Cell Differentiation. The observed patterns of increase in specific genes correlate well with the observed histologic changes. Correlating with the fact that HGF

and EGF induce appearance of cells resembling oval cells, most prominently increased gene expression was that of α-fetoprotein (AFP), a well-known marker of oval cells from whole animal models (66). AFP increased by 1269%. Oval cells are also known for expression of several biliary markers. This also correlates with the marked increase in cytokeratin 19 (592%) and DPP IV (dipeptidyl peptidase IV) (481%). Also noted was an increase in arachidonic acid epoxygenase, correlating with observed increase in prostaglandins induced by HGF in several systems (77). Increases were also seen in expression of plasminogen (400%) and iNOS (481%). There was a surprising increase in genes associated with the central nervous system. Many such genes were noted (listed in Table 2). Expression of some of these factors was so far only seen in CNS development [e.g., transcription factors Pax-6, associated with induction of the eye structures (22), and Pit-1, associated with pituitary development (54)]. Some of these proteins are associated with cell structure [e.g., synuclein (5)] or with peripheral nerves (myelin, neurofibromatosis protein). Other proteins are associated with synaptic formation, such as homer-1c (3) and neurexin III (60). There is emerging literature about convergent gene expression patterns between stellate cells of the liver

and astrocytes (9). The observed changes in these cultures may reflect enhanced proliferation and/or differentiation of stellate cells induced by HGF and EGF. This would correlate with the observed increase in connective tissue induced by addition of HGF and EGF, including enhanced expression of collagen type IV and TGF-β1 shown in our previous publication describing this system (46). Increased numbers of stellate cells would also correlate with the enhanced expression of TGF-β and HGF, known to be produced by stellate cells of the liver (68,69). On the other hand, expression of glial fibrillary acidic protein, known common marker of stellate cells and astrocytes, was suppressed (27%). The observed patterns may also reflect enhanced production of peripheral nerve elements related to peripheral ganglia. This may not be the case as expression of synuclein was localized on the hepatobiliary cells, not connective tissue cells (data not shown). The expression of several genes decreased after addition of HGF plus EGF. No clear pattern appears to emerge, other than the fact that some of the genes express proteins involved in the metabolism of fatty acids (see Table 2).

Transcription Factors.   Expression of several transcription factors was altered after addition of HGF plus EGF. Notable was the dramatic increase in HNF6-β (620%) and HNF4 (448%) as well as cyclin D1 (553%). The data from histologic analysis point to the fact that HGF and EGF are needed for the formation of the biliary monolayer (46). Recent studies have shown that HNF6 is associated with formation of the biliary epithelium (10). The increased expression of the gene under HGF plus EGF correlates well with the histologic observation from our system about the regulation of biliary epithelium appearance under these two growth factors. Also of interest is the enhanced expression of HNF4. Even though HNF4 expression is almost as enhanced as with dexamethasone, the increase in hepatocyte-associated differentiation markers is not seen with HGF and EGF, suggesting that the effects of the corticosteroid receptor are essential for achieving complete maturation of hepatocytes. In addition, enhanced expression of several other transcription factors (cyclin D1, AT-BP1, AT-BP2, REST, cJun, and DEAF1) may interfere with HNF4-mediated control of hepatocyte gene expression.

Gene Expression Patterns Induced by HGF or EGF Added Alone.   Either HGF or EGF added alone induced the characteristic histologic changes seen by the addition of the two combined. This is in agreement with our previously published results on the effects of each of the two growth factors on induction of collagen type IV and TGF-β1 (46). In view of this, it is not surprising that remarkably few differences in gene expression patterns were noted between HGF and EGF (data not shown). All genes were affected in the same direction (up- or downregulation) with differences seen being only a matter of degree (e.g., epoxide hydrolase was induced sevenfold over control by HGF but only 1.5-fold by EGF). Similar changes were also seen in a few cytochrome P450-related genes. All suppressed genes did not show significant differences between the two growth factors or from the combined addition of HGF plus EGF.

Combined Addition of Dexamethasone, HGF, and EGF.   The addition of dexamethasone and HGF + EGF brings out the mature histologic phenotype of the organoid cultures, with clear distinction between biliary epithelium and hepatocytes (46). The histology is quite different compared with that seen with addition of either dexamethasone or combined HGF + EGF alone. The histologic picture is shown in Figure 1G. The maturity of the differentiated phenotype is underscored by the pattern of distribution of HNF4, shown in Figure 1H. In the absence of dexamethasone, a variable percent of the immature epithelial cells stained positive for HNF4 in the nucleus. In cultures exposed to dexamethasone only, all hepatocytes contained HNF4 but the biliary epithelium was absent. In cultures exposed to dexamethasone, HGF, and EGF, HNF4 was seen in the nuclei of the hepatocytes only, whereas the nuclei of biliary epithelial cells did not stain positive for HNF4.

Despite the fundamental differences in histology, the gene expression patterns in dexamethasone and HGF + EGF appear as a compromise between dexamethasone alone and HGF + EGF alone (data not shown). Genes induced by both dexamethasone alone and HGF + EGF alone are induced more by the combination. Most other gene expression levels are average between the two conditions. There is a small set of genes, however, that is uniquely induced by the combination of dexamethasone and HGF + EGF than either dexamethasone or HGF + EGF alone. The glutamate receptor shown as the most elevated gene has already been described as highly expressed in liver and in central nervous system structures (36). However, the role of this receptor in the liver is not understood. VEGF-D is the member of the VEGF family produced predominately by mesenchymal cells and has effects on adjacent endothelial cells. Its expression during embryonic development is associated with organ patterning in lung and other tissues (24). There is a large literature on IGF-II expression during fetal development in the liver and expression in neoplasms (57). Adult liver expresses predominantly

IGF-I. The CC chemokine receptor has been found in the brain and in lymphoid tissues but is also expressed in hepatocytes (51). Angiotensin receptor has been known to be present in the liver. Angiotensin itself has co-mitogenic properties with EGF in rat hepatocytes (29). Neuromedin receptor has not been described in liver, though the ligand has mitogenic effects on rat hepatocytes (26). There is expression of both nerve growth factor and its low-affinity receptor in the liver. NGF is produced by hepatocellular carcinomas (35) and is expressed as an early gene after liver reperfusion injury (53). The decrease in expression of c-kit is consistent with low expression of this receptor in the liver and its upregulation during induction of oval cell formation in suppressed hepatic regeneration (20). RXR is expressed in the adult rat liver and there is an extensive literature on its functions.

DISCUSSION

The literature on signals governing the formation of hepatic tissue during embryogenesis and hepatic regeneration is quite extensive. Most of it is characterized by emphasis on unique signals and gene products, with specific single stimuli triggering a large response with linear downstream signaling cascades, culminating in mitogenesis, altered differentiation, etc. (45). The studies presented here show a complex picture of global alterations in gene expression induced by dexamethasone, HGF, and EGF, during the process of formation of organized histology in cultures derived from hepatic cells. Such complex alterations affecting global patterns of gene expression have been recently described for liver neoplasia (40) and early stages of liver regeneration (42). The histology induced by each of the specific signals (dexamethasone, HGF, and EGF and combination of the three) is quite different. The differences in patterns of gene expression correlated with histology allow for dissection of specific gene pathways associated with different aspects of hepatic histogenesis. Some of the effects observed (e.g., induction of HNF4 by dexamethasone) were only recently described and not fully understood (4). Others (e.g., induction of α-fetoprotein by HGF and EGF) constitute novel discoveries of linkage between established biomarkers (AFP) and well-studied hepatic growth factors (HGF + EGF). The results obtained allow us to understand how the specific signaling molecules of the study (dexamethasone, HGF, and EGF) contribute to the observed effects on the histology of the cultures. Because currently available genetic mouse models do not reveal the impact of these molecules on hepatic embryogen-

esis, and because it is impossible to remove the effect of the substances under study from the mouse embryo without genetic manipulations, the studies offer insights as to how the specific signals contribute to the formation of the hepatic tissue. Below, we will attempt to correlate the observed gene expression changes with the observed histology.

Effects of Dexamethasone

Inhibition of Growth of Primitive (Control Culture) Hepatocytes. The effects of corticosteroids on hepatocyte DNA synthesis have been described in several publications. This has been shown both in cell culture and in whole animal regenerating liver models (50,75). Dexamethasone inhibits the proliferative response after two thirds partial hepatectomy and liver mass is restored by hepatocyte hypertrophy. The mechanisms leading to this effect are not clear. Our results show effects on multiple families of genes associated with cell growth in general. Surprisingly, none of these genes are known to be mitogenic signaling molecules for the hepatocytes (pancreatitis-associated protein, endothelin receptor, CXC chemokine receptor, COX-2), but they have all been associated with regulation of growth in liver or other organ systems. IL-6 itself, the most suppressed gene in that category, has been associated with optimizing STAT3 activation during liver regeneration (11) and with mitogenic effects on biliary epithelial cells. Given the fact that the cells in the control cultures are immature hepatocytes, they may respond differently to IL-6 than the mature cells and have a hybrid hepatocyte and biliary response. If IL-6 is a direct mitogen for these cells (as with mature biliary epithelium), then the finding of maximal IL-6 suppression may explain in part the cessation of growth. Many other molecules suppressed by dexamethasone are also involved in liver regeneration, such as uPAR (43), FGF receptor 1 (32), etc. Of interest, one of the ligands of the mitogenic receptor EGFR (amphiregulin) is suppressed whereas another one (TGF-α) is increased. This apparently paradoxical situation suggests different effects of these ligands on the EGF receptor, or, alternatively, that the EGF receptor at that stage of differentiation does not play a mitogenic role. In fact, EGFR expression was increased by dexamethasone while growth was suppressed. The results point out that dexamethasone suppresses multiple parallel growth-related pathways in a global manner. Given the important role that many of the genes suppressed by dexamethasone play as growth regulators in liver and other organs, it is highly likely that the growth-suppressing effects of dexamethasone are mediated by suppression of these genes. Indepen-

dent immunohistochemical evidence for the effects of dexamethasone on some of these genes is shown in Figure 2.

Dexamethasone Inhibits Formation of Non-Hepatocyte-Derived Tissue Components. The dexamethasone-treated cultures have no evidence of connective tissue or growth of endothelial cells (Fig. 1C). Genetic deletion of the corticosteroid receptor in mice reveals no abnormalities in liver histology. Neonate mice die from pulmonary hemorrhage (33). This suggests that in the absence of glucocorticoid receptor, other receptors also bound by corticosteroids [e.g., PXR (37)] may play a compensating role in terms of liver development. In our system, however, the contributing effects of dexamethasone are revealed because the ligand is fully eliminated. Dexamethasone globally suppressed both the main form of VEGF and the mitogenic receptor Flk1. It also suppressed neuropilin [a co-receptor for the VEGF receptors (41)]. Most of these genes are dependent on positive inductive effects by the hypoxia-inducible factor, also suppressed by dexamethasone. The effects of dexameth-

asone on VEGF have been described sporadically for kidney (25) and keratinocytes (23), whereas its effects on liver tissue were not known. The global manner of suppression of angiogenesis is further illustrated by suppression of the gene for vascular cell adhesion molecule 1, whose expression has not been linked to the hypoxia-inducible factor. The lack of connective tissue also correlates with the gene expression patterns. TGF-β1, associated with formation of connective tissue and/or fibrosis in many systems including liver, is suppressed by dexamethasone. Several previous publications have described that dexamethasone interferes with the effects of TGF-β1. The inhibition of expression of TGF-β1 by dexamethasone, however, has not been previously described except in skin wound healing models (19). Many other connective tissue-related genes were also suppressed, however, and it is not clear whether the effects are mediated by suppression of TGF or directly, via modulation of the expression of the specific genes. Such genes included osteopontin (suppressed to 1.13% of control!), dermatan sulfate proteoglycan, versican, lumican, decorin, collagens type I and III, tenascin,
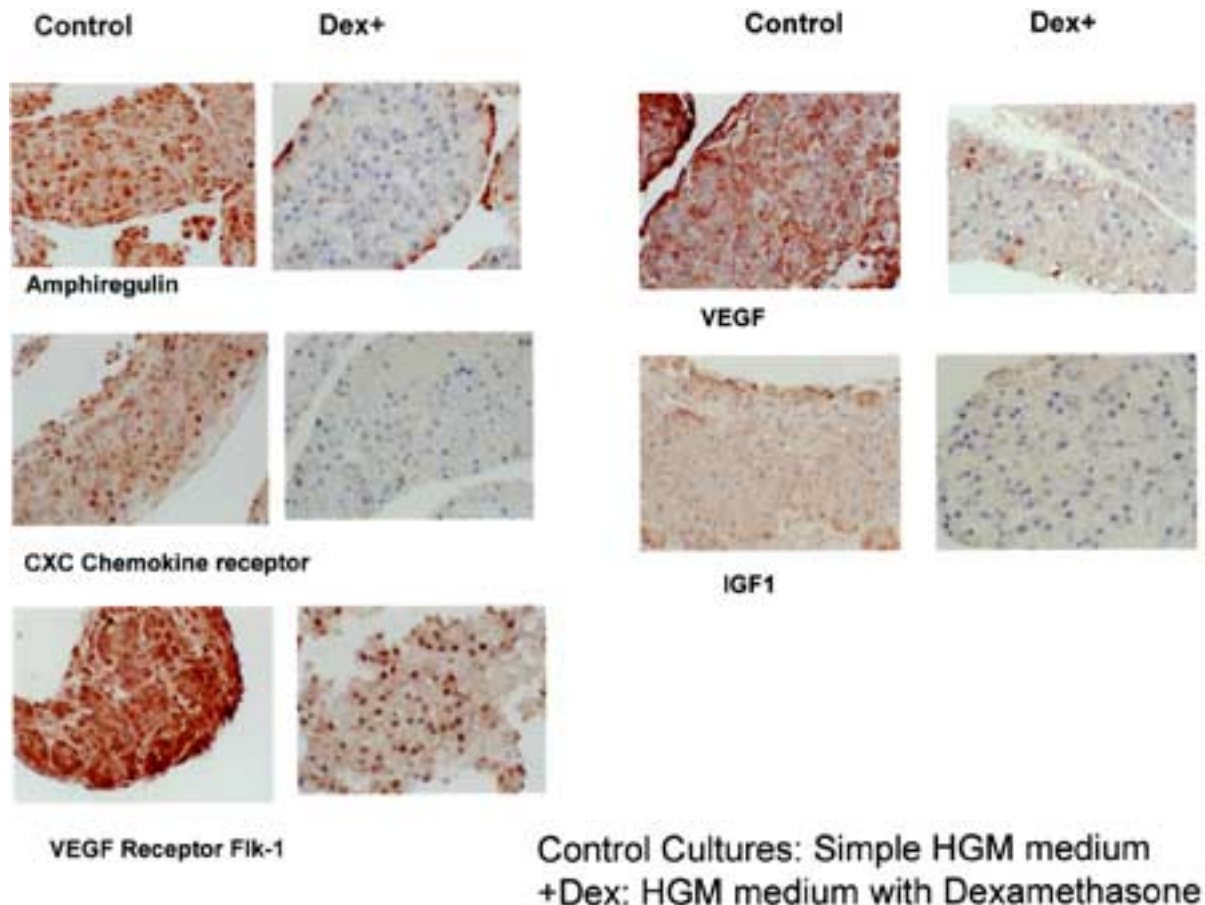

Figure 2. Immunohistochemistry for specific proteins from the list of genes suppressed by dexamethasone. Original magnifications: 400×.

fibronectin-1 osteonectin, etc. There was an increase, however, for fibronectin-2 and brevican. The increase in vitronectin was expected because for hepatocytes vitronectin is a secreted protein and differentiation marker.

Dexamethasone Induces Phenotypic Maturation of Hepatocytes. Induction of differentiation of the primitive hepatocytes to mature cells is apparent by simple histologic examination. Given the often observed association between differentiation and inhibition of growth, this is not surprising. Multiple publications from early literature on hepatocyte cultures document individual gene inductions by dexamethasone. The global effects of dexamethasone on hepatocyte maturation, however, observed in this system, have not been previously described, except for a recent publication pointing out the acquisition of the hepatocyte phenotype by pancreatic cell lines after exposure to dexamethasone (72). Key to this global effect of dexamethasone on hepatocyte maturation must be the combined induction of HNF4 (527% increase) and C/EBPα (356%). HNF4, originally described by Darnell and colleagues (70), is associated with expression of most of the genes associated with hepatocyte differentiation. It appears very early in embryogenesis (15). The mechanisms regulating expression of HNF4 are not clearly understood. Recently, a glucocorticoid-responsive enhancer element 6 kb upstream of the mouse HNF4 promoter was described. Many response elements to other transcription factors were also found in the same region (4). Other than that reference, there is no information relating expression of HNF4 to corticoids. In addition to HNF4, there was also induction of other liver-enriched transcription factors such as HNF3-β and -γ and HNF6-β, as well retinoid X receptor (RXR). Striking also was the induction of C/EBPα, another transcription factor associated with expression of most of the hepatocyte-associated genes. Numerous studies have connected this transcription factor alone or as a ratio between C/EBPα to β, as controlling hepatocyte differentiation (71). Studies from this laboratory have shown that decrease in C/EBPα is associated with dramatic decrease in differentiation in proliferating hepatocytes in monolayer culture and that matrix addition restored differentiation of hepatocytes along with reappearance of C/EBPα and increase of HNF4 in the nucleus (7.) Previous studies have also shown that dexamethasone induces enhanced expression of C/EBPα in hepatoma cells (12) and adipocytes (27). The combined induction of HNF4 and C/EBPα by dexamethasone is a powerful differentiation signal for restoration of hepatocyte differentiation.

Effects of HGF + EGF in Hepatic Tissue Formation

The role of these growth factors in hepatic tissue development has remained enigmatic. Studies with mice bearing homozygous deletion mutants for HGF (62,74) or its receptor, c-Met (14), have shown that these mutations are lethal for the embryo. Abnormalities in placental development and liver development have been cited as the cause. Yet the cited references do not provide any detail as to the nature of the hepatic abnormalities or whether these changes are secondary to the abnormalities in placental development. Histologic descriptions of the fetal livers in these strains of mice describe only smaller than normal hepatocytes. There is no reference to the hepatic histology at the time of the embryonic death. Mice with homozygous deletions for HGF were rescued by direct (in the embryo) HGF injections (73). They died shortly after birth due to abnormalities of formation of the muscle of the diaphragm. Mice with homozygous deletions of the EGF receptor that survive birth have no hepatic abnormalities (65). Even though EGF receptor is present ubiquitously in most epithelial cells, it is felt that the effects of the absence of the EGF receptor are well compensated by other receptors with overlapping function such as Her2, Neu, etc. Thus, studies with "knockout" mice cannot be used to meaningfully investigate the effects of either HGF or EGF on hepatic tissue formation. Our studies with organoid cultures show that HGF and EGF are essential in three endpoints of hepatic histology.

Formation of Connective Tissue and Stromal Elements. We showed in our previous study with this system that HGF and EGF, alone or together, induce expression of the gene for collagen type IV, probably through TGF-β1. The current studies show that HGF + EGF caused an increase in all TGF-β isoforms (1, 2, and 3). Each of these TGF-β isoforms is expressed in different tissues and they have been associated with mesenchymal development in most organs (56). Mice with homozygous deletions for the *smad* family of proteins (TGF-β signal transduction pathway) have abnormalities in hepatic development (76). In addition to their effects on induction of TGF-β family members, HGF + EGF cause an increase in gene expression in many genes associated in the liver with stellate cells. Many of these genes are also found in brain astrocytes (see below). HGF gene expression itself, a product of stellate cells, also increases. These findings suggest that HGF + EGF, directly or indirectly, increase the numbers and/or enhance the functions of the stellate cells, which are known to be the source for most of the connective tissue proteins in hepatic tissue. HGF and/or EGF appear to have a reg-

ulatory function in stellate cell maturation and gene expression, thus affecting formation of the connective tissue seen in these cultures.

Appearance of Cells With Mixed (Hepatobiliary, "Oval Cell") Phenotype. The histology of the cultures demonstrates that the epithelial cells do not have mature features of either hepatocytes or biliary epithelium. However, cytokeratin 19 is expressed on the surface. The cells, morphologically, are most similar in properties to the "oval cells," with mixed hepatocytic and biliary differentiation, described in the rodent carcinogenesis models (6). α-Fetoprotein (AFP) is used as the typical marker for oval cells (6). AFP was very highly induced in the HGF + EGF cultures (13-fold induction!). There was also increase in expression of *Notch* (4.6-fold increase). As mentioned above, enhanced expression of *Notch* is typically associated with inhibition of terminal differentiation in most systems. This finding would correlate with the observed mixed hepatobiliary phenotype, seen in the HGF + EGF cultures.

Formation of the Biliary Epithelium. We have shown in previous studies that HGF induced a ductular phenotype in hepatocytes maintained in collagen gels (7). Other studies have shown that HGF is a mitogen for biliary epithelium (31). In our studies we found that biliary epithelium is derived primarily from phenotypic transformation of the hepatocytes (47). In embryology, biliary epithelium arises from phenotypic transformation of the hepatoblasts abutting the portal nodes, rich in collagen 1 protein. There has been no demonstrated role for any growth factors in this process. In our previous studies with organoid cultures, we showed that in the absence of HGF and EGF there is no superficial expression of cytokeratin 19, a hallmark of biliary differentiation. In these studies we find that in addition to cytokeratin 19, other markers of biliary epithelium are also induced in the HGF + EGF cultures, such as dipeptidyl peptidase IV (DPP IV), cytokeratin 8, and keratin 14. Of interest, HGF + EGF also caused a 6.2-fold increase in expression of HNF6-β, a transcription factor already associated with formation of the biliary system in mice (10). In terms of formation of the biliary epithelium, however, HGF + EGF alone are not sufficient. Dexamethasone, added to HGF + EGF, induces the final maturation of the hepatocytic and biliary lineage and was associated with the appearance of both hepatocytes and biliary epithelial cells, as well as the selective expression of HNF4 on the nucleus of the hepatocytes. It should be noted that dexamethasone as well as HGF + EGF independently induce HNF6-β (see Tables 1 and 2). The histology induced by combined addition of dexamethasone and HGF + EGF

suggests that transcription factors and gene expression patterns induced or activated by dexamethasone and HGF + EGF are required for the mature biliary phenotype to emerge. *Notch* gene expression, induced 4.6-fold in the HGF + EGF cultures, decreases to 3.6-fold induction when dexamethasone is added to HGF + EGF. It is not clear, however, whether this small decrease would be sufficient to account for the observed marked separation of the differentiated phenotype between hepatocytes and biliary epithelium in the combined [Dex + HGF + EGF] cultures.

Expression of Genes Associated With the Central Nervous System. A large array of genes commonly associated with cells of the central nervous system was found induced by addition of HGF + EGF (see Results, above). The meaning of this is not clear. Some of these genes [e.g., synuclein (6.6-fold increase)] are not found in adult liver (1). Others, such as Pax-6, known as the eye transcription factor, are also expressed in pancreatic islets and have been found in models of *trans*-differentiation of hepatic progenitor cells into pancreatic islet cells (78). Most of the other genes from this group found expressed have not been described in liver. On the other hand, many recent studies have demonstrated that stellate cells of the liver express many proteins found in brain astrocytes. The total number of the proteins common to both stellate cells and astrocytes has not been fully tabulated and it is conceivable that the unexpected gene expression pattern of CNS genes in liver reflects positive trophic effects of HGF and EGF on stellate cell development. Other literature has also described neurotrophic effects of HGF, during brain development and on motor neurons of the spinal cord (16). The CNS gene expression pattern seen may reflect neurotrophic effects of HGF in susceptible cells derived from the liver, under the culture conditions. It should be emphasized that no cells with features of neuronal or glial cell type are seen in these cultures under any conditions.

Overall, the results demonstrate that hepatic histology that incorporates all the mature cells seen in liver is tightly regulated in part by corticosteroids and the receptor tyrosine kinase ligands HGF and EGF. These cytokines have been shown to play a role in liver regeneration as well. The model allows for other cytokines to be similarly investigated and derive information on the mechanisms controlling formation of hepatic tissue.

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