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REGULATION OF HEPATIC GENES AND LIVER TRANSCRIPTION FACTORS IN RAT HEPATOCYTES BY EXTRACELLULAR MATRIX

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SUMMARY: Culturing hepatocytes on different extracellular matrix (ECM) substrata including tissue culture plastic, type I collagen, Engelbreth-Holm-Swarm (EHS) gel and poly-N-p-vinylbenzyl-D-lactonamide (PVLA) regulated levels of mRNAs for cytoskeleton and liver-specific genes. In hepatocytes on EHS gel, the ratio of albumin/β-actin in mRNA levels was high and serially increased during the culture period, while the ratio was low and declined in cells on plastic substratum, collagen or PVLA. The changes in cellular levels of albumin mRNA which were regulated by ECM corresponded with those in two liver-specific transcription factors, hepatocyte nuclear factors-1 and -4, which control the transcription of liver-specific genes. These results suggest that cell-matrix interaction may determine and maintain the differentiated phenotype of hepatocytes by regulating liver-specific transcription factors. © 1995 Academic Press, Inc.

Adhesive interactions between cells and their surrounding extracellular matrix (ECM) play a vital role in tissue morphogenesis, cell migration, and the differentiation of a variety of cell types [1]. When isolated mature hepatocytes are cultured on a non-physiological substratum such as tissue culture plastic, or on a simple native substratum such as type I collagen, the cells rapidly lose their normal appearance, become flattened, and reduce liver-specific gene transcription dramatically [2]. In contrast, when hepatocytes are plated onto a model basement membrane, such as that derived from the Engelbreth-Holm-Swarm sarcoma (EHS gel) [3], or onto a synthetic substratum such as poly-N-p-vinylbenzyl-D-lactonamide (PVLA) [4], they appear to retain their normal cell polarity and structure, and the products of liver-specific genes including serum albumin continue to be secreted for prolonged periods of culture [5, 6]. Thus, the ECM may affect differentiation via distinct pathways. However, the precise mechanism(s) by which the ECM causes differentiated gene expression remains poorly defined.

It is well established that expression of liver specific serum protein genes such as albumin, transthyretin (TTR) and α_1-antitrypsin can be conferred by specific *cis*-acting DNA sequences

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located in the proximal promoter domain [7, 8]. Studies with these genes have demonstrated that sequences in their proximal promoter regions are highly conserved and that these sequences are the binding sites for *trans*-acting factors, namely liver-specific transcription factors [9, 10]. These factors, which are enriched in the mature hepatocyte, play an important role in the regulation of liver-specific gene transcriptions. Presently, four major families of "liver-specific" transcription factors, including hepatocyte nuclear factor (HNF)-1, HNF-3, HNF-4 and CCAAT/ enhancer binding protein (C/EBP), have been described [10].

To understand the cell-ECM interactions important for transcriptional activation, we assessed the abilities of different ECM substrata to activate two liver-specific transcription factors, HNF-1 and HNF-4. We also investigated the relationship between the expression of the liver transcription factors and the liver-specific gene expression or morphology of adult rat hepatocytes cultured on various ECMs.

MATERIALS AND METHODS

Cell Culture. Adult rat parenchymal hepatocytes were isolated from 6-week-old male Wistar rats by *in situ* 0.05% collagenase perfusion method as previously reported [11]. Aliquots (2ml) of the cell suspension were placed into 6-well plates at a concentration of 5 x 10^5 cells/ml in Williams' medium E (Dainippon Pharmaceutical Co., Osaka, Japan) supplemented with 50 ng/ml epidermal growth factor (Hitachi Chemical Industry, Tokyo, Japan), 10^{-7} M insulin (Wako, Osaka, Japan), 10^{-6} M dexamethasone (Wako, Osaka, Japan), 30 μg/ml kanamycin (Dainippon, Osaka, Japan) and 10 % fetal bovine serum (GIBCO, Grand Island, NY). Hepatocytes were cultured on either 35-mm tissue culture plastic, plastic dishes coated with 0.03 % type I collagen (Nitta Gelatin Co., Osaka, Japan), EHS gel (Beckton Dickinson Labware, Bedford, MA), or non-adhesive plastic dishes coated with 0.01 % PVLA solution.

Northern Blot Analysis. Total RNA was isolated from hepatocytes by the acid guanidium thiocyanide-phenol-chloroform method, separated by 1.2 % agarose gel electrophoresis, transferred to nylon membranes (Hybond-N, Amersham International, Amersham, UK), and hybridized to cDNA probes labeled with [α-^{32}P]dCTP using random primers made with the multiprime DNA labeling system (Amersham International, Amersham, UK) [12]. The various RNA blots were probed with the following; albumin, the EcoRI fragment (1.0-kb) of cDNA for rat albumin [13]; TTR, the AvaI fragment (600-bp) of cDNA for rat TTR [14]; β-actin, the EcoRI/BamHI fragment (600-bp) obtained from Japanese Cancer Research Resources Bank; HNF-1, the PstI fragment (1.0-kb) of cDNA for rat HNF-1[15]; HNF-4, the BamHI/EcoRI fragment (1.4-kb) of cDNA for rat HNF-4 [16].

Statistical Analysis. Spearman's correlation coefficient was used for determination of correlation.

RESULTS

Morphology of hepatocytes on different culture substrata. We confirmed the morphological changes of hepatocytes regulated by ECMs as previously reported [4-6]. Briefly, hepatocytes cultured on tissue culture plastic or plastic dishes coated with type I collagen assumed a flattened and extended shape. In contrast, hepatocytes cultured on EHS retained a spherical shape as single cells or as clusters. When incubated on PVLA substratum, cells formed anchored spherical multi-cell aggregates.

Effects of ECMs on mRNA levels for liver-specific genes. Northern analysis revealed that in cells on plastic substratum and type I collagen albumin mRNA increased over the first 4-day culture period but declined to the very low levels by 9 days in culture, whereas in cells on EHS gel or PVLA a strong hybridization signal for albumin mRNA was still present during the cell culture period (Fig. 1A). These albumin mRNA levels corresponded to the production of

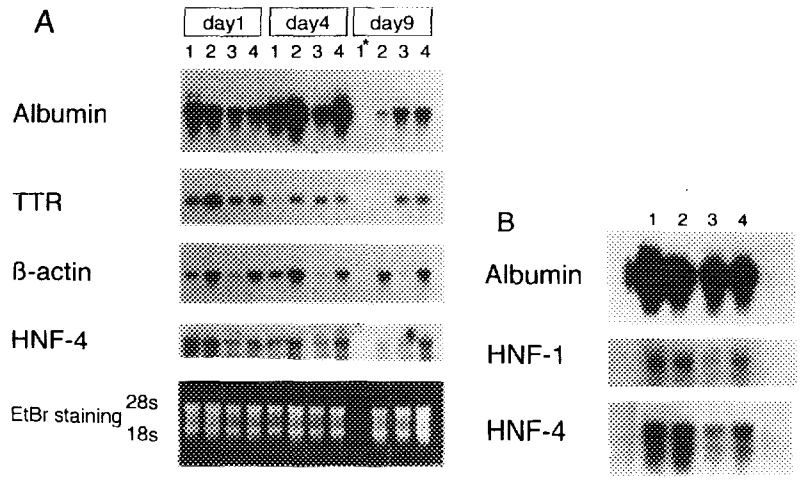
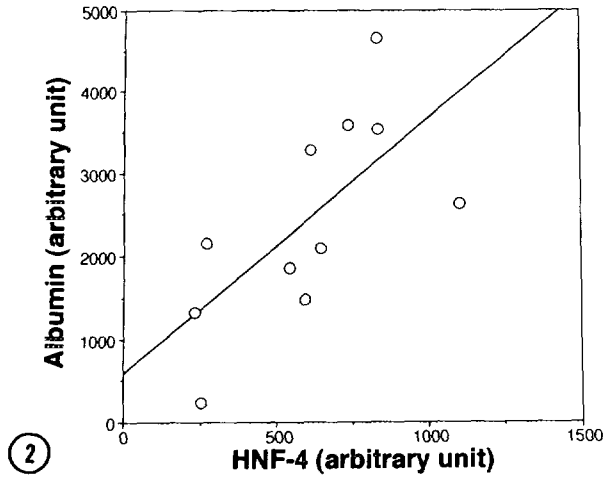


Fig. 1. (A) Effect of matrix on the time course of expression of various mRNAs in cultured hepatocytes. Total RNA was extracted at the indicated times from hepatocytes cultured on plastic (lanes 1), type I collagen (lanes 2), EHS gel (lanes 3), or PVLA (lanes 4) and assayed by Northern blot. All lanes contain 5 μg of total RNA. Ethidium-bromide-stained RNA immobilized on the nylon membrane used for the Northern blots, representing the analysis of equivalent amounts of intact total RNA isolated from the different hepatocyte cultures. * No sample on the lane. **(B) HNF-1 and HNF-4 mRNA expression in cultured hepatocytes 1 day after plating.** Total RNA extracted from hepatocytes cultured on plastic (lane 1), type I collagen (lane 2), EHS gel (lane 3), or PVLA (lane 4) at 1 day was analyzed by Northern blot. All lanes contain 10 μg of total RNA.

immunoreactive albumin protein secreted into the media by cultures derived from the same animal (data not shown). We found that alterations in TTR mRNA levels were similar to those in albumin mRNA and that cells on plastic substratum and type I collagen had lost detectable TTR mRNA by 9 days in culture.

Effects of ECMs on mRNA levels for liver-specific transcription factors. To determine whether the alterations observed in albumin and TTR mRNA expressions in response to the ECMs were regulated at the level of transcription, we measured the expressions of two liver-specific transcription factors, HNF-1 and HNF-4. Fig. 1A shows that adhesion to different ECMs caused marked changes in expression of HNF-4 mRNA in hepatocytes. The changes in HNF-1 mRNA level were coordinated with those in HNF-4 mRNA level (Fig. 1B). There was a significant positive correlation between the HNF-4 mRNA levels and albumin mRNA expressions ($r=0.80$, $P<0.01$), when the densitometric determinations of Northern blots were plotted, in hepatocytes on the various substrata (Fig. 2).

Effects of ECMs on actin mRNA levels. The expression of actin genes was also influenced by changes in cell shape regulated by the ECMs (Fig. 1A). The spherical cells on EHS gel exhibited the lowest actin mRNA levels, whereas the flattened monolayer cells on type I collagen showed the highest. To evaluate the ability of hepatocytes on various substrata to maintain the qualitative and quantitative expressions of liver-specific genes, associated with differentiated liver functions, we examined the ratio of albumin mRNA/actin mRNA (Fig. 3). The ratio was high and also serially increased in hepatocytes on EHS gel . By contrast, the ratio was constant



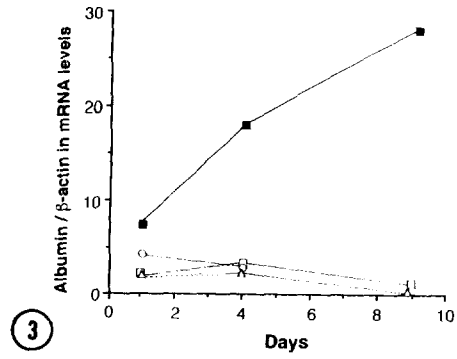


Fig. 2. Correlation between levels of albumin and HNF-4 mRNA. Quantitative analysis of results of Fig. 1A.

Fig. 3. Effect of matrix on the time course of albumin/β-actin mRNA ratio in cultured hepatocytes. Hepatocytes were cultured on plastic (open circles), plastic coated with type I collagen (open triangles), EHS gel (closed squares), or PVLA (open squares).

in hepatocytes on PVLA, or gradually decreased in hepatocytes on plastic substratum or type I collagen.

DISCUSSION

In the present studies, we have demonstrated that liver-specific genes and liver-specific transcription factors were coordinately modulated, probably through morphological changes, by ECM to which hepatocytes were attached. On collagen, cells formed a cobblestone arrangement of well-spread cells, exhibited higher actin mRNA levels and rapidly lost liver-specific mRNAs such as albumin and TTR mRNA, whereas on EHS gel they formed small aggregates of round cells, exhibited lower actin mRNA levels and maintained abundant levels of liver-specific gene expression in the culture period. We further evaluated the ratio of albumin mRNA/actin mRNA in hepatocytes on various ECMs. The data (Fig. 3) resulted in our understanding that EHS gel was the most effective at the maintenance of the differentiated phenotype of hepatocytes in cell culture. Recent studies have indicated the importance of overall cell morphology in regulating tissue-specific gene expression. Ben-Ze'ev et al. demonstrated that the organization of the cytoskeleton, which was dictated by the extent of cell-cell and cell-matrix interaction, was intimately associated with mechanisms that regulated tissue-specific gene expression [17]. Yuasa et al. have found that hepatocytes proceed through the cell cycle with suppression of their differentiated functions on conversion from spheroid to monolayer culture [18]. Other study, however, has demonstrated that the addition of dilute soluble EHS to dedifferentiated hepatocytes grown on type I collagen can increase albumin mRNA levels without altering cell shape or ultrastructure [19].

Some investigators have demonstrated that culturing hepatocytes on an ECM can stimulate albumin gene transcription [19-21]. To gain an insight into the mechanisms by which ECM

caused differentiated gene expression, we analyzed the activities of two liver transcription factors, HNF-1 and HNF-4, which control the transcription of hepatic genes. We have shown that HNF-1 and HNF-4 mRNA levels were influenced by ECM and that albumin mRNA which was regulated by ECM positively correlated with HNF-4 mRNA in hepatocytes on various culture substrata. This finding strongly suggested that ECM could stimulate cell differentiation by activating HNF-4 gene expression inasmuch as HNF-4 can *trans*-activate the HNF-1α promoter [22] and HNF-1 is important for synergistic activation of human albumin promoter with C/EBPα [23]. However, we cannot exclude the possibility that ECM could modulate other higher-ordered nuclear transcription factors. It is likely that there are more complex transcriptional regulatory networks on the activation of liver-specific genes. In fact, DiPersio et al. have shown that culturing a hepatocyte-derived cell line H2.35 on a collagen gel caused a selective increase in a liver transcription factor HNF-3α at the levels of mRNA [24]. Further studies are required to elucidate which liver-specific transcription factor(s) is primarily responsible for the regulation of cell differentiation by ECM and to define signal transduction pathways from ECM proteins that regulate liver-specific transcription factors.

In conclusion, our study suggests that ECM can regulate liver-specific gene by activating hepatic transcription factors, HNF-1 and HNF-4.

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