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Rapid communication

Hepatocyte nuclear factor-4α and -1 small interfering RNA inhibits hepatocyte differentiation induced by extracellular matrix

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Abstract

Liver-specific genes and hepatocyte nuclear factor (HNF)-4α and -1 are coordinately regulated by extracellular matrix (ECM). However, still are unclear interactions between liver-specific genes and these liver-enriched transcription factors in the mechanism of hepatocyte differentiation regulated by ECM. To elucidate the relationship, we used small interfering RNA (siRNA), which obtains strong and specific knockdown of gene expression in cell culture. Treatment with siHNF-4α and siHNF-1 declined the expression levels for HNF-4α mRNA and HNF-1 mRNA in primary rat hepatocytes, respectively. The mRNA expressions of albumin, transthyretin, and apolipoproteins that were up-regulated in hepatocytes cultured on a basement membrane matrix, Engelbreth-Holm-Swarm (EHS) gel, were decreased in the presence of siHNF-4α or siHNF-1. Moreover, siHNF-4α and siHNF-1 did not affect the morphology and actin assembly of hepatocytes. These findings demonstrated that HNF-4α and HNF-1 directly regulate liver-specific gene expression and might be downstream of cytoskeletal organization in the mechanism by which the differentiated phenotype of hepatocytes is regulated by EHS gel.

Keywords: Collagen; Engelbreth-Holm-Swarm; RNA interference; Transcription factor

1. Introduction

Cell adhesion to surrounding extracellular matrix (ECM) plays a pivotal role both in morphogenesis and in the regulation of gene expression in a variety of cell types. When isolated mature hepatocytes are cultured on dried type I collagen, they appear as a flattened monolayer and express low levels of liver function-specific mRNA and proteins [1,2]. In dramatic contrast, when hepatocytes are cultured on a model basement membrane, Engelbreth-Holm-Swarm (EHS) gel, hepatoytes retain their normal polarity and structure, and the products of liver-specific genes continue to be secreted for prolonged periods of culture [1,2]. Thus, the ECM may influence hepatocyte differentiation and the assembly of endocytic components.

Differentiation of mammalian cells is associated with changes in gene expression that are primarily controlled at the level of transcription. Tissue-specific gene transcription is regulated based on the recognition of *cis*-elements of their target genes, and accomplished by transcription factors that have restricted tissue distributions. Liver-specific gene expression is governed by the combinatorial action of a small set of liver-enriched transcription factors as follows: hepatocyte nuclear factor (HNF)-1, -3, -4α, and -6, and CCAAT/enhancer binding protein family, that are capable of modulating hepatocyte gene expression in hepatoma cells [3–5]. A series of independent findings have shown that HNF-4α may act the furthest upstream as a master gene in transcriptional factor cascade that would drive the differentiation of hepatic lineage [6,7]. We and other investigators have demonstrated

Abbreviations: ECM, extracellular matrix; EHS, Engelbreth-Holm-Swarm; HNF, hepatocyte nuclear factor; RNAi, RNA interference; dsRNA, double-stranded RNA; siRNAs, small interfering RNAs; RISCs, RNA induced silecing complexes; DIG, digoxigenin

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that liver-specific genes and liver-enriched transcription factors such as HNF-4α and HNF-1 are coordinately modulated by ECM to which hepatocytes are attached [2,8,9]. We have shown that well-differentiated hepatoma derived cell lines and primary hepatocytes transfected with HNF-4α cDNA restore differentiated gene expressions and liver-specific function by the overproduction of HNF-4α [10,11]. However, it is still unknown whether hepatocyte differentiation induced by ECM is directly regulated by HNF-4α or HNF-1.

RNA interference (RNAi) is the process by which double-stranded RNA (dsRNA) directs sequence-specific degradation of mRNA [12]. These long dsDNAs are processed by a RNase III-like enzyme Dicer into small interfering RNAs (siRNAs), short RNA duplexes of 21–23 nucleotides with two nucleotide 3′ overhangs on each strand [13]. siRNAs are incorporated into RNA induced silecing complexes (RISCs), which recognize and cleave target mRNA [14]. Transfection of mammalian cells with synthetic siRNAs 21–23 nucleotides in length obtains strong and specific suppression (knockdown) of gene expression by RNAi in cell culture [15,16].

To understand the involvement of HNF-4α and HNF-1 in the cell–ECM interactions that regulate liver cell differentiation, we investigated hepatocyte-specific gene expressions and cell morphology of hepatocytes cultured on EHS gel or type I collagen by inhibiting HNF-4α or HNF-1 using HNF-4α siRNA or HNF-1 siRNA, respectively.

2. Materials and methods

2.1. Hepatocyte isolation and culture

Adult rat parenchymal hepatocytes were isolated from 8- to 12-week-old male Wistar rats by an in situ 0.05% collagenase perfusion method as previously reported [17]. The viability of the hepatocytes was tested using trypan blue dye exclusion and found to be always above 85%. The freshly isolated rat hepatocytes were plated into 100 mm tissue culture dishes coated with type I collagen (Iwaki/Asahi Techno Glass, Tokyo, Japan) or Engelbreth-Holm-Swarm sarcoma (EHS) gel (Beckton Dickinson Labware, Bedford, MA) at a concentration of 5×10^5 cells/ml in Williams' medium E (Gibco, Carlsbad, CA) supplemented with 10^{-7} M insulin (Biosourse, Camarillo, CA), 10^{-8} M dexamethasone (Wako, Osaka, Japan), 1% penicillin and streptomycin (Gibco BRL, Life Technologies, Inc., Rockville, MD), and 10% heat-inactivated fetal bovine serum (Wako). Culture medium was changed after 4 h, and 1 day later, hepatocytes were cultured in the presence of control siRNA (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), HNF-4α siRNA (Santa Cruz Biotechnology, Inc.), or HNF-1 siRNA (Santa Cruz Biotechnology, Inc.) for 48 h. Transfection of siRNA was performed using target-specific siRNAs (Santa Cruz Biotechnology, Inc.) according to the manufacture's instructions. Briefly, 100 μl of 10 μM control siRNA, 10 μM HNF-4α siRNA, or 10 μM HNF-1 siRNA was added to 1200 μl siRNA transfection medium (Santa Cruz Biotechnology, Inc.), and then was gently mixed and kept at room temperature for 5 min. Sixty microliters of siRNA transfection reagent (Santa Cruz Biotechnology, Inc.) was added to 350 μl of siRNA transfection medium, and then was gently mixed and kept at room temperature for 5 min. To form siRNA–siRNA transfection reagent complex, siRNA transfection medium and siRNA transfection reagent were combined and incubated at room temperature for 20 min. The transfection mixtures were added directly to preincubated cells in 8.3 ml of antibiotic-free Williams' medium E with 10% serum.

2.2. Actin filament staining

Hepatocytes were fixed for 20 min with 4% paraformaldehyde followed by three washes with PBS. Cells were permeabilized with 0.1% Triton X-100 for 5 min and washed. To reduce nonspecific binding of antibody, cells were stained for 30 min with 1.0 U/100 μl FITC-conjugated phalloidin (Molecular Probes, Eugene, OR). After three washes with PBS, cells were examined by fluorescence microscopy.

2.3. Northern blot analysis

Total RNA (30 μg) was subjected to electrophoresis on a 1% agarose-formaldehyde gel and transferred to positive-charged nylon membranes (GeneScreen Plus; PerkinElmer Life Sciences, Inc., Boston, MA). The membranes were hybridized with cDNA probes for rat HNF-4α (sense, 5′-GGAATTCCGCCGACATGGACAGGCTG-3′; antisense, 5′-CGTCTAGAGCCTAGATGGCTTCCTGCTTGG-3′), HNF-1 (sense, 5′-GGAGGCCAGACCAATAACCA-3′; antisense, 5′-AACCGGCGCAAGGAAGAAGC-3′), albumin (sense, 5′-GCTGCTGACTTTGTTGAGGA-3′; antisense, 5′-GTTTGCTTCTTTATCTGCTT-3′), transthyretin (TTR) (sense, 5′-CTTGCTGGACTGGTATTTG-3′; antisense, 5′-GGAACCCTAACCACTGCTGT-3′), apolipoprotein AI (ApoAI) (sense, 5′-CAACTGGGACACTCTGGGTT-3′; antisense, 5′-AGCGACATCTCGGGTTTGAA-3′), and apolipoprotein CIII (ApoCIII) (sense, 5′-ACATGGAACAAGCCTCCAAG-3′; antisense, 5′-TGCCGAGTTCTCAACCACAA-3′), each of which was created by the PCR method using a digoxigenin (DIG) luminescent labeling kit (Roche Diagnostics, Mannheim, Germany).

2.4. Data analysis

All values in the figures are expressed as the means ± S.D. The significance of differences among mean values was evaluated by Student's *t*-test.