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Adenovirus-mediated hepatocyte nuclear factor-4α overexpression maintains liver phenotype in cultured rat hepatocytes ☆,☆☆

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Abstract

Hepatocyte nuclear factor 4α (HNF-4α) is a transcription factor that controls embryonal liver development and that maintains and regulates gene expression in adult liver cells. We have previously demonstrated that transient overexpression of HNF-4α up-regulates a number of liver-specific genes in hepatoma cell lines. In this study, we extend these studies by assessing the functional role of HNF-4α in regulating cellular viability and liver-specific functions of primary rat hepatocytes. In cells transfected with an adenovirus vector carrying rat HNF-4α cDNA, induction and maintenance of liver-specific genes and functions were observed over a long-term culture, which might be associated with the prevention of a rapid loss of the mitochondrial membrane potential. In addition, we demonstrated that transthyretin mRNA was up-regulated by HNF-4α in primary hepatocytes, but not in hepatoma cells. These results indicate that HNF-4α plays a role in the maintenance of morphologically and biochemically functional hepatocytes and that the difference in expression of liver-specific genes induced by HNF-4α may depend on a differentiation state of cells.

Keywords: Bioartificial liver; Hepatocyte differentiation; Transcription factor

Fulminant hepatic failure (FHF) is a condition in which the functions of a normal liver become severely impaired in a very short amount of time, leading to the development of an encephalopathy within 8 weeks of onset of the illness [1], or, in the case of late onset hepatic failure, at between 8 and 24 weeks of illness [2]. The two conditions are potentially reversible, but their mortality rates are still high despite recent advances in medical treatment. With the introduction of orthotropic liver transplantation as a therapeutic option for patients with FHF, the survival rate has improved [3], but due to limited organ availability only 10% of those afflicted by FHF receive transplantation. Under the development of biotechnology and tissue culture technology, artificial liver support systems or liver cell transplantation, which uses living hepatocytes, have been currently assessed in animal [4] and human trials [5,6].

Hepatocyte differentiation is associated with changes in gene expression that are primarily controlled at the level of transcription. Studies on the transcriptional

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☆☆ *Abbreviations:* HNF, hepatocyte nuclear factor; FHF, fulminant hepatic failure; C/EBP, CCAAT/enhancer binding protein; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; EHS gel, Engelbreth–Holm–Swarm gel; Apo, apolipoprotein; TTR, transthyretin.

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regulatory elements of genes expressed in hepatocytes have identified a number of liver transcription factors, including hepatocyte nuclear factor (HNF)-1, -3, -4, and -6, and CCAAT/enhancer-binding protein (C/EBP) family, that are capable of modulating hepatocyte gene expression in hepatoma cells [7–9]. HNF-4α is a member of the steroid hormone receptor superfamily [10]. A series of independent findings have shown that HNF-4α may act the furthest upstream as a master gene in a transcriptional factor cascade that would drive the differentiation of hepatic lineage. During development, HNF-4α mRNA is first detected in the primitive endoderm of the blastocyst [11]. This expression of HNF-4α early in the genes of the hepatic lineage precedes that of HNF-1, which has also been implicated in the regulation of liver gene expression [12]. In adults, HNF-4α is a positive regulator and activator of HNF-1 expression [10]. Genome-scale location analysis has revealed that the numbers of genes enriched in HNF-4α chromatin immunoprecipitation is much larger than that observed with typical site-specific regulators, and that HNF-4α is bound to about 12% of the genes represented on a human DNA microarray in hepatocytes, although no other transcription factors bind more than 2.5% of the promoter regions [13].

Recently, we constructed an adenovirus vector carrying rat HNF-4α cDNA and transfected HNF-4α into two well-differentiated hepatoma derived cell lines, HepG2 and HuH-7 cells. We have shown that these transfected cell lines restore differentiated gene expressions and liver-specific function by the overproduction of HNF-4α [14,15]. In the present study, we demonstrate that the overproduction of HNF-4α resulted in the maintenance of the cell viability and the maintenance a differentiated liver phenotype of primary hepatocytes as well as transformed liver cells.

Materials and methods

Hepatocyte isolation and culture. Adult rat parenchymal hepatocytes were isolated from 6-week-old male Wistar rats by an in situ 0.05% collagenase perfusion method as previously described [16]. Aliquots (10 ml) of the cell suspension were placed into 10 cm dishes coated with type I collagen (IWAKI, Chiba, Japan) at a concentration of 5×10^5 cells/ml in Williams' medium E (Gibco-BRL, Rockville, MD, USA) supplemented with 10^{-9} M insulin (Wako, Osaka, Japan), 10^{-8} M dexamethasone (Wako), 1% penicillin, 1% streptomycin (Gibco-BRL), and 5% fetal bovine serum (Sigma–Aldrich, Irvine, UK).

Recombinant adenovirus vector. Recombinant adenoviruses carrying rat HNF-4α cDNA or LacZ cDNA were constructed as previously reported [14,17]. After the overnight inoculation of isolated rat hepatocytes, the medium was changed to serum free medium containing the purified recombinant adenoviruses at 1 multiplicity of infection (m.o.i.) for 1 h.

β-Galactosidase cytochemical assay. Determination of LacZ gene expression was carried out according to Miyake et al. [17]. Briefly, the fixed cells were rinsed twice with phosphate-buffered saline (PBS), incubated in a reaction mixture containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$ and 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in PBS for 1 h at 37 °C, and then washed three times with PBS.

Crystal violet assay. Cell viability was measured by Crystal violet assay. In brief, cells cultured in 12-well dishes were fixed by 25% glutaraldehyde for 10 min, gently rinsed twice by PBS, and stained by 1 ml of 4% crystal violet (Sigma–Aldrich) solution for 5 min. After gentle rinsing with distilled water, cells were dissolved with 1 ml of 1% SDS (sodium dodecyl sulfate) solution. Concentration of crystal violet in the cell lysis solution was determined colorimetrically at 570 nm.

Mitochondrial membrane potential assay. The mitochondrial membrane potential was visualized by vital staining of mitochondria with 10 μM Rhodamine 123 (Sigma–Aldrich). Rhodamine 123 was added and the solution was incubated for 30 min. pH-sensitive fluorescence of Rhodamine 123 was observed in a Bio-Rad MRC-1024 laser scanning confocal imaging system (Bio-Rad Laboratories, Hercules, CA), with a Kr–Ar ion laser equipped with a Nikon Diaphot 300 inverted fluorescence microscope (Nikon, Kawasaki, Japan).

Northern blot analysis. Total RNA (10–40 μg) was separated by electrophoresis on a 1% agarose–formaldehyde gel and transferred onto GeneScreen Plus, positively charged nylon membranes (Perkin Elmer Life Sciences, Boston, MA). The membranes were hybridized with specific cDNA probes for human HNF-4α, apolipoprotein AI (ApoAI), α1-antitrypsin, and transthyretin (TTR), each of which was made by a PCR method using a digoxigenin (DIG) luminescent labeling kit (Roche Diagnostics, Mannheim, Germany). The primers and PCR conditions have been reported previously [15].

Ammonia metabolism analysis. Forty-eight hours after HNF-4α transfection, the medium was changed to DMEM (Sigma–Aldrich) supplemented with 0.25 mM NH_4Cl and the cells were incubated for 2 h. The ammonia concentration of the medium was determined colorimetrically at 630 nm using a Wako Chemical kit (Wako).

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

Results

Cell viability was maintained by HNF-4α overexpression

To determine the viral infection in primary cultured rat hepatocytes, we first assessed the expression of β-galactosidase in the cells infected by the control adenovirus AdLacZ. AdLacZ infection at 1 m.o.i. induced β-galactosidase expression in almost all rat hepatocytes (data not shown). Phase-contrast photomicrographs revealed that no apparent changes in cell shape were observed in primary rat hepatocyte transfected at 1 m.o.i. by AdLacZ or by AdCAGHNF4 over a 24 h culture period (Fig. 1).

Ninety-six hours after the treatment with adenoviruses for 1 h, cell viability of HNF-4α-transfected hepatocytes was maintained to a twofold greater extent than cells with AdLacZ or cells without adenovirus transfection (Fig. 1).

Mitochondrial function was maintained in HNF-4α overexpressing hepatocytes

To investigate the mechanism by which the cell viability of HNF-4α-overexpressing hepatocytes was