Involvement of Egr-1 in HGF-induced elevation of the human 5α-R1 gene in human hepatocellular carcinoma cells

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INTRODUCTION

HCC (hepatocellular carcinoma), one of the most common malignancies, has a striking increased predominance in males [1]. Not only do males develop HCC more often than females, but also males die sooner than females once they develop the cancer. Various studies in experimental animal models have also shown that androgens are associated with an increased incidence of liver neoplasms. Agnew and Gardner [2] reported the increased incidence of hepatic tumours in mice chronically exposed to androgens. The elevation of hepatic androgen content has been observed in chemically induced hepatocarcinogenesis in rats [3]. Moreover, the growth and proliferation of hepatic tumour cell lines have been shown to be enhanced by DHT (dehydrotestosterone) and testosterone [4]. Furthermore, long-term use of androgenic steroids is associated with an increased risk of developing liver neoplasms, including HCC [5–8].

The enzyme 5α-R (steroid 5α-reductase) is a key enzyme in the conversion of steroids into their respective 5α-reduced derivatives, plays a key role in some hormone-dependent tumours and is abundant in the liver, although it is also widely distributed throughout the body. HGF (hepatocyte growth factor) is a pleiotropic cytokine/growth factor involved in the progression of hepatocellular carcinoma. In the present paper, we report the stimulatory effect of HGF on human 5α-R1 transcription in hepatocellular carcinoma cells. Pre-treatment with actinomycin D or cycloheximide blocked the up-regulation of 5α-R1 mRNA expression by HGF, indicating that the increased level of 5α-R1 mRNA expression is regulated by transcriptional activation and was dependent on de novo protein synthesis. Functional analysis of the 5′-flanking region of the 5α-R1 gene by transfection analysis showed that the −79 to −50 region functioned as the HGF-responsive region. Mutagenesis and electrophoretic mobility-shift assays demonstrated that induction of 5α-R1 by HGF is mediated by an Egr-1 (early growth-response gene 1)-binding site at −60/−54. In addition, overexpression of Egr-1 was sufficient to transactivate 5α-R1 promoter activity, and knockdown of Egr-1 with gene-specific small interfering RNA resulted in inhibition of HGF-induced up-regulation of endogenous 5α-R1 expression. These data provide the first evidence that HGF stimulates 5α-R1 expression through up-regulation of the transcription factor Egr-1, thus suggesting the possibility that regulation of steroid metabolism by HGF represents a mechanism for high risk of hepatocellular carcinogenesis in males.

Key words: early growth-response gene 1 (Egr-1), hepatocellular carcinoma, hepatocyte growth factor (HGF), steroid 5α-reductase 1 (5α-R1), transcriptional regulation.

Abbreviations used: 5α-R, steroid 5α-reductase; ACE, angiotensin-converting enzyme; Act D, actinomycin D; CHX, cycloheximide; DHT, dehydrotestosterone; Egr-1, early growth-response gene 1; EMSA, electrophoretic mobility-shift assay; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; NGF, nerve growth factor; RDA, cDNA representational difference analysis; RT, reverse transcription; siRNA, small interfering RNA.

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MATERIALS AND METHODS

Cell lines and reagents

Human HCC cell lines HepG2, Hep3B, Bel-7402 and SMMC-7721 were purchased from the Type Culture Collection of The Chinese Academy of Sciences, Shanghai, China. All cell lines were maintained in DMEM (Dulbecco’s modified Eagle’s medium) (Gibco®) with 10% (v/v) fetal calf serum. Recombinant human HGF, Act D (actinomycin D) and CHX (cycloheximide) were purchased from Sigma–Aldrich.

RDA (cDNA representational difference analysis)

Double-stranded cDNA was prepared by RT (reverse transcription) of 2 μg of poly(A) mRNA using the Universal Ribocloner® cDNA Synthesis System (Promega). RDA was performed as described by Xu et al. [25]. Briefly, the driver representation consisted of cDNA generated from the pooled mRNA of normal HepG2 cells. This representation was subtracted from tester cDNA representation of the mRNA repertoire of HepG2 cells treated with HGF for 1 h. After three rounds of subtraction (driver excess: 50×, 400× and 10000× in successive rounds) and amplification, the entire third difference products were subcloned into the pGEM-T easy vector (Promega) and sequenced using the ABI Prism 3100 Genetic Analyzer (Applied Biosystems). A homology search of genes was performed using the online-based BLAST program at the NCBI.

RT–PCR

Total RNA was isolated using TRIzol® (Gibco) and was reverse-transcribed using RT–PCR kits (Promega) according to the manufacturer’s instructions. The 5α-R1 forward primer was 5′-ATGGCAACGGCAGCGGAGGTGGCGGAGG-3′, and the reverse primer was 5′-CAGGGCATACCACACCTCCATGATT-3′. G3PDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal control (G3PDH forward primer was 5′-ACCACGTCTCAGCCTCCA-3′, and the reverse primer was 5′-CAGGGGACCCAGG-3′). The PCR products were electrophoresed on 1% agarose gels and photographed under UV light.

Northern blotting

Northern blotting was performed as described previously [25]. Briefly, total RNA was fractionated on a 1% agarose gel that contained 0.6 mol/l formaldehyde, transferred to a GeneScreen™ Plus membrane (PerkinElmer) and hybridized to 32P-labelled cDNA probes. Hybridization to all probes was carried out at 68°C in 6× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate), 5× Denhardt’s solution (0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA), 1% SDS and 100 mg/ml single-stranded DNA. The blots were washed in 2× SSC/0.05% SDS for 20 min at room temperature (25°C), followed by 20 min washes in 0.1× SSC/0.1% SDS at 50°C before exposure to an X-ray film. The probes for human 5α-R1 and G3PDH were generated by PCR.

Western blotting

Cells cultured on the plate were lysed with M-PER® Mammalian Protein Extraction Reagent (Pierce) according to the manufacturer’s instructions. Then, 20 μg of protein from each sample was loaded on to the gel. After separation by SDS/PAGE (10% gels), proteins were transferred on to PVDF membranes and probed with various antibodies (all from Santa Cruz Biotechnology) at the following concentrations: anti-Egr-1 (1:1000), anti-5α-R1 (1:500), and anti-β-actin (1:1000). Chemiluminescence detection was conducted using SuperSignal substrate (Pierce) according to the manufacturer’s specifications.

Enzyme activity assay

The enzyme activity assay of 5α-R1 in cells was performed as described in [26]. Briefly, 105 cells were seeded in a six-well tissue culture plate and incubated overnight to allow the cells to attach. Then the cells were serum-starved for 12 h followed by treatment with 20 ng/ml HGF. After 8 h, 1 μM testosterone (PerkinElmer) was added at a concentration of 7.5 nM. Samples were taken at 60 min and measurement was performed. Sample treatment and HPLC analysis was performed as described by Reichert et al. [26].

Plasmid constructions

The −2647 to +240 region relative to the transcription start site of 5α-R1 was PCR-amplified using primers: P−2647, 5′-CCCAAGCTTTGTTAATTGCGAGCCGCC-3′, and P+240, 5′-CGACGCGTACACTGAGTTCGTCGACATG-3′. The PCR product was sequenced using HindIII and NcoI and inserted in a promoterless luciferase reporter vector pGL3-basic (Promega), to generate pGL3−(−2647/+240). A series of 5′-truncated constructs, pGL3−(−1810/+240), pGL3−(−1151/+240), pGL3−(−840/+240), pGL3−(−514/+240), pGL3−(−168/+240), pGL3−(−79/+240) and pGL3−(−50/+240), were generated by PCR using P+240 as the 3′-end primer and the following primers: P−1810, 5′-CCCAAGCTTTGTTAATTGCGAGCCGCC-3′; P−1151, 5′-CCCAAGCTTTGTTAATTGCGAGCCGCC-3′; P−840, 5′-CCCAAGCTTGTAATTGCGAGCCGCC-3′; P−514, 5′-CCCAAGCTTGTAATTGCGAGCCGCC-3′; P−168, 5′-CCCAAGCTTGTAATTGCGAGCCGCC-3′; P−79, 5′-CCCAAGCTTGTAATTGCGAGCCGCC-3′; and P−50, 5′-CCCAAGCTTGTAATTGCGAGCCGCC-3′ respectively. The numbers in parentheses indicate the boundaries of the inserts relative to the transcription start site. The pCMV-Egr-1 vector was kindly provided by Dr Eileen Adamson (Cancer Research Center, Burnham Institute, La Jolla, CA, U.S.A.).

Mutagenesis

Oligonucleotide-directed mutagenesis with the overlap-extension PCR amplification method was used to generate a mutant Egr-1 binding site in the context of pGL3−(−79/+240) by transversion of GGGCC to GATTC at −60/−54. The mutated segments furnished with HindIII and NcoI sites were subcloned directly into pGL3-basic vector [pGL3-m(−79/+240)].

EMSA (electrophoretic mobility-shift assay)

The nuclear proteins were prepared using the NE-PER Nuclear and Cytoplasmic Extraction reagents (Pierce). The sequences of the upper strands of the oligonucleotides used for EMSA were Egr-1−(−70/−45), 5′-CCCCCGCCCCGGGGGCGGACCACA-3′; and mEgr-1−(−70/−45), 5′-CCCCGGCCGGGGAATTCGACCACA-3′. The oligonucleotide containing the Egr-1 consensus binding site 5′-CCCCGGCCGGGGAATTCGACCACA-3′ was used as reported previously [27]. Labelling, binding reactions, electrophoresis and detection were carried out with a Gel Shift Assay System (Promega) according to the manufacturer’s instructions. For the competitor assay, a 50-fold excess of unlabelled double-stranded DNA was added to the reaction.
expression was very low in untreated cells, increased gradually and was extracted and subjected to RT–PCR (Figure 1A) and Northern blot analysis. To isolate genes induced directly by HGF in human hepatoma cells, HepG2 cells were serum-starved for 12 h and then treated with 20 ng/ml recombinant human HGF for 1 h. Total RNA was extracted and an RDA was performed. After three rounds of subtraction and amplification, a total of 38 subtracted clones were extracted and an RDA was performed. After three rounds of

Table 1 Genes up-regulated in HepG2 cells treated with HGF

<table>
<thead>
<tr>
<th>Gene name</th>
<th>NCBI definition</th>
<th>Accession no.</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKHD1</td>
<td>Polycystic kidney and hepatic disease 1</td>
<td>NM_138694</td>
<td>5</td>
</tr>
<tr>
<td>DAP2</td>
<td>Cytoskeleton-associated protein 2</td>
<td>NM_001098525</td>
<td>7</td>
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<td>Steroid-5α-reductase 1</td>
<td>NM_001047</td>
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<tr>
<td>DKK2p6496p09249</td>
<td>None</td>
<td>BX641993</td>
<td>6</td>
</tr>
<tr>
<td>GH1</td>
<td>Growth hormone 1</td>
<td>NM_000515</td>
<td>7</td>
</tr>
</tbody>
</table>

siRNA (small interfering RNA) transfection

The siRNA oligonucleotides of Egr-1 were synthesized by GenePharma Biotechnology as follows: sense, UUCUCCGAAACGUGUCACGCUdTdT; and antisense, ACGUGACACGUUCGAGAAdTdT. The negative control (non-silencing) siRNA was obtained from Qiagen. RNAs were transfected into HepG2 cells using TransIT-TKO® (Roche) at a concentration of 20 nM according to the manufacturer’s instructions.

Transfection and luciferase assay

Cells were plated in six-well plate at a density of 2 × 10⁵ cells/well. Transfections were performed with LipofectamineTM 2000 reagent (Invitrogen). The pRL-TK vector was used as an internal control and co-transfected. At 24 h after transfection, cells were harvested, and activities of firefly and Renilla luciferases were measured using the Dual-Luciferase Assay system (Promega). The activity of firefly luciferase was normalized to the activity of Renilla luciferase. All transfections and reporter assays were carried out each at least three times.

Statistical analysis

All experiments were performed at least three times. Data are reported as means ± S.E.M. and the statistical significance was assessed by one-way ANOVA followed by Students–Newman–Keuls tests. P ≤ 0.05 was considered to be significant.

RESULTS

5α-R1 can be induced transiently by HGF in human HCC cell lines

To isolate genes induced directly by HGF in human hepatoma cells, HepG2 cells were serum-starved for 12 h and then treated with 20 ng/ml recombinant human HGF for 1 h. Total RNA was extracted and an RDA was performed. After three rounds of subtraction and amplification, a total of 38 subtracted clones were randomly picked for sequencing analysis and a BLAST database search. Table 1 summarizes all the up-regulated genes. Among the 38 clones, eight clones were identical with 5α-R1, suggesting that 5α-R1 can be induced by HGF in HepG2 cells.

To confirm the effect of HGF on 5α-R1 expression, HepG2 cells were serum-starved for 12 h and then treated with 20 ng/ml HGF for various time periods as indicated (Figure 1). Total RNA was extracted and subjected to RT–PCR (Figure 1A) and Northern blot analysis (Figure 1B). As Figure 1A shows, 5α-R1 mRNA expression was very low in untreated cells, increased gradually after HGF treatment, reaching a maximum level after 60 min, and decreased at 2 h. Western blot analysis was performed to analyse the effect of HGF on the protein level of 5α-R1. As shown in Figure 1C, HGF caused a pronounced induction of 5α-R1 protein expression, with a peak after 8 h. To confirm that the induction of the 5α-R1 protein correlated with the increased functional level, the enzyme activity of 5α-R1 was assayed. As shown in Figure 1(D), the enzyme activity of 5α-R1 was also induced by HGF in HepG2 cells. To demonstrate further that the induction of 5α-R1 by HGF is a general phenomenon in hepatoma cells, several other human HCC cell lines such as Hep3B, Bel-7402 and SMMC-7721 were investigated and similar results were obtained (Figures 1D and 1E).

To probe into the mechanism of HGF-dependent 5α-R1 gene induction, HepG2 cells were pre-treated with the transcription inhibitor Act D (10 μM) or the protein synthesis inhibitor CHX (15 μg/ml), and the cells were then incubated in the presence or absence of HGF. Total RNA was prepared and subsequently RT–PCR analysis was performed. As Figure 1(F) shows, induction of 5α-R1 mRNA expression by HGF was blocked by pre-treatment with Act D, suggesting that the induction of 5α-R1 by HGF was regulated at the transcriptional level. In addition, the lack of increased 5α-R1 mRNA expression in the presence of CHX suggested that stimulation of 5α-R1 expression by HGF was dependent on new protein synthesis. Therefore these observations indicate that the up-regulation of 5α-R1 mRNA expression by HGF is mediated at the transcriptional level and requires de novo protein synthesis.

Isolation of the human 5α-R1 promoter

To analyse the molecular mechanism of HGF-dependent 5α-R1 gene induction in detail, we isolated a 2887 bp fragment containing a 2647 bp upstream sequence and a 240 bp downstream sequence from the transcription start site of 5α-R1 from human genomic DNA by PCR, and generated a reporter plasmid, pGL3−(−2647/+240), by introducing the 2887 bp fragment into the promoterless plasmid pGL3-basic.

The pGL3−(−2647/+240) vector was transfected into HepG2 cells, and the cells were treated with HGF at the concentration indicated (Figure 2). After 24 h, the luciferase activities were measured. As shown in Figure 2, the luciferase activity increased dramatically with increasing treatment with HGF compared with that exhibited by the HGF-un-treated control. The results indicate that exposure to HGF resulted in increased 5α-R1 promoter activity and the potential cis-elements responsible for the HGF effect were present in the region − 2647/+ 240.

Identification of the HGF-responsive region in the 5α-R1 promoter

To identify specific DNA regions of the promoter that are responsible for HGF-induced stimulation of 5α-R1 promoter activity, a series of 5′-truncated plasmids were transiently transfected into HepG2 cells. After 24 h of HGF treatment, the luciferase activity was measured. As Figure 3 shows, the deletion of fragments from 5′ to 2647 showed significant responsiveness to HGF with a 3-fold increase in promoter activity, whereas the deletion of −79 to −50 lost the responsiveness to HGF. The results indicate that the region between −79 and −50 contains essential elements for 5α-R1 induction by HGF.

HGF stimulation of 5α-R1 promoter activity through the Egr-1-binding site at −60/−54

To elucidate further the molecular mechanism by which HGF stimulates 5α-R1 expression, we investigated the potential involvement of candidate transcription factors. Several studies have
Figure 1  Induction of 5α-R1 by HGF treatment in HepG2 cells

HepG2 cells were serum-starved for 12 h and then treated with 20 ng/ml HGF for the indicated times, and levels of 5α-R1 mRNA were assessed by RT–PCR (A) and Northern blot analysis (10 μg per lane) (B). G3PDH was used as an internal control. (C) 5α-R1 protein was detected by Western blot analysis (20 μg per lane). β-Actin was used as an internal control for protein loading. Molecular masses are indicated in kDa (kD). (D) Enzyme activity of 5α-R1 was increased in the cell lines indicated when treated with HGF. The liver cells were serum-starved for 12 h and treated with HGF. After 8 h, the enzyme activity of 5α-R1 was calculated. The percentage conversion was calculated as DHT radioactivity (c.p.m.)/testosterone (c.p.m.) radioactivity × 100. Results are means ± S.E.M. of triplicate measurements. *P ≤ 0.05; **P ≤ 0.001. (E) The cells indicated were pre-treated with 10 μM Act D or 15 μg/ml CHX for 30 min before HGF addition. The levels of 5α-R1 mRNA were assessed by RT–PCR. G3PDH was used as an internal control. Lanes M in (A), (E) and (F) are molecular-mass markers.

Figure 2  HGF induces the promoter activity of 5α-R1

HepG2 cells were transiently transfected with 500 ng of pGL3(-2467/+240) plasmid/well of a six-well plate and then treated with HGF at the concentrations indicated for 24 h. Then the relative luciferase activity was measured. The transfections were normalized according to Renilla luciferase activity. Results are means ± S.E.M. for three independent experiments. *P ≤ 0.05; **P ≤ 0.001.

shown that HGF up-regulates many genes, including PDGF (platelet-derived growth factor) and VEGF (vascular endothelial growth factor) [28], CD44v6 [29], ACE (angiotensin-converting enzyme) [30] and fibronectin [31], through Egr-1. A recent paper reported that, in HepG2 cells, HGF can induce Egr-1 [32]. Egr-1 is a nuclear zinc-finger transcription factor capable of binding to specific GC-rich DNA sequences containing the consensus binding site GCG(G/T)GGGCG [33]. A survey of the region
site abolished the response to HGF, clearly indicating that the Egr-1 motif GGGGCC to GAA TTC. The mutant constructs were transiently transfected into HepG2 cells, which were then treated with HGF, we next examined the regulation of Egr-1 by HGF. HepG2 cells were made quiescent by serum starvation and then stimulated with HGF for up to 2 h. Results show that Egr-1 mRNA expression with HGF for 60 min. Specific antibody against Egr-2 and normal rabbit IgG were used as negative controls.

To test whether the Egr-1-binding site at position −54 was critical for the induction of 5α-R1 expression by HGF was associated with increased binding activity of Egr-1 protein to the Egr-1 site at −60/−54.

HGF-induced Egr-1 binding to the Egr-1 consensus site at −60/−54

To examine whether Egr-1 bound to the site at −60/−54, EMSAs were performed using nuclear extracts from HGF-treated or untreated HepG2 cells and 32P-labelled double-stranded fragments of −70/−45. As shown in Figure 5A, nuclear extracts from HGF-treated cells formed a stronger DNA–protein complex with the probe compared with extracts from control cells, suggesting that nuclear proteins recognizing the Egr-1-binding site were present in nuclear extracts of HepG2 cells treated with HGF.

Transactivation of 5α-R1 promoter by Egr-1 protein

To determine directly whether Egr-1 protein could functionally modulate 5α-R1 promoter activity, pGL3−(−79/+240) vector was co-transfected with the different doses of pCMV-Egr-1 vector indicated in Figure 6(A) into HepG2 cells. The luciferase activity assay suggested that pCMV-Egr-1 stimulated the 5α-R1 promoter activity in a dose-dependent manner (Figure 6A). Moreover, overexpression of Egr-1 did not stimulate the luciferase activity of pGL3−m(−79/+240) (Figure 6B) in which the Egr-1-binding site at −60/−54 was mutated. These results indicate that overexpression of Egr-1 transactivated the 5α-R1 promoter via the Egr-1-binding site at position −60/−54.

Egr-1 was induced by HGF stimulation in HepG2 cells

Since Egr-1 is an inducible intracellular signal transducer for HGF, we next examined the regulation of Egr-1 by HGF. HepG2 cells were made quiescent by serum starvation and then stimulated with HGF for up to 2 h. Results show that Egr-1 mRNA expression was very low in untreated HepG2 cells and increased gradually after HGF treatment, reaching a maximum level after 15 min and

Figure 4. HGF stimulation of 5α-R1 promoter activity through the Egr-1-binding site at −60/−54

(A) DNA sequence of the 5′-flanking region of the human 5α-R1 gene. The transcription initiation site is indicated by the arrow, the translation start site is boxed, and the putative transcription factor-binding sites are underlined. Note the Egr-1-binding site at −60/−54.

(B) Gel–(−79/+240) or the mutant pGL3−m(−79/+240) was transiently transfected HepG2 cells and treated with 20 ng/ml HGF. The assay for relative luciferase activity was performed 24 h post-transfection. The transfections were normalized according to Renilla luciferase activity. Results are means ± S.E.M. for three independent experiments. **P < 0.001;

−79/−50 using a program TFSEARCH (http://www.cbrc.jp/research/db/TFSEARCH.html) also identified a putative Egr-1-binding site at position −60/−54 (Figure 4A).

To test whether the Egr-1-binding site at −60/−54 is functional, mutagenesis on the site was performed to alter the Egr-1 motif GGGGCC to GAATTC. The mutant constructs were transiently transfected into HepG2 cells, which were then treated with HGF. The luciferase activity assay showed that the mutation abolished the response to HGF, clearly indicating that the Egr-1 site at −60/−54 was critical for the induction of 5α-R1 by HGF (Figure 4B).

Figure 5. HGF induces Egr-1 binding to the Egr-1 consensus site at −60/−54

(A) Nuclear extracts from HepG2 cells treated with HGF for the times indicated were prepared for EMSA analysis. Nuclear protein (10 μg) was combined with radiolabelled oligonucleotide −70/−45. For supershift assay, anti-Egr-1 antibodies were added to the nuclear extract of HepG2 cells treated with HGF for 60 min. Specific antibody against Egr-2 and normal rabbit IgG were used as negative controls.

(B) Competition of Egr-1–DNA complex with the Egr-1 consensus oligonucleotide or the fragment of −70/−45. Nuclear extracts (NE) of HepG2 cells treated with HGF for 1 h were isolated for EMSA, and the oligonucleotide −70/−45 was radiolabelled as a probe. Competition experiments were performed in the presence of 50-fold excess of unlabelled oligonucleotide as indicated.

To confirm whether the protein complexes that bound to the site in the HepG2 nuclear extracts were indeed Egr-1, gel–supershift assays were undertaken with the Egr-1 antibody and nuclear extracts from HepG2 cells treated with HGF. As expected, the band disappeared upon addition of anti-Egr-1 antibody (Figure 5A). However, the antibody against Egr-2 and a normal rabbit IgG did not inhibit the formation of the DNA–protein complex. Competition experiments showed that the binding of Egr-1 was effectively and specifically inhibited by a 50-fold molar excess of either Egr-1 consensus oligonucleotide or the fragment −70/−45. In contrast, an excess of an oligonucleotide with a targeted mutation in the Egr-1 site (m−70/−45) did not affect the intensity of this band (Figure 5B).

All of these results suggest that up-regulation of 5α-R1 expression by HGF was associated with increased binding activity of Egr-1 protein to the Egr-1 site at −60/−54.
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Gradually decreasing at 2 h (Figure 7A). Western blot analysis demonstrated that HGF caused a pronounced induction of Egr-1 protein expression with a peak after 1 h (Figure 7B). These data suggest that HGF-induced 5α-R1 expression is mediated by the up-regulation of Egr-1 protein.

Knockdown of HGF-induced Egr-1 leads to down-regulation of HGF-induced 5α-R1 mRNA expression

To confirm the involvement of Egr-1 in HGF-induced endogenous 5α-R1 expression, we investigated the effect of Egr-1 knockdown by using an Egr-1 siRNA duplex. HepG2 cells were transfected with either a control non-silencing siRNA or Egr-1-specific siRNA, followed by treatment with or without HGF. To examine the silencing effect of Egr-1 siRNA, Western blot analysis was performed using anti-Egr-1 antibody and whole-cell lysates from untransfected, control siRNA- and Egr-1 siRNA-transfected cells supplemented with or without HGF. As shown in Figure 8(A), in untransfected cells and cells transfected with the control siRNA, Egr-1 was highly expressed, reaching a maximum level at 1 h, and decreasing at 2 h, which is consistent with the results shown in Figure 7(B). However, in cells transfected with Egr-1 siRNA, the Egr-1 level was reduced at all time points compared with the controls. The effect of Egr-1 knockdown on HGF-induced 5α-R1 expression was determined by Western blot analysis using whole-cell lysates from cells treated with HGF as described above. As presented in Figure 8(B), the transfection of HepG2 cells with the control siRNA did not show any inhibitory effect on HGF-induced 5α-R1 expression, whereas transfection with the Egr-1 siRNA led to down-regulation of HGF-induced 5α-R1 expression. These results indicate that the blockade of Egr-1 overexpression by siRNA prevented HGF-stimulated endogenous 5α-R1 expression and provided further evidence for the involvement of Egr-1 in the HGF-induced up-regulation of human 5α-R1 expression.

DISCUSSION

In the present study, we showed that HGF is a strong inducer of 5α-R1 in HepG2 cells. Luciferase activity and EMSA revealed that an Egr-1-binding site at −60/−54 was critical for the induction of 5α-R1 by HGF. Overexpression of Egr-1 significantly transactivated the promoter activity of 5α-R1. After HGF treatment, Egr-1 gene expression peaked by 1 h, followed by the peak in 5α-R1 gene expression 8 h later, consistent with the need for Egr-1 protein expression for induction of it. Furthermore, the blockade of Egr-1 overexpression by Egr-1-specific siRNA blocked endogenous HGF-induced 5α-R1 expression and provided further evidence for the involvement of Egr-1 in the HGF-induced up-regulation of human 5α-R1 expression.

Enzymes involved in hormone metabolism can influence hormonal activities and risk of hormone-dependent cancers. 5α-R1 is well known as the enzyme that catalyses the conversion of testosterone into the more potent androgen DHT. The conversion of testosterone into DHT by 5α-R1 is implicated in various disease states, including benign prostatic hyperplasia, hirsutism and cancer. Metabolism studies have shown that mammary tissues from several species including humans [34,35] and several human breast cell lines [36,37] exhibit...
involved in the elevation of 5α-R1 induced by HGF also suggested that 5α-R1 might act as a target gene of Egr-1 in mediating the progression of HCC induced by HGF. Although the Egr-1 element appeared to be essential in 5α-R promoter activity, this does not exclude the possibility of an involvement of other regulatory elements. The deletion mutant pGL3-514 showed a 2-fold increase in promoter activity compared with pGL3-728, indicating that the region between −728 and −514 may contain other critical negative regulatory elements. Identification of these transcription factors may facilitate understanding of the mechanism for transcriptional regulation of the 5α-R1 gene in liver cells.

In conclusion, data presented in this study are the first to demonstrate that the up-regulation of 5α-R1 in response to HGF is dependent on an Egr-1-binding site on the promoter at −60/−54, suggesting a correlation between HGF and the enzyme involved in androgen metabolism in HCC.

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