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• BASIC RESEARCH •

Identification and characterization of a novel isoform of hepatopoietin

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Abstract

AIM: **To isolate a novel isoform of human HPO (HPO-205) from human fetal liver Marathon-ready cDNA and characterize its primary biological function.**

METHODS: **5'-RACE (rapid amplification of cDNA 5' ends) was used to isolate a novel isoform of hHPO in this paper. The constructed pcDNA$^{HPO-205}$, pcDNA^{HPO} and pcDNA eukaryotic expression vectors were respectively transfected by lipofectamine method and the stimulation of DNA synthesis was observed by ^{3}H-TdR incorporation assay. Proteins extracted from different cells were analyzed by Western blot.**

RESULTS: **A novel isoform of hHPO (HPO-205) encoding a 205 amino acid ORF corresponding to a translated production of 23 kDa was isolated and distinguished from the previous HPO that lacked the N-terminal 80 amino acids. The dose-dependent stimulation of DNA synthesis of HepG2 hepatoma cells by HPO-205 demonstrated its similar biological activity with HPO *in vitro*. The level of MAPK (Mitogen-activated protein kinase) phosphorylation by Western blot analysis revealed that HPO-205 might have the stronger activity of stimulating hepatic cell proliferation than that of HPO.**

CONCLUSION: **A novel isoform of hHPO (HPO-205) was isolated from hepatic-derived cells. The comparison of HPO-205 and HPO will lead to a new insight into the structure and function of hHPO, and provide the new way of thinking to deeply elucidate the biological roles of HPO/ALR.**

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INTRODUCTION

Hepatic stimulatory activity was identified from human fetal liver lysate, which was named as hepatopoietin (HPO)[1]. Later we proved that HPO was encoded by mRNA of human fetal liver[2]. Recombinant human hepatopoietin (rhHPO) showed its activity of specifically stimulating DNA synthesis of hepatic cells and promoting healing after liver injury *in vitro* and *in vivo*[3-6]. Recently, HPO/ALR/EVR1 homologous cDNAs (including EST sequences) have been isolated in many laboratories[7,8]. Sequence analysis of these homologous cDNAs revealed that the same 3' sequence was presented in all reported sequences, however, the 5' sequence varied in these cDNAs[9]. Furthermore, The analysis of genomic sequences of HPO/ALR also suggested there might exist different transcripts in nature. In this paper, we reported the identification and characterization of a novel isoform of human HPO in hepatic-derived cells. This cDNA with a 205 amino acid ORF was named HPO-205 to distinguish it from the previous HPO that lacked the N-terminal 80 amino acids.

MATERIALS AND METHODS

Sequence analysis and RNA extraction

HPO sequences and their proteins were analyzed by using DNA tools, Biosoft software and GenBank blast program. The primer for 5'RACE was: 5'-GGT CTT CAG GTT CAG ACA CAT GTT GGC-3'. The human fetal liver Marathon-ready cDNA Clontech) served as template to amplify 5' end of HPO by using a kit (Clontech). The PCR products were ligated to pGEM-T vector and sequenced using T7 and SP6 primers by PE (ABI PRISM) DNA Sequencer. Cos7 (African green monkey kidney cell line), HepG2 and HLE (human hepatoma cell line) were purchased from the American Type Culture Collection. BEL-7402 (epithelial hepatoma cell lines, derived from male, 75 years) and SMMC-7721 (epithelial hepatoma cell lines, derived from male, 50 years) were purchased from the Cell Institute of Chinese Academy of Science. All cell lines were cultured in Dulbecco's Modified Eagle Media (DMEM) (GiBco BRL, Life Technologies.) supplemented with antibiotics and 100mL·L^{-1} heat-inactivated fetal bovine serum in a humidified atmosphere of 50mL·L^{-1} CO_2.Total RNA was isolation from the cells using TRIzol™ reagent (GiBco).

RT-PCR

Forward and reverse primers for human HPO-250 and G3PDH genes were designed and applied to amplify transcripts. Briefly, Total RNAs were extracted with Trizol (GIbco) reagent. Reverse-transcribed and amplified for 30 cycles, using RT and PCR kit (TaKaRa LA Taq with GC buffer, TaKaRa Biotech, Dalian,China), according to the manufacturer's instructions. The expression of G3PDH mRNA was detected as an internal control. Primer pairs for RT-PCR were: HPO-250 sense: 5'- ATG GCG GCG CCC GGC GAG CGG GGC CGC TT-3'; antisense:5'- CTA GTC ACA GGA GCC ATC CTT CCA-3'; G3PDH sense:5'-ACC ACA GTC CAT GCC ATC AC-3'; antisense: 5'-TCC ACC ACC CTG TTG CTG TA-3'. The expected size of the amplified DNA fragments for HPO-250 and G3PDH were 618 and 1000 bp respectively. The PCR products were separated on 10g·L^{-1} agarose gel with ethidium bromide staining and photographed under UV. The photographs of the gels were analysed using Bio-Print (M&S Instruments Trading Inc, Tokyo). The values of these DNA fragments were calculated as relative intensity against G3PDH mRNA.

Construction of HPO expression vectors and Transfection

Both *Eco*R I and *Bam*H I sites were introduced by PCR *in vitro* mutagenesis, and the full length cDNA encoding human HPO and HPO-250 were inserted into pcDNA 3.1(+) vector downstream of CMV promoter. The recombinant pCDNA 3.1 plasmids were isolated and used for transfection. Cos-7 or Bel-7402 cells were subsequently harvested and reseeded at a density of 1×10^{6}cells/100-mm plate.

The cells were transfected the next day with different plasmids (2μg) using lipofectamine reagent (Gibco). For transient expression, the conditional medium of Cos-7 cells transfected with pcDNA$^{HPO-250}$ or pcDNA plasmid was harvested 48h after transfection. For stable expression, the Bel-7402 cells after being transfected with pcDNA$^{HPO-250}$ or pcDNA^{HPO} or pcDNA plasmid by 72h were selected in DMEM medium containing 400mg·L^{-1} G418 for 14d. G418-resistant clones were isolated and grown in DMEM medium containing G418 to maintain the phenotype.

Western blot analysis

Protein was extracted from different cells, and the protein concentration was determined by Coomassie Brilliant Blue G-250 staining. Samples containing equivalent amounts (50ìg) were separated on a 100g·L^{-1} SDS polycocrylamide gel under reducing conditions and transferred to a Hybond-N nitrocellulose membrane (Amersham, Arlington Heights, Illinois). The membrane was incubated respectively with rabbit polyclonal anti-human HPO antibody or rabbit polyclonal anti-pERK or ERK antibody (Santa Cruz) and developed with an ECL western blotting detection system (Santa Cruz) using horseradish peroxidase-conjugated second antibody (Santa Cruz).

^{3}H-TdR incorporation assay

HepG2 hepatoma cells were counted and adjusted to 1×10^{8}cells·L^{-1}, then 100μL of cell suspension was inoculated into 96-well plates and incubated at 37℃, 50mL·L^{-1} CO_2 for 12h. Then the fresh medium containing prepared samples was added. After 48h of culture in presence of various medium supplements, 37kBq/well ^{3}H-TdR was added and incubated for 3h. The cells were collected to filters, and radioactivity was determined in an LKB liquid scintillation counter,and results were expressed as median counts per minute from triplicate cultures.

RESULTS

Sequence analysis of HPO

Searching against GenBank with human HPO sequence, this sequence showed homology with 11 human cDNAs encoding HPO-like proteins, Protein sequence alignment showed that all of these encoding proteins were highly conserved in the 125 amino acid of C terminal but varied in the N terminal. Further analysis of their nucleotide sequences demonstrated no stop codon was found before the same open reading frame (ORF). This result indicated that deposited 125 amino acid HPO might be incomplete.

Isolation of HPO-205 cDNA and sequence analysis

To investigate whether a novel isoform of HPO exists in nature, a Marathon-ready cDNA from human fetal liver served as the template to amplify 5' ends of hHPO. The PCR products containing different sizes of 375, 500, 750 and 1200 bp bands were cloned into pMD18-Tvectors and transformed *Ecoli*. DH5α. Then the clones were screened by hybridization with ^{32}P-HPO cDNA as a probe, and more than 50 positive clones were obtained. After we sequenced 10 positive clones, we obtained the novel isoform HPO cDNA that encoded a 205 amino acid ORF. This cDNA with a 205 amino acid ORF was named HPO-205 (GenBank CI 11559825) to distinguish it from the previous HPO that lacked the N-terminal 80 amino acids (Figure 1).

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1   MAAPGERGRF HGGNLFFLPG GARSEMMDDL ATDARGRGAG RRDAAASAST
51  PAQAPTSDSP VAEDASRRRP CRACVDFKTW MRTQQKRDTK FREDCPPDRE
                                     MRTQQKRDTK FREDCPPDRE
101 ELGRHSWAVL HTLAAYYPDL PTPEQQRDMA QFIHLFSKFY PCEECAEDLR
    ELGRHSWAVL HTLAAYYPDL PTPEQQRDMA QFIHLFSKFY PCEECAEDLR
151 KRLCRNHPDT RTRACFTQWL CHLHNEVNRE LGKPDFDCSK VDERWRDGWK
    KRLCRNHPDT RTRACFTQWL CHLHNEVNRE LGKPDFDCSK VDERWRDGWK
201 DGSCD*
    DGSCD*
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Figure 1 Amino acid sequence alignment of HPO-205 and HPO(GenBank CI 11559825)

Expression of HPO-205 in hepatoma cell lines

The expression of HPO-205 was determined by semi-quantitive RT-PCR and Western blotting. As shown in Figure 2 and Figure 3, the cell lines showed interesting and revealing differences in the levels of HPO-205. The levels of hHPO-205 mRNA in HepG2 and HLE cell lines were significantly higher (4-5 folds) than in BEL-7402 and 7721 cell lines (Figure 2). Similarly, we also observed HPO-205 protein in HepG2 and HLE cell lines were significantly higher (4-5 folds) than in BEL-7402 and 7721 cell lines (Figure 3) by Western blot analysis. The M_r of HPO-205 in these four hepatoma cell lines is 23000 identical to the predicted molecular weight (Figure 3).

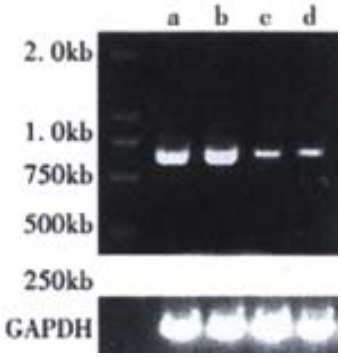

Figure 2 The mRNA expression of HPO-205 was determined by RT-PCR in hepatoma cell lines. a: HepG2 cell line; b: HLE cell line; c: SMMC-7721 cell line; d: Bel-7402cell line; GAPDH as a control.

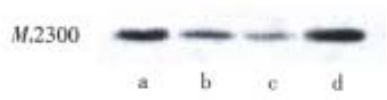

Figure 3 HPO-205 protein was detected by Western blot in hepatoma cell lines. a:HepG2 cell line; b:Bel-7402 cell line; c:SMMC-7721 cell line; d: HLE cell line

HPO-205 stimulated HepG2 cells proliferation in vitro

We demonstrated that biological activity of HPO-205 could be expressed from its cDNA in transient expression experiment in Cos7 cells. As shown in Figures 4A and 4B, the conditional medium from transfected cells with the pcDNA$^{HPO-205}$ revealed the dose-dependent stimulation of DNA synthesis of HepG2 hepatoma cells. However, as a negative control, the conditional medium from mock-transfected did not show any activity.

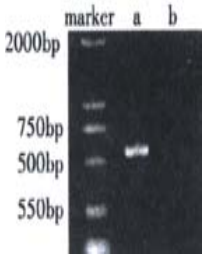

Figure 4A The HPO-205 mRNA expression in transfected Cos7 cells was detected by RT-PCR method. a: DNA marker;b: pcDNAHPO-205 vector; c: pcDNA vector.

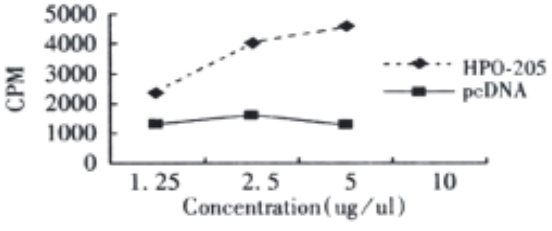

Figure 4B The effect of DNA synthesis of HepG2 cells stimulated by the different expressed protein.

Activition of Mitogen-activated protein kinase (MAPK) by expression of HPO-205 in Bel-7402 hepatoma cells

MAPK could be activated by a number of growth factors and cytokines. To assess whether HPO-205 activated the MAPK pathway, the pcDNA$^{HPO-205}$, pcDNA^{HPO} and pcDNA plasmids were transfected into Bel-7402 cells with low level expression of HPO. Expression of HPO-205 and HPO protein were analyzed by western blotting using antibody against HPO protein. As shown in Figure 5A, a protein band with an apparent M_r of 23000 only was detected in the HPO-205 transfected Bel-7402 cells, and a protein band with an apparent M_r of 15000 only was detected in the HPO transfected Bel-7402 cells. As shown in Figure 5B, the level of MAPK phosphorylation in BEL-7402 cells could be elevated by HPO and HPO-205 (P<0.01), and the level of MAPK phosphorylation activated by HPO-205 was higher than that by HPO (P<0.01).

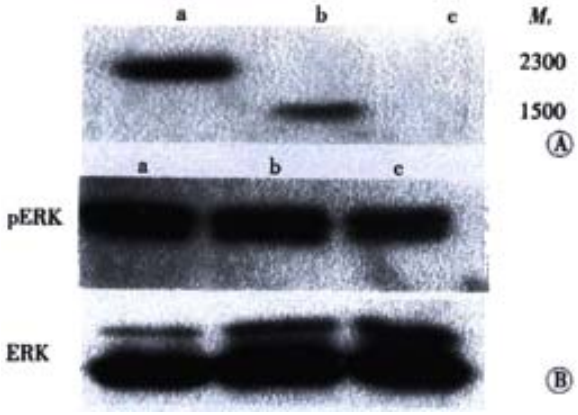

Figure 5 The stably transfected cell lines were confirmed and MAPK activation was examined by Western Blot.
A. HPO and HPO-205 protein in transfected Bel-7402 cells;
B. MAPK levels in different transfected cell lines.

DISCUSSION

We isolated and characterized a novel isoform of HPO (HPO-205) cDNA that encoded a 205 amino acid ORF. This cDNA with a 205 amino acid ORF was named HPO-205 (GenBank CI 11559825) to distinguish it from the previous HPO that lacked the N-terminal 80 amino acids. The molecular weight of HPO-205 was 23 kDa identified in four human hepatoma cell lines by Western blot, and the HPO-205 mRNA expressions were detectable in human hepatoma cell lines including HepG2, HLE, 7402 and 7721 by RT-PCR method. Our sequence analysis of HPO-205 shows presence of very rich GC bases in 5' end of HPO mRNA (more than 75%). Such high amount of GC bases will affect the efficiency of mRNA transcription. Therefore, it will lead to obtaining different kinds of incompleted HPO mRNA under different conditions in many laboratories[10].

As we know, human HPO is a specific growth factor and plays an important role in liver regeneration *in vivo*[11-14]. Many experiments demonstrated that HPO could stimulate the proliferation of hepatic cells *in vitro* and *in vivo*[1,2,15]. Therefore, identification and characterization of the biological function of HPO-205 and the relationship between HPO-205 and HPO are very important. Some data have shown they are different in intracellular location, tissue distribution and mRNA expression under different pathological conditions, which have suggested their different biological functions[16]. In this paper, we find, as human HPO, HPO-205 also stimulated the DNA synthesis of HepG2 cells. However, whether HPO-205 can specifically stimulate DNA synthesis of hepatic cells and promote healing after liver injury *in vitro* and *in vivo*, just like HPO, remain to be under investigation.

Our previous study demonstrated that HPO stimulated the hepatic cell proliferation through its specific receptor[4]. Recently, another report indicates that HPO/ALR assigns a FAD-linked sulfhydryl oxidase activity, which plays a role in modifying some molecules *in vivo*[17], and regulates the expression of some mitochondrial genes[18-21]. These data suggest that there are many different mechanisms of HPO/ALR involved in stimulating cell proliferation and improving hepatic repair. Recently, Li *et al*[22] have reported that HPO promotes the proliferation of hepatic cells through activating MAPK signal pathway. Here we have also found that both of HPO-205 and HPO could increase the activation of MAPK phosphorylation compared with the control. Moreover, the result also showed that the level of MAPK phosphorylation in HPO-205 transfected cells was significantly higher than that in HPO transfected cells by Western blot. These data indicate that HPO-205 might have the stronger activity of stimulating hepatic cell proliferation than that of HPO.

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