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Single chain precursor prohaptoglobin promotes angiogenesis by upregulating expression of vascular endothelial growth factor (VEGF) and VEGF receptor2

Mi-Kyung Oh [a], Hyo-Jung Park [a], Joo-Hyun Lee [a], Hyun-Mi Bae [b,1], In-Sook Kim [a,*]

[a] Department of Medical Lifescience, College of Medicine, The Catholic University of Korea, 222 Banpo-daero, Seocho-gu, Seoul 137-701, Republic of Korea
[b] Department of Internal Medicine, Incheon St. Mary's Hospital, The Catholic University of Korea, Incheon 403-720, Republic of Korea

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ABSTRACT

Prohaptoglobin (proHp) is processed into mature haptoglobin *via* site-specific cleavage. Although haptoglobin has been well studied, the functions of proHp remain unclear. We investigated the angiogenic action of proHp in endothelial cells, demonstrating that proHp upregulated vascular endothelial growth factor (VEGF) and VEGF receptor 2 (VEGFR2) expression and endothelial sprouting and branching. ProHp-induced sprouting was attenuated by a VEGFR2 inhibitor. Moreover, proHp was detected in sera of cancer patients by immunoprecipitation and Western blot. These findings indicate that proHp promotes angiogenesis via VEGF/VEGFR2 signalling, and serum proHp level may be a useful biomarker for diseases associated with angiogenesis.

1. Introduction

Haptoglobin (Hp) is an acute phase plasma protein [1–3] that can act either as an anti-inflammatory modulator or a pro-inflammatory activator during an inflammatory response [4–7]. In addition, Hp appears to be involved in arterial restructuring and vascular disease [8–10]. Hp protein consists of two chains, the α-chain and the β-chain, but is synthesised from a single mRNA. After synthesis of the polypeptide pre-prohaptoglobin, which contains a signal peptide and the α- and β-subunits, prohaptoglobin (proHp) is generated by removal of the N-terminal signal peptide. In the endoplasmic reticulum, proHp is specifically cleaved at the α–β junction site by a serine protease, separating the α- and β-chains [11,12]. In mature Hp, the separated α- and β-chains are connected by disulphide bonds, and eventually form a tetramer or polymer [13–15]. Although most Hp is released into circulation as mature protein, considerable amounts of proHp are also secreted [11,12]. However, the biological significance of proHp secretion and its function(s) remain largely unknown.

Complement C1r-like protein (C1r-LP), a serum protein of hepatic origin containing the serine protease domain of C1r, has been identified as a specific proHp-cleaving enzyme [16]. Although Hp is synthesised mainly in the liver, which is also a major site of C1r-LP synthesis, non-hepatic Hp expression has been observed in the arteries, lung, adipose tissue, and leukocytes [17–21]. ProHp may be secreted from non-hepatic cells lacking C1r-LP or under pathological conditions in which C1r-LP activity is suppressed.

Recently we found that endothelial progenitor cells (EPCs) transfected with the gene encoding Hp exhibit improved angiogenic/vasculogenic potential and greater ability to restore blood perfusion in a mouse model of hindlimb ischemia [22]. However, in that study it was not identified which Hp isoform was expressed. Since the proHp-processing enzyme C1r-LP is primarily expressed

Abbreviations: Hp, haptoglobin; proHp, prohaptoglobin; Clr-LP, complement Clr-like protein; HUVECs, human umbilical vein endothelial cells; EPC, endothelial progenitor cells; EGM, endothelial growth medium; EBM, endothelial basal medium; CM, conditioned medium; VEGFR, vascular endothelial growth factor receptor

Author contributions: M.-K. Oh performed the most of the experiments and analyzed data. H.-J. Park and J.-H. Lee helped in preparation of plasmid constructs and mouse aortic ring assay. H.-M. Bae contributed to obtain sera of patients and healthy individuals. I.-S. Kim planned the experiments, discussed data and wrote the manuscript.

* Corresponding author. Fax: +82 2 2258 7761.
E-mail address: ikim@catholic.ac.kr (I.-S. Kim).
[1] Present address: Seoul-Bae Clinic, 1192 Gaepo-dong, Kangnam-gu, Seoul 135-244, Republic of Korea.

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in hepatic cells [16], there is a possibility that the EPCs secreted the precursor form of Hp (proHp), which contributed to angiogenesis. Therefore, in the present study we evaluated the angiogenic action of proHp and its presence in the serum of patients with liver cancer and healthy individuals.

2. Materials and methods

2.1. Plasmid constructs and site-directed mutagenesis

The open reading frame of human *Hp2* cDNA [18] was subcloned into the EcoRI and KpnI sites of the pcDNA3.0 vector containing a C-terminal *FLAG* gene (gift from H.S. Rhim at The Catholic University of Korea, Seoul, Korea). To replace arginine (CGG) with glutamine (CAG) at amino acid 143 (R143Q) in proHp2, a single point mutation in the *Hp2* gene was generated using an oligonucleotide QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The specific mutation was confirmed by DNA sequencing (Macrogen Inc., Seoul, Korea). Hereafter, *Hp2* and proHp2 will be referred to as *Hp* and proHp, respectively.

2.2. Cell culture and conditioned medium collection

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh umbilical veins, as previously described [22,23]. The cells were cultured in endothelial growth medium (EGM-2 Bullet Kit™ medium; Clonetics, San Diego, CA) supplemented with 5% FBS (Gibco Laboratories, Gaithersburg, MD) and used at passages 3 through 7. COS-7 cells were maintained in DMEM (JBI WelGENE, Daegu, Korea) supplemented with 10% FBS.

To generate proHp-overexpressing cells, COS-7 cells were transfected with plasmid DNA containing the wild type or mutant *Hp* gene using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Stably transfected COS-7 cells were then selected by exposure to 1 mg/ml G418 (Invitrogen). For knockdown of *Hp* gene expression, small interfering RNA (siRNA; Dharmacon Inc., Lafayette, CO) against *Hp* was introduced into stably *Hp*-transfected COS-7 cells using DharmaFECT 4 reagent (Dharmacon), according to the manufacturer's protocol.

For conditioned medium (CM) collection, *Hp*-transfected COS-7 cells were grown to approximately 80% confluency in 150-mm culture dishes. The culture medium was then replaced with fresh

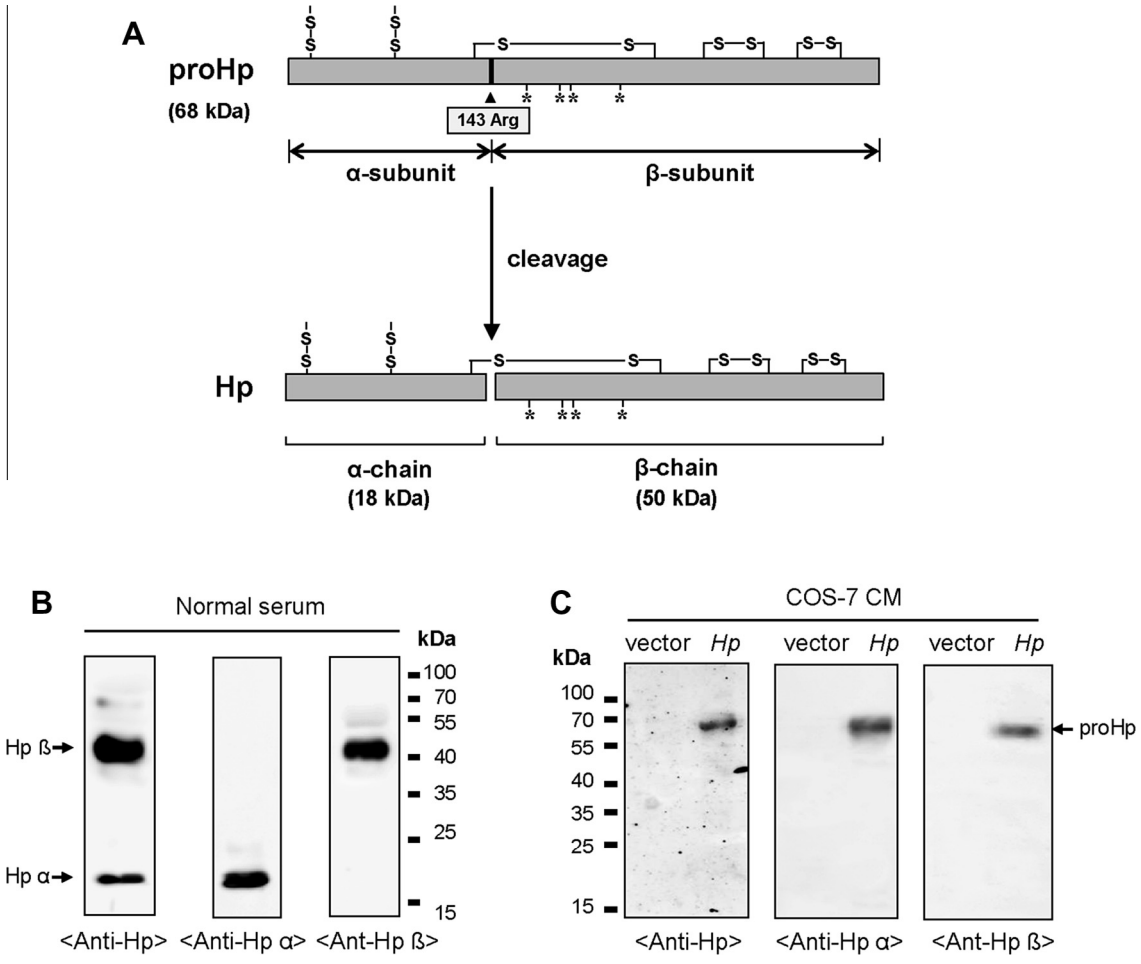

Fig. 1. Secretion of proHp from *Hp*-transfected COS-7 cells. (A) Structural difference between proHp and Hp isoforms is schematically shown. ProHp is the single-chain precursor protein, and mature Hp consists of α- and β-chains, which are generated by cleavage at Arg143 of proHp. *Glycosylation site. (B) Normal human serum (0.2 μl) was electrophoresed on a 12% SDS–polyacrylamide gel under reducing conditions, and Hp was detected with antibodies against Hp, Hp α-chain, and Hp β-chain. (C) COS-7 cells were stably transfected with empty vector or vector expressing human *Hp* cDNA. After cells were cultured for 48 h in serum-free DMEM, the conditioned medium (CM) was collected, and the presence of proHp in the CM was verified by Western blot using the same antibodies.