Recombinant hemangiopoietin promotes cell adhesion and binds heparin in its multimeric form

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Abstract. Hemangiopoietin (HAPO) is a novel growth factor stimulating the proliferation of hematopoietic and endothelial progenitor cells in vitro and in vivo. The native protein is a 294-amino acid multimodular protein. The N-terminus constitutes of two somatomedin B (SMB) homology domains that contain 14 cysteines. The central region is a putative heparin-binding domain (pHBD) and the C-terminus contains mucin-like repeats. In the present study, we demonstrated that prokaryotic recombinant human HAPO (rhHAPO) self-assembles into a multimeric form with a mass weight of ~129 kDa, suggesting a homologous tetramer. rhHAPO in its multimeric form was found to be more stable and more potent in promoting HESS-5 cell adhesion. Multimeric rhHAPO had a higher affinity to heparin compared with its dimeric form, although there was no significant conformational change. C-terminal repeats-truncated rhHAPO (rhHAPOΔmucin) was also found to be assembled into a multimer, while deletion of pHBD (rhHAPOΔmucin-pHBD) caused the protein to remain in a dimeric form, demonstrating that SMB domains participate in self-aggregation of the molecule and that the pHBD region promotes the tetramerization.

Introduction

Hemangiopoietin (HAPO), a 294-amino acid protein, stimulates the proliferation and hematopoiesis and/or endothelial differentiation of human bone marrow mononuclear cells and of purified CD34+, CD133+, kinase domain receptor-positive (KDR+) or CD34+/KDR+ cell populations (1). The native protein is one of the alternative splicing products of proteoglycan 4 (PRG4), which consists of 12 exons. The coding sequence of native HAPO includes part of exon 2, full-length exons 3 and 4 and part of PRG4 exon 6. Research performed to date supports the assignment of at least three domains within HAPO: i) The N-terminal domain encoded by PRG4 exons 2 and 3 consists of two somatomedin B (SMB) homology domains (SMB₁₂Hapo), while HAPO lacks N-terminal 15 amino acids in exon 2. SMB, with the consensus sequence CX₁₉CX₂₉CX₃₉CX₁₉CCX₁₉CX₁₉, is homologous to the cysteine-rich region of vitronectin (Vn) and the sequence identity is 45% (2). All 14 cysteines of HAPO are distributed over the two SMB domains. ii) The center region of HAPO encoded by PRG4 exon 4 is a lysine (K)-rich region with the probable function of heparin binding and is known as putative heparin-binding domain (pHBD) (2). The interaction between protein and heparin is one of the most investigated topics in previous studies, particularly the mechanism of promoting cell adhesion. iii) The C-terminus encoded by PRG4 exon 6 is a mucin-like O-linked oligosaccharide-rich repeat region composed of KEPATTT/P and XXTTTX consensus sequences (2). These repeats are highly diverse in length among species (3).

PRG4, an extracellular matrix (ECM) protein, has been identified as megakaryocyte-stimulating factor (MSF) or cartilage superficial zone proteoglycan (SZP) encoded by the DOLS4 gene. Two tissue-specific alternative splice variants of PRG4 have been detected in human pathological tendon by polymerase chain reaction (PCR) with forward primers specific for exon 3 in combination with a reverse primer specific for exon 6. One splice variant, similar to HAPO, lacks exon 5, while the other lacks exons 4 and 5 (3-6).

The recombinant HAPO expressed in E. coli supported the survival of MO7e cells through a PI3K-Akt pathway following deprivation of granulocyte-macrophage colony stimulating factor (7). The transfection of the HESS-5 mouse bone marrow stromal cell line with a eukaryotic HAPO-expressing vector
supported the rapid generation of primitive progenitor cells and maintains reconstitution of CD34+ hematopoietic stem cells in vitro (8).

HAPO has clinical potential in the management of various cytopenias and radiation injury. However, there is limited knowledge with regard to its biophysical characteristics. In this study, we cloned and expressed the full-length protein and two deletion mutants. Biophysical experiments were also performed in order to gain knowledge of the protein structure.

Materials and methods

Expression and purification of rhHAPO and the mutation variants. The complementary DNA (cDNA)-encoding full length HAPO was amplified by PCR from human fetal liver cDNA and inserted into plasmid pET22b (+) (Novagen, Madison, WI, USA) which had been digested with NcoI and XhoI as previously described. The human fetal liver was obtained from aborted fetuses of 17-22 weeks' gestation after informed consent was obtained (1).

The protein was extracted using the one-step extraction method. The cells were suspended into three volumes of 20% sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA) and 20 mM Tris-HCl (pH 7.9). Seven times volume ice water was then added. Following centrifugation at 12,000 rpm for 30 min at 4°C, the supernatant was loaded to a nickel-chelating column. The fraction washed from the affinity column containing His-bind resin with 0.5 M NaCl, 200 mM imidazole and 20 mM Tris-HCl (pH 7.9), was dialyzed into 20 mM citrate sodium buffer (pH 5.5) and then applied to a fast flow SP Sepharose™ column. The target protein was eluted at the same buffer with 0.5 M NaCl.

The cDNA of rhHAPOmucin and rhHAPOmucin-pHBD was amplified from human fetal liver cDNA using the 5' (5'-CATgCCATgATgCCACCTgCAACTgTgA-3') and 3' (5'-CTagCTgAgTGACCTgACCTTgAAgTC-3') oligonucleotide primers. The amplified cDNA products were digested with NcoI and XhoI. The rhHAPOmucin cDNA was inserted into pET22b (+) and the rhHAPOmucin-pHBD cDNA was inserted into pET32 (+). The purification of rhHAPOmucin mutant was performed as described above for the full-length HAPO. For the purification of the rhHAPOmucin-pHBD, the protein was eluted by 160 mM imidazol, 0.5 M NaCl and 50 mM Tris-HCl (pH 8.0), dialyzed into 20 mM Tris-HCl (pH 8.0) and excised with enterokinase (3 U/ml protein; Invitrogen, Carlsbad, CA, USA) at 4°C for 16 h. The peptide and the enzyme were separated with Resource Q (GE Healthcare Bioscience Corp., Piscataway, NJ, USA) at 0-0.5 M NaCl. The protein concentration was determined by measuring absorbance (A) at 280 nm with a U-3010 spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) and using a calculated extinction coefficient given by ExPASy-ProtParam tool (http://kr.expasy.org/tools/protparam.html).

Size-exclusion chromatography. Following dialyzation into 20 mM Tris-HCl (pH 8.0), rhHAPO eluted from Reasource Q was applied to a Superdex 200 10/300 GL column (Amersham Pharmasia Biotech, Piscataway, NJ, USA) at 0.5 ml/min. Purified rhHAPOmucin and rhHAPOmucin-pHBD were dialyzed into phosphate-buffered saline (PBS) and applied to a Superdex 200 10/300 GL column. The column had a void volume (Vo) of 8.3 ml and the standards IgG1 (150 kDa), bovine serum albumin (BSA; 67 kDa) and lysozyme (14.4 kDa) eluted at 12.305, 14.345 and 17.147 ml (Ve), respectively. The elution volumes of the standards were divided by the elution volume of the thyroglobulin (Ve/Vo) and plotted against the log of the molecular weights of the standards. Then, the molecular masses of the peaks of rhHAPO could be measured.

Western blot analysis. Western blot analysis was routinely performed. Following electrophoresis and transfer, the blotted membrane was blocked in Tris-buffered saline (TBS) buffer (PBS, 0.02% Tween-20) containing 5% non-fat dried milk for 1 h at room temperature. The membrane was then incubated with anti-rhHAPO MoAb prepared by the laboratory of National Research Center for Stem Cell Engineering and Technology in TBS buffer overnight at 4°C and horseradish peroxidase-conjugated goat anti-mouse IgG (Beijing Zhongshan Biotechnology Co., Beijing, China) for 1 h at room temperature.

Mass spectrum assay. The second fraction (1 mg/ml) of rhHAPO eluted from Superdex 200 was dialyzed into water and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) was performed for molecular weight analysis (National Center of Biomedical Analysis, Beijing, China).

Isoelectrofocusing. The pl values of rhHAPO and rhHAPOmucin-pHBD were calculated using the ExPASy-ProtParam tool. Isoelectrofocusing was performed to examine the purification and check their pl with the method suggested by the manufacturer (Instruction 1818-A, LKB-Produkter AB, Bromma, Sweden) using a 0.55-mm thin-layer polyacrylamide gel, ampholine carrier ampholytes (pH 3.5-9.5; LKB-Produkter AB). rhHAPO and pHBD were dialyzed into water and 20 µl 1 mg/ml protein was loaded into the loading filter paper. The marker was broad pl calibration kit (pL 3.5-9.3; Amersham Pharmasia Biotech). Following electrofocusing, the gel was stained with Coomassie blue and the pl was monitored.

Cell adhesion assay. The murine bone marrow stromal cell line HESS-5 was routinely cultured in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% fetal calf serum, 1% Gln, 1% penicillin-streptomycin. Ninety-six-well plates were coated with 0.5% BSA at 4°C overnight and washed with PBS twice to prevent non-specific adhesion. HESS-5 cells (5x10^4) were plated into every well with IMDM medium containing 2% fetal calf serum, 1% Gln and 1% penicillin-streptomycin. rhHAPO in dimeric or mulimeric forms (1,000 ng/ml) was added. The recombinant proteins were separated from rhHAPO by size-exclusion chromatography with
Superdex 200. Cells were cultured at 37°C for 2 h, the supernatant was then removed and the cells were washed twice with PBS. The crystal violet method was performed to measure A at 596 nm. Ratio of relative adhesion = [(A_HAPO - )/(A_PBS)] x100.

Heparin binding assay. Heparin binding assay was performed as previously described (9). Protein was applied to a 1x5-cm heparin agarose column in 20 mM Tris-HCl buffer (pH 7.4) and eluted at a flow rate of 1 ml/min with a NaCl gradient in the same buffer. A was monitored at 280 nm. NaCl concentration was determined by conductivity.

Circular dichroism (CD). The CD spectrum was performed on a Jasco J720 spectropolarimeter (Jasco, Easton, MD, USA). Far UV measurements were taken in a 0.1-cm path length utensil with rhHAPO dialyzed into PBS buffer. Time constants were 4 sec and 5 scans were averaged for each measurement. CD was expressed in terms of ellipticity [θ] in degree x cm²/dmol. Low molecular weight (LMw) heparin was purchased from Sigma-Aldrich (St. Louis, MO, USA). To determine whether rhHAPO binds to heparin, solutions containing protein plus heparin were prepared at various protein:heparin ratios (w/w). CD was performed to analyze the conformational change. The K2D program (http://kal-el.ugr.es/k2d/spectra.html) was used for the prediction of protein secondary structure from CD spectra.

Results and Discussion

rhHAPO is a stable tetramer. In the gel filtration chromatogram by Superdex 200 300/10 GL column chromatography, there were four A peaks of rhHAPO purified from SPFF with the first peak eluted at void volume suggesting matter with a high molecular weight (Fig. 1A). Western blot analysis with anti-rhHAPO MoAb was performed to identify the peaks (Fig. 1B). The first two peaks did not contain the protein recognized by the rhHAPO antibody and after the endotoxin was removed from the sample, fraction 1 disappeared (data not shown), suggesting that this peak was mostly composed of endotoxin. According to the retention volume of standards, the calculated molecular mass of fractions 3 (principal component) and 4 was 129.8 and 65.2 kDa (Fig. 1A), respectively. Since the theoretical molecular weight deduced from amino acid sequence of rhHAPO with (His)₆-tag was 32,742.6 Da, rhHAPO is likely to be a homotetramer with a trace amount of the dimeric form. Fraction 3 was expected to be a 137-kDa protein calculated by DLS. This was consistent with the results from gel filtration, suggesting a tetrameric form of rhHAPO.

Multimeric rhHAPO is more potent in promoting cell adhesion than the other form. HESS-5 cell adhesion assay was performed to determine the different efficacy between the monomer, dimer and tetramer structures of rhHAPO. HESS-5 is a murine bone marrow stroma cell line that supports the reconstituting ability of ex vivo-generated hematopoietic stem cells from human bone marrow and cytokine-mobilized peripheral blood (10). When HESS-5 cells were cultured in the presence of the tetrameric HAPO, increased adhesion was observed compared with the presence of the dimeric HAPO (relative adhesion value =54.3±11.1 vs. 33.2±12.1%; P=0.033). Platelet Vn is another protein known to promote endothelial
cell adhesion and the conformationally altered multimeric Vn is more potent compared with the monomeric form (11). Binding efficiency of multimeric Vn with porcine endothelial cells monolayer is 3-4 times higher compared with monomeric Vn (11). A possible explanation for this different behavior is the steric influence due to aggregation. A number of studies suggest that the SMB domain is indeed cryptic in native monomeric Vn (12). Highly sulfated glycosaminoglycans (GAGs) bind to native plasma Vn and induce Vn multimerization at physiological ionic strength, resulting in the exposure of the SMB domain. Although the principal site for cell attachment in Vn is the Arginine-Glycine-Aspartic (RGD) sequence located in the SMB domain, it has been confirmed that the purified recombinant SMB domain, which does not contain the RGD sequence, is able to promote the attachment of HT-1080 and U937 cells (13). Multimeric Vn is able to bind the endothelial cells through the heparin-binding domain, not the RGD sequence (14). Due to the high sequence homology between Vn and HAPO, it is possible that dimeric HAPO self-associates into the tetrameric form, which may cause a structural change and consequently SMB or heparin-binding domain exposure, thus facilitating binding to their receptors.

Multimeric rhHAPO is more potent in binding heparin compared with the monomeric rhHAPO in the absence of significant conformational change. Heparin-binding peptides from ECM proteins have been identified to promote cell adhesion (8,15). Direct binding of the peptide to heparin may be assessed by affinity chromatography on a heparin-agarose column. High binding activity was evaluated from rhHAPO tetramer interactions with heparin-agarose column at 1 M NaCl elution (Fig. 2A). However, during the elution procedure with a linear salt gradient of 0-1.0 M NaCl, dimeric rhHAPO was eluted with loading buffer and did not bind heparin (Fig. 2B). There was a molecular weight dependence of rhHAPO binding to heparin. This notable observation was similar to Vn (16). Vn association into a multivalent form contains proximal aligned heparin-binding sites exposed on its surface and has been shown to have a stronger binding activity due to the increased number of heparin-binding sites (17). A similar phenomenon may explain why the tetrameric rhHAPO was more effective in promoting cell adhesion compared with the monomeric rhHAPO.

The far-ultraviolet CD spectrum of multimeric rhHAPO is shown in Fig. 3. There was no significant structural change after binding with heparin (Fig. 2C). This spectrum differs significantly from CD spectra of proteins known to assume disordered structure. The spectrum gives a strong negative ellipticity maximum at 200 nm, while weak positive ellipticities are not observed at 218 nm. Although a negative signal near 200 nm may be associated with disordered structure, the broad nature of the band suggested some structural contribution. This spectrum was characteristic of rhHAPO with predominant β-sheets and random coils, with limited α-helical content (18). Analysis of this CD spectrum using K2D online software indicated that rhHAPO is 61% random coils, 27% α-helices and the remainder is β-sheets.

SMB and pHBD is the oligomerization region of multimeric rhHAPO. Denaturation and renaturation of Vn under physiological solution conditions is invariably accompanied by self-association of the protein into a multimeric form (19). Intermolecular disulfide cross-linking occurs primarily at the multimeric Vn. We predicted a three-dimensional structure model of the first SMB domain with swissmodel (http://swissmodel.expasy.org/) (20) to investigate the disulfide model. The proposed disulfide linkage was Cys5-Cys9, Cys99-Cys11,
Cys\textsuperscript{21}-Cys\textsuperscript{32} and Cys\textsuperscript{25}-Cys\textsuperscript{39}. In this pattern, the first two cysteines form an independent disulfide bond. The first SMB domain of the native HAPO purified from patients and rhHAPO lack the N-terminal 15 amino acids containing these two cysteines. Thus, absence of these 15 amino acids does not appear to form dissociated cysteines in the molecule.

The results of reducing and non-reducing SDS-PAGE further supported our hypothesis. On SDS-PAGE under reducing and non-reducing conditions, the multimeric protein migrated as a single band with a molecular mass of \( \sim 42 \) kDa (Fig. 3A and B). The results showed that disulfide bond was not involved in the aggregation. There was no free sulfhydryl in rhHAPO as well as the result of recombinant SMB determined by Ellman's method (21). Under non-reducing conditions, the mobility of rhHAPO was slightly increased. This was caused by 14 cysteines being distributed over the SMB domain (22). The protein amount of positive charges or cysteines does not have a linear correlation with the relative migratory ratio and molecular weight. Meanwhile, there was a high content of positively charged amino acids throughout the full length HAPO. Sequence analysis showed that is a K-rich protein with 14.3% Lysine amino acid residues. The ratio of positively charged residues (K+R) to negatively charged residues (D+E) was 50/38. To identify the oligomerization sequence, we investigated the consequences of the absence of the mucin-like domain. According to the amino acid sequence, the predicted molecular weight of rhHAPO\textsubscript{Δmucin} was 18.2 kDa, whereas the calculated molecular weight was 72.4 kDa in Superdex 200 size-exclusion chromatography (Fig. 3D), suggesting that the protein was also a stable tetramer. We found that deletion of the mucin-like repeat did not hamper the ability of rhHAPO to form a tetramer. C-terminal mucin-like repeats did not participate in the oligomerization.

### Table I. Comparison of characteristics of rhHAPO and rhHAPO\textsubscript{Δmucin-pHBD.}

<table>
<thead>
<tr>
<th>Protein</th>
<th>Monomer (kDa)</th>
<th>Oligomer (kDa)</th>
<th>pI</th>
<th>Predicted extinction coefficients(^a) (M(^{-1})cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>rhHAPO</td>
<td>32.7</td>
<td>129.8</td>
<td>8.52</td>
<td>5,960</td>
</tr>
<tr>
<td>rhHAPO\textsubscript{Δmucin-pHBD}</td>
<td>12.9</td>
<td>28.6</td>
<td>6.39</td>
<td>6,835</td>
</tr>
</tbody>
</table>

\(^a\)Values were computed at 280 nm assuming the Cys residues appear as half cysteines. rhHAPO, recombinant human hemangiopoetin.
Based on the alternative splicing of PRG4 mRNA, a cDNA lacking the center region PHBD was amplified with the same primers as those of rhHAPOΔmucin. In Superdex 200 300/10 GL column chromatography, the retention volume of rhHAPOΔmucin-pHBD was 17.98 ml (Fig. 3D) with the calculated molecular mass 28.2 kDa, validating the dimeric form of rhHAPOΔmucin-pHBD. The recombinant SMBΔs with C-terminal of thioredoxin had monomeric and dimeric form (23). The two N-terminal SMB domains of nucleotide pyrophosphohate/phosphodiesterases I (NPP1) were disulphide-linked homodimers and dimers could even be detected after reducing SDS-PAGE (24). However, we found that recombinant SMBΔs was a non-covalent dimer by reducing and non-reducing SDS-PAGE (Fig. 3C). Expression of this deletion mutant protein resulted in structural alternations. Thus, SMB and PHBD consisted of an intact tetramer. The absence of pHBD caused the protein to be a dimer and changed certain physical characteristics of the protein (Table 1). It has been proposed that the heparin-binding domain in Vn mediates the protein association, but this conclusion has been disproved in biochemical and biophysical studies (19). Intermolecular disulphide cross-linking close to the C-terminal heparin-binding domain is the oligomerization force of Vn. This study showed that pHBD mediated the association of the rhHAPO dimer into a tetramer. We proposed a potential model to account for the oligomerization of rhHAPO (Fig. 4). The subunits of rhHAPO were assembled post-translationally into a dimer by interactions between SMB domains, and this interaction caused a conformational change of pHBD which mediated the association of the rhHAPO dimer into a tetramer.

Taken together, these observations demonstrate that rhHAPO is a stable 129-kDa noncovalent homologous oligomer. Initially self-associated rhHAPO dimers are formed by interactions between SMB domains, whereas the formation of a tetramer is a secondary event mediated by pHBD sequences. Tetrameric rhHAPO is more potent in promoting the adhesion of HESS-5 cells compared with dimeric rhHAPO. Dimeric rhHAPO does not bind heparin, while tetrameric rhHAPO has a high affinity for heparin although there are no clear conformational changes.

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