Biotechnol Lett
DOI 10.1007/s10529-013-1198-2

ORIGINAL RESEARCH PAPER

Functional analyses of recombinant mouse hepcidin-1 in cell culture and animal model

Yaghoub Yazdani · Neda Keyhanvar · Hamid Reza Kalhor · Abbas Rezaei

Received: 29 August 2012 / Accepted: 28 March 2013
© Springer Science+Business Media Dordrecht 2013

Abstract Hepcidin is a peptide hormone that plays an important role in iron metabolism. We have produced a recombinant mouse hepcidin-1 by using baculovirus expression system. Its expression yield was 25 μg/ml when cell culture media were supplemented with a protease inhibitor cocktail. The recombinant mouse hepcidin-1 and synthetic human hepcidin-25 had similar effects on reducing ferroportin expression in J774A cell line and in peritoneal macrophages. However, synthetic human hepcidin-25 was more efficient than recombinant mouse hepcidin-1 in reducing iron concentration in blood circulation ($p < 0.01$).

Keywords Baculovirus expression system (BES) · Ferroportin · Hepcidin · Iron metabolism

Y. Yazdani (✉)
Infectious Diseases Research Center and Laboratory Science Research Center, Golestan University of Medical Sciences, Gorgan, Iran
e-mail: yazdani@goums.ac.ir

N. Keyhanvar
Faculty of Advanced Medical Technologies, Golestan University of Medical Sciences, Gorgan, Iran

H. R. Kalhor
Biochemistry Research Laboratory, Department of Chemistry, Sharif University of Technology, Tehran, Iran

A. Rezaei
Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran

Introduction

Hepcidin is a cysteine-rich, antimicrobial peptide (Park et al. 2001). In addition to its antimicrobial properties, hepcidin has an iron regulatory function (Nemeth et al. 2003). Iron is an important component of O_2-carrier proteins such as hemoglobin and myoglobin (Cairo et al. 2006). Hepcidin transcript is translated into an 84-amino acid prohepcidin that subsequently undergoes enzymatic cleavage to form mature hepcidin-25 (Park et al. 2001). Mice have two forms of hepcidin: hepcidin-1 and hepcidin-2. Human hepcidin-25 and mouse hepcidin-1 are involved in iron storage and regulation (Lou et al. 2004). An excess of free iron can generate harmful reactive oxygen species that increase the rate of cancer and tissue damage (Hershko 2007). Hepcidin only binds to a transmembrane iron exporter channel called ferroportin. This results in ferroportin internalization and lysosomal degradation. As a result, hepcidin can decrease iron levels in blood circulation (Nguyen et al. 2006; Vyoral and Petrak 2005).

Iron chelators, such as deferoxamine, are used to balance iron overloading conditions (Brittenham 2003). Although deferoxamine is the preferred drug, it is associated with several side effects such as deafness, visual defects, or growth retardation (Hoffbrand 2005). Therefore, there is a demand for alternative drugs that are not associated with the aforementioned disadvantages. Hepcidin as a natural peptide can be a more efficient drug for treating iron overload. Moreover, it is biodegradable and has low cytotoxic effects.

Published online: 04 April 2013

We have already produced recombinant mouse hepcidin-1 in insect cells and showed that it resulted in iron accumulation in J774A macrophage cells (Yazdani et al. 2011). In the present study, we have shown that a protease inhibitor cocktail increases the yield of hepcidin production up to 25 μg/ml in a baculovirus expression system (BES). Our results showed a loss or inaccessibility of the His-tag during hepcidin expression suggesting that anion exchange chromatography is more suitable than nickel column chromatography for purifying recombinant hepcidin. Finally, functional assays confirmed that effects of recombinant hepcidin on ferroportin were similar to those of its synthetic human counterpart. However, synthetic human hepcidin-25 was more efficient than recombinant mouse hepcidin-1 in reducing iron concentration in blood circulation ($p < 0.01$).

Materials and methods

Large-scale production of recombinant mouse hepcidin-1

Molecular cloning and expression of mouse hepcidin-1 in a BES was reported earlier (Yazdani et al. 2011). For optimal expression and for decreasing the different effects of cell proteases on recombinant mouse hepcidin-1, cell culture medium was supplemented with a protease inhibitor cocktail (Sigma-Aldrich) at 1.04 mM. This comprised 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 0.8 μM aprotinin, 0.04 mM bestatin, 0.014 mM E-64, 0.02 mM leupeptin, and 0.015 mM pepstatin A.

Recombinant mouse hepcidin-1 expression was investigated at 48–96 h after infection using 15 % (v/v) SDS-PAGE and western blot analyses with polyclonal anti-His-tag and anti-mouse hepcidin antibodies (Alpha Diagnostic). Finally, Ni–NTA and DEAE columns were used for purifying mouse hepcidin-1. For functional assays, the His-tag was cleaved from the recombinant mouse hepcidin-1 by using tobacco etch virus (TEV) enzyme.

Effects of hepcidin on ferroportin expression in cell culture

J774A cell line was used to study the effects of hepcidin on ferroportin expression. The cells were treated with ferric nitrilotriacetate (100 μM $FeCl_3$ and 400 μM NTA) for 16 h to increase ferroportin expression (Delaby et al. 2005). Next, the cells were separately incubated with 25 or 50 μg recombinant mouse hepcidin-1 and synthetic human hepcidin-25 for 24 h. After incubation, the cells were washed 3 times with phosphate buffered saline (PBS) and lysed in 2 % (w/v) SDS solution containing protease inhibitor cocktail. Ferroportin expression was examined by 12 % (v/v) SDS-PAGE and western blot analyses.

Rabbit anti-mouse ferroportin antibody (Alpha Diagnostic) and anti-beta-actin antibody (Cell Signaling Technology) were used for western blot analysis. Ferroportin and beta-actin bands were detected using ECL advance western blotting system.

Effects of hepcidin on iron circulation in animal model

Three-to four-week-old male C57BL/6 mice were from the Pasteur Institute of Iran. This study was approved by the animal research committee of the Golestan University of Medical Sciences (G/P/35/1084). Mice were maintained on a normal iron diet for 2 weeks. The mice were then given a single intraperitoneal dose of 10, 25, or 50 μg of native mouse hepcidin-1. Refolded synthetic human hepcidin-25 (Yazdani et al. 2011) in the same concentration was used as a control. Each group included six mice. The treated mice were sacrificed 24 h after the injection to evaluate serum iron levels. Blood was collected using cardiac puncture technique. Hemolysed sera were excluded from the study, and serum iron levels were evaluated using iron assay kit.

Effect of hepcidin on ferroportin expression in peritoneal macrophages

Peritoneal macrophages were isolated and washed with 2 mM EDTA in PBS containing the protease inhibitor cocktail. The macrophages were centrifuged at $500\times g$ and lysed with lysis buffer [150 mM NaCl, 20 mM Tri/HCl, 2 mM EDTA, 2 % (w/v) SDS, and protease inhibitor cocktail]. The lysates were centrifuged at $2{,}000\times g$ for 10 min to eliminate cell debris, and supernatants were tested using BCA protein assay kit to estimate the total protein concentration in all groups. Ferroportin expression was examined by 12 % (v/v) SDS-PAGE and western blotting. The details of the experiments are mentioned in the preceding section.

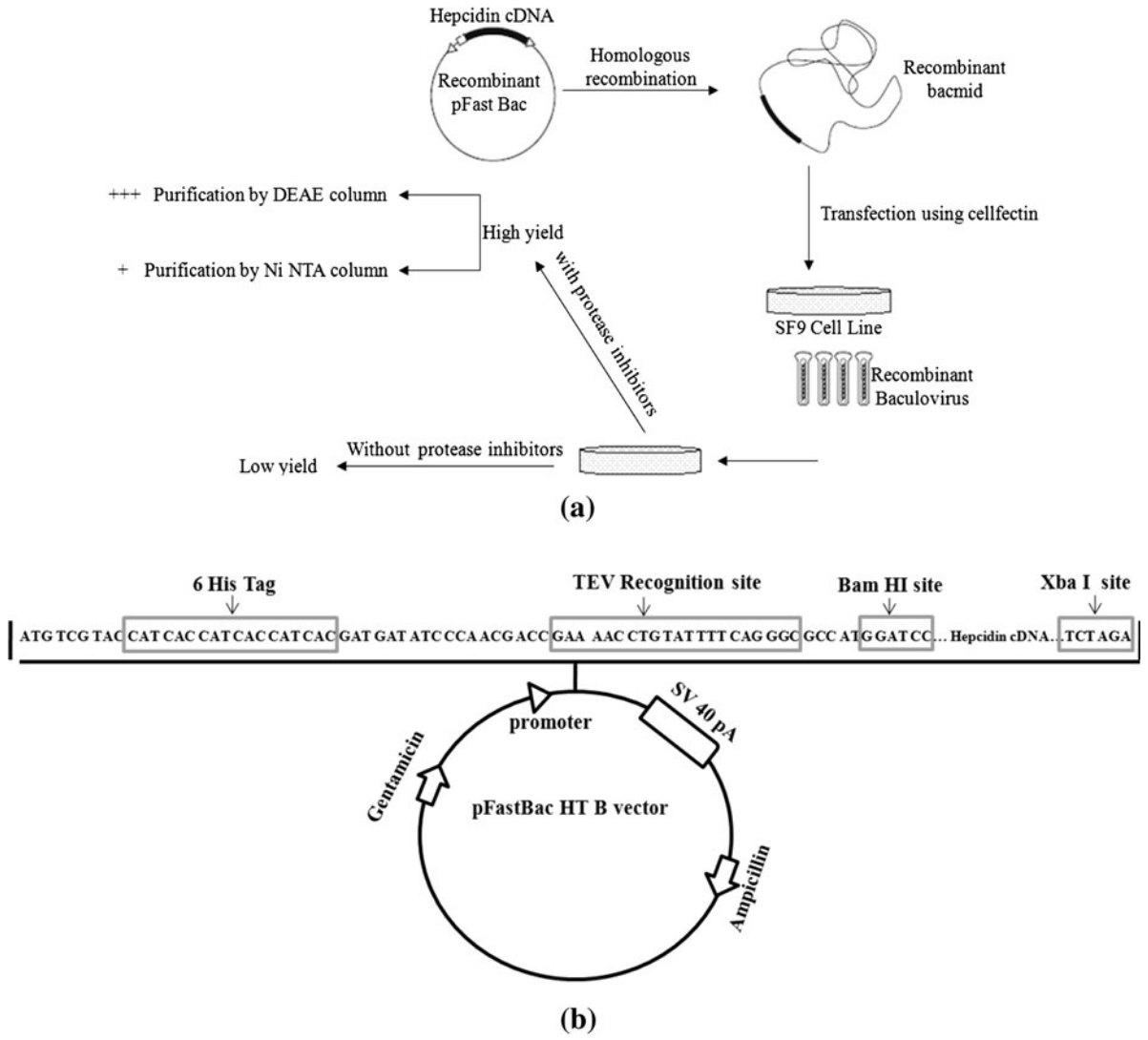

Fig. 1 **a** Schema for the production of recombinant mouse hepcidin-1 in baculovirus expression system. Hepcidin cDNA was cloned into pFastBac HT B vector, and recombinant bacmid was produced using homologous recombination in *DH10* bacteria. Insect *Sf*9 cell line was used for producing recombinant baculovirus. For hepcidin expression, MOI 10 was used in monolayer *Sf*9 cells. Infected insect cells in cell culture media with or without protease inhibitor cocktail were harvested at 48 h after infection. Finally, Ni–NTA and DEAE columns were used to purify the recombinant mouse hepcidin-1. The amount of purified recombinant mouse hepcidin is labeled as +. **b** The diagram of the complete coding sequence of recombinant mouse hepcidin-1. Restriction enzyme sites were used for direct cloning of hepcidin cDNA into a multiple cloning site of vector. The His-tag sequence allowed the expressed hepcidin to be purified using Ni–NTA column. The TEV recognition site encoded a TEV protease site for His-tag removal after hepcidin purification

Results and discussion

Expression and increase in the yield of recombinant mouse hepcidin-1

In our previous study (Yazdani et al. 2011), we produced recombinant mouse hepcidin-1 in the BES (Fig. 1a). The modified coding sequence of the recombinant mouse hepcidin-1 is a 333-bp segment that encodes a small secretory peptide having a theoretical molecular mass of 12.5 kDd (Fig. 1b).

During secretion, the produced recombinant mouse hepcidin-1 is susceptible to protease activities within the expressing cells and within the cell culture medium.

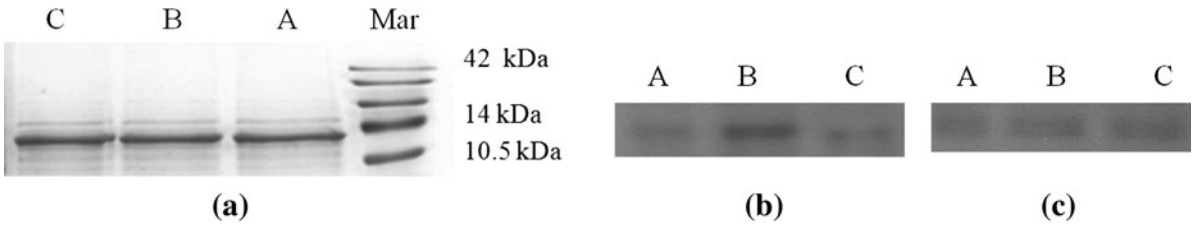

Fig. 2 Expression analysis of the recombinant mouse hepcidin-1. Expressed proteins in cell culture medium were electrophoresed on SDS-PAGE (**a**) and then confirmed using anti-His-tag (**b**) and anti-hepcidin (**c**) polyclonal antibodies. Insect cells infected with MOI 10 and production of recombinant mouse hepcidin-1 was studied at 48, 72 and 96 h after infection. *Lanes A*, *B* and *C* indicate protein analysis at 48, 72, and 96 h after infection, respectively

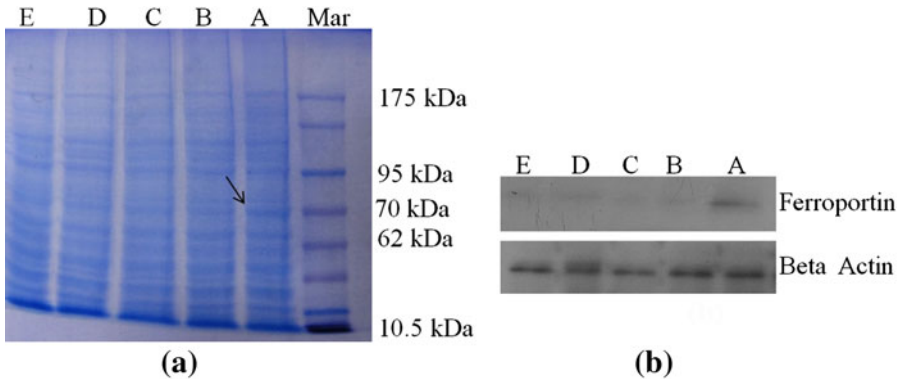

Fig. 3 Effects of hepcidin on ferroportin expression in iron-treated J774A cell line. Proteins were examined on SDS-PAGE (**a**). The position and sizes (in kDa) of molecular markers are indicated on the right as Mar. *Lane A* corresponds to the total protein in J774A cell line treated only with Fe-NTA as a negative control. Ferroportin band is clearly observed to be ~68 kDa. *Lanes B* and *C* indicate cells cotreated with iron and 25 and 50 μg of the recombinant mouse hepcidin-1. *Lanes D* and *E* show the results of the same treatment with synthetic human hepcidin-25. After electrophoresis, proteins were transferred to a nitrocellulose membrane and ferroportin was confirmed using rabbit anti-mouse ferroportin antibody (**b**). *Lane A* indicates cells treated with Fe-NTA as negative control. Other lanes are similar to part (**a**)

Several studies on the BES have shown that the viral genome expresses several families of proteases such as carboxyl peptidases (Gotoh et al. 2001; Licari and Bailey 1991). Baculoviruses that have deletions in the p26, p10, and p74 genes show higher expression of the recombinant peptide (Hitchman et al. 2010). Greenshields et al. (2008) reported that recombinant Sal1 hepcidin was not detected by western blot of the BES even though hepcidin mRNA was isolated from the infected insect cells. These reports indicated that baculoviruses and insect cells have different factors that affect the production of the recombinant peptide.

In the absence of protease inhibitors, there was no correlation between the results obtained from SDS-PAGE and those obtained from western blot analysis. Signals obtained by western blot analysis using anti-His-tag antibody significantly decreased. The yield of hepcidin expression was between 15 to 20 μg/ml (Yazdani et al. 2011). When the cell culture medium was supplemented with a protease inhibitor cocktail to minimize the effects of proteases on the yield of recombinant mouse hepcidin-1, the yield increased to 25 μg/ml.

Optimum production time is another important factor that affects the yield of hepcidin production. Our previous report showed that optimum production time of recombinant hepcidin is 72 h after infection (Yazdani et al. 2011). In this study, we took samples at 48, 72, and 96 h after infection to determine optimal production time for recombinant mouse hepcidin-1. Insect cells were therefore transfected with recombinant baculovirus at multiplicity of infection (MOI) 10 in the presence of the protease inhibitor cocktail. Hepcidin expression was then investigated using SDS-PAGE and western blot analyses with anti-His-tag and anti-mouse hepcidin antibodies (Fig. 2).

SDS-PAGE analysis showed the same band corresponding to the recombinant mouse hepcidin-1. However, detailed assessments with western blot analysis showed that the protease inhibitor cocktail did not completely protect the hepcidin structure (Fig. 2). At 48 h after infection, western blot analysis with anti-His-tag antibody showed a high-intensity band corresponding to the recombinant mouse hepcidin-1 (Fig. 2b). Reduction in western blot signals can be explained by the removal or degradation of His-tag from some of recombinant hepcidin molecules during or after the expression in the insect cell line. Another possibility is that the His-tag might be hidden by the 3-dimensional structure of the hepcidin protein. Thus, the present data indicates that the optimum time for native recombinant mouse hepcidin-1 expression is 48 h after infection. Because of the loss of His-tag or His-tag inaccessibility, purification method such as nickel column chromatography, which is based on the affinity of histidine for nickel ion, was not suitable for hepcidin purification. Therefore, anion exchange chromatography, which depends on charge–charge interactions between the recombinant mouse hepcidin-1 and the charges immobilized on the resin, was used for hepcidin purification.

Functional studies on recombinant mouse hepcidin-1

For functional analysis, recombinant mouse hepcidin-1 was purified using a DEAE column and the His-tag was cleaved using TEV protease. Next, the synthetic human hepcidin was refolded as a control (Yazdani et al. 2011). Functional assessments were performed by evaluating the effects of hepcidin on ferroportin expression in iron-treated J774A cell line. The effects of hepcidin on iron concentration in blood circulation and ferroportin expression in peritoneal macrophage cells were also studied.

The effects of hepcidin on ferroportin expression in cell culture are summarized in Fig. 3. Both recombinant mouse and synthetic human hepcidins significantly decrease the ferroportin band in cells cotreated with iron and hepcidin peptides. Reduction in ferroportin expression was dose dependent; hence, 50 µg both hepcidins had higher effects on ferroportin expression. The difference between the two types of hepcidins was not significant.

For in vivo assays, mice maintained on normal iron diet were given a single doses of 10, 25 or 50 µg recombinant mouse hepcidin-1 and synthetic human hepcidin-25 separately. The serum iron levels in treated mice, assessed 24 h after injection, were decreased in both groups. Mice treated with synthetic human hepcidin-25 also showed a progressive decrease in serum iron levels (Fig. 4). Moreover, our findings showed an increase in hepcidin concentration resulted in further reduction in serum iron levels in both the treated groups ($p < 0.01$). However, no significant difference was observed between 25 and 50 µg of recombinant mouse hepcidin-1 with respect to the reduction in serum iron levels ($p = 0.167$).

The differences in the effects of recombinant mouse and synthetic human hepcidins on iron concentration could be explained by two scenarios. First, because of the larger size and presence of four disulphide bonds, recombinant mouse hepcidin-1 may become aggregated after infusion into the mice peritoneal cavity. However, the refolded synthetic human hepcidin-25 has a smaller size (2.5 kDa) and is diffused after peritoneal injection. Second, the recombinant mouse hepcidin-1 may require additional post-translational

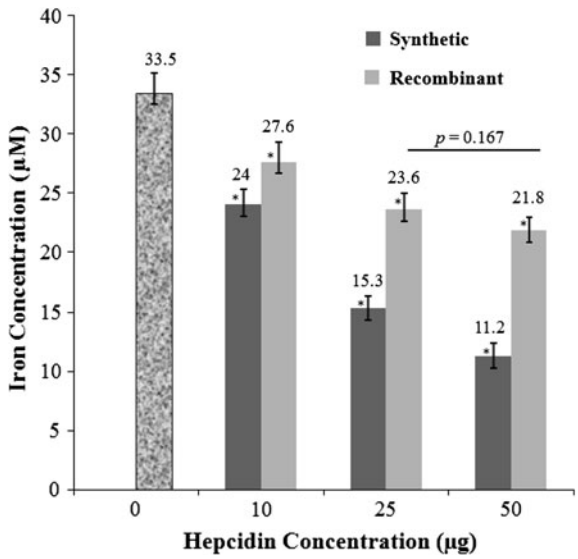

Fig. 4 Effects of hepcidin on iron concentration. Mice received a single dose of either 10, 25 or 50 µg recombinant mouse and synthetic human hepcidins separately. Data are expressed as the mean ± SEM of 6 mice, and p values are obtained using t test. The significant level for p value is $p \leq 0.05$(* $p < 0.05$). No significant difference was observed between 25 and 50 µg recombinant mouse hepcidin-1 ($p = 0.167$)

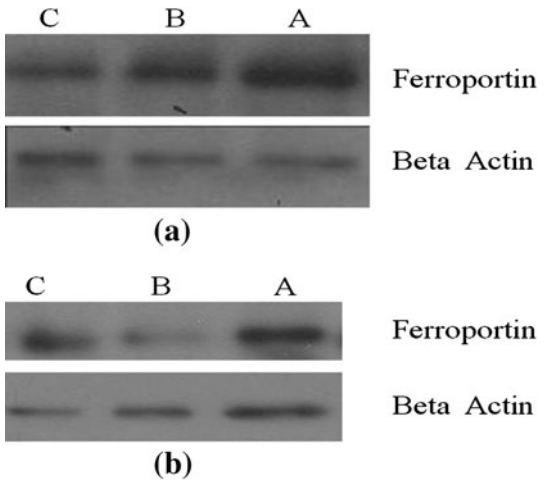

Fig. 5 Effects of hepcidin on ferroportin expression in resident peritoneal macrophages. **a** Different effects of the recombinant mouse and synthetic human hepcidins on ferroportin expression. *Lane A* represents the control mice treated with normal saline. *Lanes B* and *C* represent mice treated with 50 μg recombinant mouse and synthetic human hepcidin-25, respectively. **b** Result of hepcidin effect on ferroportin expression in mice treated with 50 μg (*lane B*) and 25 μg (*lane C*) of the recombinant mouse hepcidin-1. *Lane A* represents control mice treated with normal saline

modifications such as enzymatic cleavage during secretion to attain its complete function (Ravasi et al. 2012).

To investigate the underlying differences between recombinant mouse hepcidin-1 and synthetic human hepcidin-25, we assessed the effects of hepcidin on ferroportin expression as a target molecule. When mice were treated with 50 μg hepcidin, no significant difference was observed with respect to the reduction of ferroportin expression between the two forms of hepcidins (Fig. 5a). Moreover, these results showed that reduction in ferroportin expression was dose-dependent; it was higher in mice treated with 50 μg recombinant mouse hepcidin-1 than in mice treated with 25 μg recombinant mouse hepcidin-1 (Fig. 5b).

Conclusion

A protease inhibitor cocktail can increase the yield of recombinant mouse hepcidin-1 to up to 25 μg/ml in BES. Further, the optimum production time for recombinant mouse hepcidin-1 was 48 h. Because of the loss of His-tag or His-tag inaccessibility during hepcidin expression, anion exchange chromatography was considered to be a more suitable approach for purifying recombinant mouse hepcidin-1. Recombinant mouse hepcidin-1 significantly decreased ferroportin expression in the J774A cell line although the difference between recombinant mouse hepcidin-1 and synthetic human hepcidin-25 was not significant. However, after infusion into the mouse peritoneal cavity, recombinant mouse hepcidin-1 mainly showed a local activity on peritoneal macrophages. Synthetic human hepcidin-25, that has a tendency to form an aggregated peptide in biological samples, not only had a local effect but also had effects throughout the body and significantly decreased iron levels in blood circulation in comparison to the recombinant mouse hepcidin-1.

Acknowledgments This study has been funded by the Golestan University of Medical Sciences and Isfahan University of Medical Sciences.

References

Brittenham GM (2003) Iron chelators and iron toxicity. Alcohol 30:151–158

Cairo G, Bernuzzi F, Recalcati S (2006) A precious metal: iron, an essential nutrient for all cells. Genes Nutr 1:25–39

Gotoh T, Miyazaki Y, Kikuchi K, Bentley WE (2001) Investigation of sequential behavior of carboxyl protease and cysteine protease activities in virus-infected Sf-9 insect cell culture by inhibition assay. Appl Microbiol Biotechnol 56:742–749

Greenshields AL, Knickle LC, Syvitski R, Douglas SE (2008) Strategies for recombinant expression of small, highly disulphidebonded, cationic antimicrobial peptides. Protein Pept Lett 15:985–994

Hershko C (2007) Iron loading and its clinical implications. Am J Hematol 82:1147–1148

Hitchman RB, Possee RD, Crombie AT, Chambers A, Ho K, Siaterli E, Lissina O, Sternard H, Novy R, Loomis K, Bird LE, Owens RJ, King LA (2010) Genetic modification of a baculovirus vector for increased expression in insect cells. Cell Biol Toxicol 26:57–68

Hoffbrand A (2005) Deferiprone therapy for transfusional iron overload. Best Pract Res Clin Haematol 18:299–317

Licari P, Bailey JE (1991) Factors influencing recombinant protein yields in an insect cell-bacuiovirus expression system: multiplicity of infection and intracellular protein degradation. Biotechnol Bioeng 37:238–246

Lou DQ, Nicolas G, Lesbordes JC, Viatte L, Grimber G, Szajnert MF, Kahn A, Vaulont S (2004) Functional differences between hepcidin 1 and 2 in transgenic mice. Blood 103:2816–2821

Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T (2003) Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 101:461–2463

Nguyen NB, Callaghan KD, Ghio AJ, Haile DJ, Yang F (2006) Hepcidin expression and iron transport in alveolar macrophages. Am J Physiol Lung Cell Mol Physiol 291: L417–L425

Park CH, Valore EV, Waring AJ, Ganz T (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 276:7806–7810

Ravasi G, Pelucchi S, Trombini P, Mariani R, Tomosugi N, Modignani GL, Pozzi M, Nemeth E, Ganz T, Hayashi H, Barisani D, Piperno A (2012) Hepcidin expression in iron overload diseases is variably modulated by circulating factors. PLoS One 7:e36425

Vyoral D, Petrak J (2005) Hepcidin: a direct link between iron metabolism and immunity. Int J Biochem Cell Biol 37: 1768–1773

Yazdani Y, Sadeghi H, Alimohammadian M, Andalib A, Moazen F, Rezaei A (2011) Expression of an innate immune element (mouse hepcidin-1) in baculovirus expression system and the comparison of its function with synthetic human hepcidin-25. Iran J Pharm Res 10:559–568