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آموزش مهارت های کاربردی در تدوین و چاپ مقاله
Expression of an Innate Immune Element (Mouse Hepcidin-1) in Baculovirus Expression System and the Comparison of Its Function with Synthetic Human Hepcidin-25

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Abstract

Hepcidin is an innate immune element which decreases the iron absorption from diet and iron releasing from macrophage cell. In contrast to the chemical iron chelators, there has been limited effort applied to the specific use of hepcidin as a new drug for decreasing the iron overload.

Hepcidin is produced in different biological systems. For instance, E-coli is used for human hepcidin expression, however, post-translational modification is impaired. We have used a simple baculovirus expression system (BES) to improve the hepcidin folding and activity. Hepcidin Messenger Ribonucleic acid (mRNA) was isolated from mouse liver cells and its complementary Deoxyribonucleic acid (cDNA) was produced and amplified. PFastBac HTB vector was used for recombinant bacmid production. Recombinant baculovirus was produced using SF-9 cell line. The mouse hepcidin-1 protein was expressed in a large quantity and functional tests were performed for this recombinant peptide. The yield of hepcidin in BES was 20 µg/mL and anti-histidine (anti-His) tag antibody was used for the confirmation of hepcidin on western blot nitrocellulose paper. Functional tests showed that mouse hepcidin accumulates iron in the macrophage cell line J774A.1 up to 63%. In addition, our data showed that the mouse hepcidin-1 has less toxicity compared to the synthetic human hepcidin-25 (p = 0.000).

Keywords: Hepcidin; Baculovirus expression system; Functional study; J774A.1.

Introduction

Hepcidin was originally isolated from the human serum and urine (1, 2). Hepcidin is predominantly found in liver cell and weekly express in stomach, intestine, colon, heart, thymus and alveolar macrophages cells (3, 4). Sequential analysis of protein has shown that hepcidin is a cysteine rich peptide with several disulphide bonds (5). It is conserved among species from fishes to mammals (2). Humans have only one copy of the hepcidin gene, however, mice have two copies named hepcidin-1 and hepcidin-2 (3). Mouse hepcidin gene consists of 3 exons and 2 introns which is located on chromosome-7 (3). Reports show that mouse hepcidin-1 (but not hepcidin-2) involve
in iron regulation, iron storage and hemoglobin level (6, 7). Hepcidin causes internalization and degradation of iron exporter ferroportin, which is present on the cell surface of macrophage and enterocyte cells. Thus, hepcidin inhibits the release of iron by macrophages and iron uptake by enterocytes (8).

Antimicrobial activity against several microorganisms were also pointed in hepcidin derived from different species (9, 10). Moreover, hepcidin as an iron inhibitor represents an important class of anti-tumor agents since iron is an essential trace element that is vital for DNA synthesis (11).

Iron overload is the dangerous side effect of blood transfusion that occurs in hemoglobinopathies, such as thalassemia (12, 13). Deterprine and deferoxamine are routinely used for removal excess iron (14). In contrast to the chemical iron chelators, there has been limited effort applied to the specific use of hepcidin for decreasing iron overload in serum.

In spite of the reports describing the effect of hepcidin on iron regulation in the literature, there is little information about mouse hepcidin-1 effect on iron accumulation and also cell viability on macrophage cells, as important cells that participate in iron metabolism.

Hepcidin can be extracted through purification from plasma and urine, but the yield of purified crude hepcidin in this way is low (2). The second way is synthesis of hepcidin. Preparation plasmid encoding the synthetic human hepcidin is difficult and refolding procedures are required (15). Alternatively, hepcidin can be produced in bacterial expression system. The advantage of this expression system is its low cost and high productivity, but its post-translational modifications can be impaired in comparison with the expression in mammalian cells (16).

We tried to use a simple biological expression system that might have more similarity to the expression in mammalian cells. Therefore, we hypothesize that the baculovirus expression system (BES) would be efficient for this purpose (17, 18). The other advantage of this system is the lack of contamination with bacterial component such as LPS and hence, applying this protein would not activate immune responses (19).

We decided to use BES for cloning and expression of mouse hepcidin-1 peptide. In this study, isolation and cloning of the mouse mRNA encoding hepcidin protein was carried out and its production in baculovirus expression system was considered. For functional assessment, we compared mouse hepcidin-1 and synthetic human hepcidin-25 effect on iron concentration and cell viability in 3774A.1 cells line. To the best of our knowledge, the present study is the first work that considers all the above aspects for expression and functional assessment of mouse hepcidin-1 in the baculovirus expression system.

Experimental

Cloning and production of recombinant baculovirus

The liver of sacrificed male C57Bl6 mice was isolated and its total mRNA was extracted from liver cells using RNeasy Mini Kit (Qiagen, Germany). Then, the hepcidin cDNA was amplified utilizing Qiagen one-step RT-PCR Kit. The primer sequences were as follows: forward primer (BamHl), 5'-GGATCCTATGTTTTGCGCTCTAAG-3' and reverse primer (XbaI), 5'- TGATGATGGTATGACTGTAATGCGTTTGTCG-3'.

Patterns of digestion with ALU I enzyme was used for the preliminary assessment of PCR product.

The purified PCR product was ligated into the "PZ57R/T" vector. One of the obtained recombinant plasmids, was then sequenced (Macrogen, Korea). Subsequently, this plasmid was digested with BamHl and XbaI restriction enzymes and then the insertion was subcloned into the "PFastBac HTB" plasmid (Invitrogen, USA). The insertion caused the addition of a hexa histidine sequence into the 5' end of the hepcidin cDNA. The fidelity of the insertion in PFastBac B vector was confirmed by PCR analysis using polyhedrin primer and reverse primer of hepcidin cDNA and digestion with Neol enzyme.

Four selected recombinant plasmids were then sequenced (Macrogen, Korea) to verify the orientation and proper framing of insertion.

The selected recombinant vector was used for the production of recombinant bacmid. Afterward, the bacmids were isolated utilizing midiprep kit (Invitrogen, USA) from the selected colonies.

Insect SF9 cell line (Invitrogen, USA) was used for the production of recombinant baculovirus. Before the transfection, cells were grown (27°C) using "SF-900 II SFM" cell culture medium (Invitrogen, USA) supplemented with 1x penicillin/streptomycin/neomycin (Gibco BRL, USA) in order to adopt them to this growth condition. According to manufacturer's instruction, the transfection of these cells occurred using cellfectin reagent. After the cells showed signs of late stage of infection, the medium containing the baculovirus was collected and the amount of it was calculated.

Expression and analysis of mouse hepcidin-1

For hepcidin expression, multiplicity of infections (MOIs) 5, 10 and 20 were utilized in monolayer SF-9 cell (20). Infected SF-9 cells were harvested 48, 72 and 96 h post-infection for hepcidin expression analysis. Expression of the recombinant mouse hepcidin-1 in the SF-9 cell was examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Samples were electrophoresed in 15% SDS PAGE gels. After the electrophoresis, the proteins were transferred to a nitrocellulose membrane (Sartorius AG-USA) at 400 mA for 45 min using an electroblot system (Bio-Rad, USA). Membranes were then washed extensively with PBS buffer (0.15M, pH 7.4) and blocked with 3% of skim milk (Sigma, USA). The membranes were washed for 1.5 h at 37°C with 0.15M phosphate buffer (Sigma, USA) and then incubated with the recombinant mouse hepcidin-1 in the SF-9 cell line (20). The His-tag antibody (Calbiochem, USA) at final concentration of 4 µg/mL in PBS buffer, containing 2% bovine serum albumin (BSA) (Sigma, USA).

After stringent washing using PBS-Tween-20 buffer (0.05% Tween, 2% BSA), the membranes were incubated with 1 : 1000 HRP conjugated sheep anti mouse (Sigma, USA) for 1.5 h. After washing the membranes, hepcidin was detected using DAB as the substrate for HRP and appearance of brown colored protein bands on the membrane (Sigma, USA).

The hepcidin effect on cell viability

Cell viability for hepcidin was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium) proliferation assay (21). The SF-9 cell line was cultured in 24 well plate at a density of 4 × 105 cells/well in 1 mL DMEM medium. Recombinant mouse hepcidin-1 was added to the wells with the concentrations of 2, 3, 4, 5, 6, 7 and 8 µg/well. The MTT solution (Promega, USA) was used for the production of recombinant bacmid. Afterward, the bacmids were isolated utilizing midiprep kit (Invitrogen, USA) from the selected colonies.

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The hepcidin effect on iron concentration

The His-tag bounded at recombinant mouse hepcidin-1 was cleaved with enterokinase enzyme according to manufacturer’s instruction (Promega, USA). Human hepcidin refolded using refolded buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 100 mM KCl, 5 mM GSH and 0.5 mM GSSG) at final concentration of 100 µg/mL (incubated 24 h in 4°C). 3774A.1 cell line (Pasteur Institute, Iran), was utilized for evaluating the hepcidin effect on iron export. The cells were cultured in DMEM medium (Sigma, USA) supplemented with 1x penicillin/streptomycin, neomycin (Invitrogen, USA) and 10% fetal bovine serum (Sigma, USA).

The cells were seeded into a 6-well plate with 1 × 106 cells/well in 2 mL DMEM medium and incubated for 24 h simultaneously with 4 or 8 µg of hepcidin. In another groups, synthetic human hepcidin-25 (PRIMM, Italy) and BSA (dissolved in refolding buffer) were used with the same concentration. After incubation, cells were washed three times with PBS (0.15 M, pH 7–7.5) and then lysed in 2% SDS. Eventually, total proteins were precipitate with 20% trichloroacetic acid and free iron was measured in supernatant using atomic absorption tool (Perkin Elmer, 2380, USA).

The hepcidin effect on cell viability

Cell viability for hepcidin was examined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium) proliferation assay (21). The selected recombinant vector was used for the production of recombinant bacmid. Afterward, the bacmids were isolated utilizing midiprep kit (Invitrogen, USA) from the selected colonies.

Expression of mouse hepcidin-1

Hepcidin cDNA was amplified using specific primers (mentioned in materials and methods). After the gel electrophoresis, the amplified cDNA was appeared as a clear band at 260
Nucleotide sequential determination of hepcidin cDNA indicated that the sequence pattern had the position of 112 bp and one with 157 bp were confirmed that the amplified segment would be hepcidin cDNA. The Lane 2 in Figure 1 indicate a digested pattern of amplified cDNA after the treatment with ALU-I restriction enzyme. Nucleotide sequential determination of hepcidin cDNA indicated that the sequence pattern had only one restriction site for the ALU-I enzyme at 112 bp. As a result of digestion, one restriction site for the ALU-I enzyme was selected for sequencing. Afterward, recombinant bacmid was produced when DH110 cells were transformed by recombinant "PFastBac HTB" vector (Figure 3).

M13 forward and reverse primers were utilized for the evaluation of recombination bacmid. The Pattern of gel electrophoresis illustrates the occurrence of proper homologous recombination between recombinant vector and bacmid genome (Figure 3: Lanes 2, 5 and 6). Subsequently, SF-9 cells were transfected with the recombinant bacmid and the baculoviruses were successfully produced as a result of this transfection. Ninety-nine h post-transfection the recombinant bacmid and the baculoviruses were collected from the lysed cells. After the baculoviruses got expression in MOIs 5, 10 and 20 were used for protein purification of hepcidin on nickel column. An illustrated a pure 10 KDa band of mouse hepcidin-1 after passing the total protein products through nickel columns for the purification process.

Functional assessment was performed by purification of hepcidin on nickel column. An amount of 1 × 10^7 J774A.1 cells were cultured in 12-well plates containing 2 mL of DMEM medium. Then, the cells were incubated with 4 and 8 µg of purified mouse hepcidin-1. Synthetic human hepcidin-25 and bovine serum albumin (BSA) were added with the same concentrations for positive and negative control groups, respectively.

Intracellular iron concentration was determined using atomic absorption device 24 h post-incubation. Results of iron concentration are summarized in Table 1. The obtained data showed that in concentration of 4 µg, the effect of the mouse recombinant hepcidin-1 was lower than the synthetic hepcidin-25 on iron concentration (p = 0.016). However, the effect of both types of hepcidin (Table 1) on iron accumulation in the cells was significantly higher than BSA as the control group (p = 0.000).

Toxicity assay
Both types of hepcidin with the concentration of 2-8 µg/mL as well as BSA were added separately to the tissue culture wells containing 4 × 10^6 J774A.1 cells. The viability of the cells was evaluated by MTT assay. The results are
summarized in Table 2. The analysis indicates that the viability of the cells with the mouse hepcidin is much higher than that of human hepcidin-1 with the p-value of 0.000-0.007 for all the MOIs. In the baculovirus expression system, the effect of hepcidin on iron accumulation in J774A.1 cells was achieved after 72 h post-infection by recombinant baculovirus (42). Our results show that 72 h would be the optimum condition for production of intact and functional hepcidin. In addition, infection time period was tested for more than 72 h (i.e. 96 h) and the hepcidin expression was not different from 72 h. On the western blot analysis, anti-His tag antibody was used for the indirect confirmation of hepcidin expression. Our result revealed that this antibody does not have direct correlation with the relative amount of hepcidin expression. For this finding, one possibility is considerable. His-tag epitope is hidden inside the protein, thus, its epitope is not properly in access with the anti-His tag antibody (43).

Researchers have reported that the yield of protein expression was 5-15 µg/mL in baculovirus expression system (44), 55-60 µg/mL in bacterial expression system (45) and 100 µg/mL in fungal expression system (29). The yield of our hepcidin product was 20 µg/mL and this is more appropriate for baculovirus expression system.

According to the reports, if hepcidin binds to ferroportin channel, the channel will become degrade and will inhibit the iron export from the cells (46). Chaston et al. indicate that ferroportin levels decreased in hepcidin treated cells (47). In our experiments, the highest amount of hepcidin was produced in SF-10 after 24 h post-infection. However, when cells were incubated for 96 h, no significant difference was observed between the MOI.5 and most of the MOIs. In the baculovirus expression system reported by Kim et al. (2007), the highest protein expression was in MOI.5. On the other hand, Posse et al. (2008) used MOI.5 for optimum conditions, as well (38, 39). The details of our results can be clarified as follows:

The existent baculovirus in MOI.5, used all capacity of insect cell line for the protein expression, therefore, most of MOIs had no effect on this process (40).

The produced hepcidin is a foreign protein in SF-9 cell line. Synthesis of any foreign protein consumes cell sources such as amino acids and may decrease the protein expression in host cell when its concentration increases (41).

Ruan et al. (2008) have reported that maximum yield of exogenous protein in SF-9 cell was achieved after 72 h infection by recombinant baculovirus (42). Our results show that 72 h would be the optimum condition for production of intact and functional hepcidin. In addition, infection time period was tested for more than 72 h (i.e. 96 h) and the hepcidin expression was not different from 72 h. On the western blot analysis, anti-His tag antibody was used for the indirect confirmation of hepcidin expression. Our result revealed that this antibody does not have direct correlation with the relative amount of hepcidin expression. For this finding, one possibility is considerable. His-tag epitope is hidden inside the protein, thus, its epitope is not properly in access with the anti-His tag antibody (43).
iron precipitate accumulation in macrophage cells and thus, it a high concentration and it was revealed that functional mouse hepcidin-1 as a new drug at mouse hepcidin-1 or BSA has a higher cytotoxic effect in vitro. This cell line may express distinct levels of hepcidin genes in the redbanded seabream (Spondyliosoma cantharus), characterization and gene expression analysis of four hepcidin cDNA of the Atlantic cod (Gadus morhua) and Arosio P. Recombinant human hepcidin expressed in Escherichia coli isolates as an iron containing protein. Cells Mol. Dis. (2005) 57: 177-181.

The data is mean ± SD of optical density (OD) resulted from J774A.1 cells treated with hepcidin. The p-value 0.000

The effect of hepcidin on cell viability in J774A.1 cell line.

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The difference between the two types of hepcidin was not statistically significant (p = 0.44). These could also be explained by two possibilities. First, this cell line may express distinct levels of ferroportin channel, so the increase in hepcidin uptake during iron overload. J. Biol. Chem. (2001) 276: 7811-7819.

Second, cell culture medium has limited the source of the cells and they have restriction for the iron uptake. Thus, due to the limitation of iron, increasing hepcidin would not effect on iron concentration in the cells.

Physiological and biochemical effects of hepcidin in biological systems are under investigation. Jyh-Yih Chen et al. stated that fish hepcidin TH2-3 product have cytotoxic effect on HT1080 cell line (51). Our data show that synthetic hepcidin-25 in comparison with recombinant hepcidin-1 or BSA has a higher cytotoxic effect.

In conclusion, during this study, we produced functional mouse hepcidin-1 as a new drug at a high concentration and it was revealed that mouse hepcidin-1 has considerable effect on iron accumulation in macrophage cells and thus, it can decrease iron overload in serum and prevent iron precipitate formation from a marine fish (Pseudosciara crocea) and the antimicrobial activity of its synthetic peptide. Peptides (2009) 30: 638-646.
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