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Original Article

Design and production of a novel chimeric human growth hormone superagonist fused to human Fc domain

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Abstract

Background and purpose: Growth hormone (GH) has been known as a crucial metabolic hormone expressed at the pituitary and the other number of cells and tissues and responsible for body growth. Because of the short half-life of GH, daily subcutaneous injections were shown to be more effective for GH therapy. This represents a burden for patients. So, there is a strong effort from the industry to create a long-acting form of GH and lots of technologies like GH fusion proteins are used to increase GH half-life.
Experimental approach: In this study, the Fc domain of human IgG1 with serine-glycine linkers was attached to the C-terminal of a GH superagonist *via* molecular cloning. The presence of recombinant vector in *E. coli* host was confirmed by PCR. SDS-PAGE and western blot analysis showed the expression of recombinant proteins in the bacterial lysate. The binding ability to growth hormone receptors is determined by ELISA.
Findings / Results: Our results showed that the novel SupGH-Fc has a good binding affinity to its receptor in ELISA in comparison to standard GH, although it has a big size.
Conclusion and implications: Our data in this study clearly demonstrated the expression of the SupGH-Fc in a recombinant protein expression system. It is an introduction to the production of the new recombinant GH, which can bind to its receptor more effectively than commercial growth hormones and also might have a longer half-life.

Keywords: Fc fusion proteins; Growth hormone; Growth hormone superagonist; Long half-life.

INTRODUCTION

Growth hormone (GH) is a metabolic hormone expressed at the pituitary with the multi biological functions. The number of cells and tissues, which cause lipolysis, protein anabolism, and expression of many other growth factors like insulin-like growth factor I. It has direct effects on cellular proliferation and differentiation too (1,2). Till the 1950s, with the start of recombinant human GH (rhGH) production, GH has been used as a therapeutic protein with low cost and high efficiency in the treatment of a variety of diseases such as GH deficiency, smallness for gestational age, and idiopathic short stature (3-6). In 1985, rhGH was approved for clinical use (7). In numerous experimental studies, daily subcutaneous injections of rhGH can stimulate the growth of stature, similar to endogenous hGH.

GH molecular weight is just about 22 kDa and due to its small size, usually cleared *via* the kidneys (8). So, its plasma half-life after subcutaneous and intravenous injection is about 3.4 h and 20 min, respectively. Daily subcutaneous injections of GH are inconvenient because not only increase the cost of health care but also hurt and worry many patients and make patients refuse to use it.

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Accordingly, with the purpose of the production of rhGH with high efficiency and safety and fewer total injections to patients, various types of long-acting formulations of GH have been developed (9).

The hypothesis of long-term GH preparation has been proposed to reduce the frequency of injections and improve adherence to GH receptors leading to a longer half-life and better results to help patients (10). In recent times, protein fusion technology has caused the development of long-acting rhGH (4). With great effort, major advances were made in the production of Nutropin Depot® as a long-acting GH for the treatment of GH deficiency in 1999 (4,10). Polyethylene glycol, a hydrophilic polymer with low immunogenicity, is another approach used to adjust therapeutic proteins and peptides by increasing molecular weight and solubility, less toxicity, and prolonging the half-life of the circulating protein (10). Several early releases of polyethylene glycol-bound GH have induced lipoatrophy at the site of subcutaneous injection, which appears to be due to a delay in absorption of long-acting high-molecular-weight GH (4,10). As long as hGH is eliminated from the body by both kidney filtration and receptor-mediated clearance, a long-acting hGH-XTEN fusion protein is developed to treat GH deficiency (11). The benefits of using *Escherichia coli* (*E. coli*) as the host system are well known. It has exclusive fast growth and can easily grow to high cell densities (12).

Evidence from therapeutic monoclonal antibodies studies may provide useful information on how Fc-fusion proteins progress, and it will be clear that there is a close relationship between the desired effect of these drugs and the extent of interference with effective Fc systems (13).

Fc-based fusion proteins are homodimers, that they directly and covalently linked to any kind of biologically active protein or peptide molecule from the Fc domain of an immunoglobin G (IgG) (4,14). Following the binding of a peptide to the Fc of the IgG domain, the plasma half-life is intensely extended because the Fc portion binds to the neonatal Fc receptor in the endosome and its degradation is reduced. At present, several Fc-peptide complement proteins have been developed for clinical uses that offer fewer benefits as long-term GH therapy with less complexity of structure and less immune nature (4).

While fusion Fc-protein technologies have been extensively used, based on our knowledge and our teamwork, there are no reports that this technology has been applied to superagonist GH (SupGH) creation. In this study, a novel chimeric superagonist of rhGH is prepared by molecular cloning. The sequence of SupGH was designed in our previous work based on some changes of amino acids sequence of hGH to increase the affinity of SupGH to its receptor. For this purpose, the genetic structure of the GH was evaluated through NCBI and the essential amino acids for changing the hGH affinity (without affecting the attachment efficiency) selected. According to the conducted research studies, the substitution of the amino acids of arginine-167 to asparagine, lysine-168 to alanine, aspartic acid-171 to serine, lysine-172 to arginine, glutamine-174 to serine, histidine-21 to asparagine, isoleucine-179 to threonine, and histidine-18 to aspartic acid was down using gene design and the synthetic DNA sequence encoding SupGH constructed in pCold-I vector. Its engineered protein is proved by dot blotting, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and western blotting. To compare its affinity to the receptors with hGH, sandwich enzyme-linked immunosorbent assay (ELISA) was performed too.

MATERIAL AND METHODS

E. coli strains and plasmid

The *E. coli* Bl21 strain DE3 (Novagen) was used in this study for the production of rhGH protein. The construct of SupGH-Fc (long-acting hGH superagonist), was cloned in a pCold-I vector (Takara Cat # 3361). pCold-I plasmid contains cspA promoter, lac operator, translation enhancing element (TEE), HIS-Tag, factor Xa site, and the engineered gene (SupGH-Fc) in the cloning site. Moreover, the plasmid containing *Nde*I restriction enzyme in the N-terminal of cloning site targeted protein that followed by stop codon and *Hind*III as a restriction enzyme.

Construction of the SupGH-Fc vector

For long-acting superagonists, the recombinant chimeric protein was generated by fusing SupGH to the Fc fragment of human IgG1. In this structure, SupGH fused to a part of the flexible linker (3 glycines), *Bam*H1 restriction enzyme that not only played a role of the enzymatic property but also is a part of the flexible linker, 2 glycines is another part of the flexible linker put right after restriction site and in following Fc domain of human IgG1 (CH2 + CH3 without hinge region). Schematic diagram of the expression vector and recombinant proteins showed in Fig. 1. Schematic diagram of plasmid (pCold-I) showed in Fig. 2 .

To clone both SupGH fragment and Fc fragment in one vector, we used the following primers designed by Gene Runner software. SupGH primers (forward: 5′GCACATATGAT GTTCCCAACCATTCCGTTAAGCCG3′ and for reverse: 5′GAGGATCCACCACCACCAA AGCCACAGCTACCTTCCACAGAGCG3′) and Fc primers (forward: 5′GAGGATCCGGT GGTCCGTGCCCAGCACCTGAACTCCTG3′ and for reverse: 5′CGAAAGCTTTTATTTAC ACGGAGACAGGGAGAGGCTCTTCTG3′). The expected size of the final DNA products for SupGH-Fc was assessed with polymerase chain reaction (PCR).

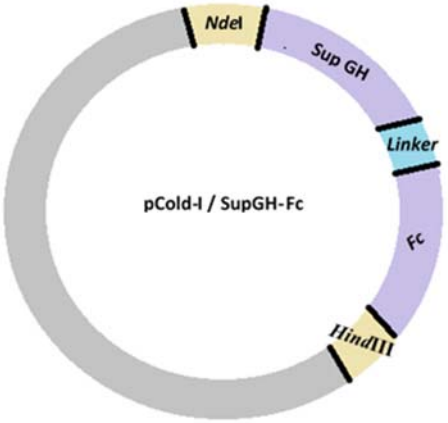

Fig. 2. Schematic diagram of plasmid (pCold-I) and recombinant protein (SupGH-Fc). SupGH, Growth hormone superagonist.

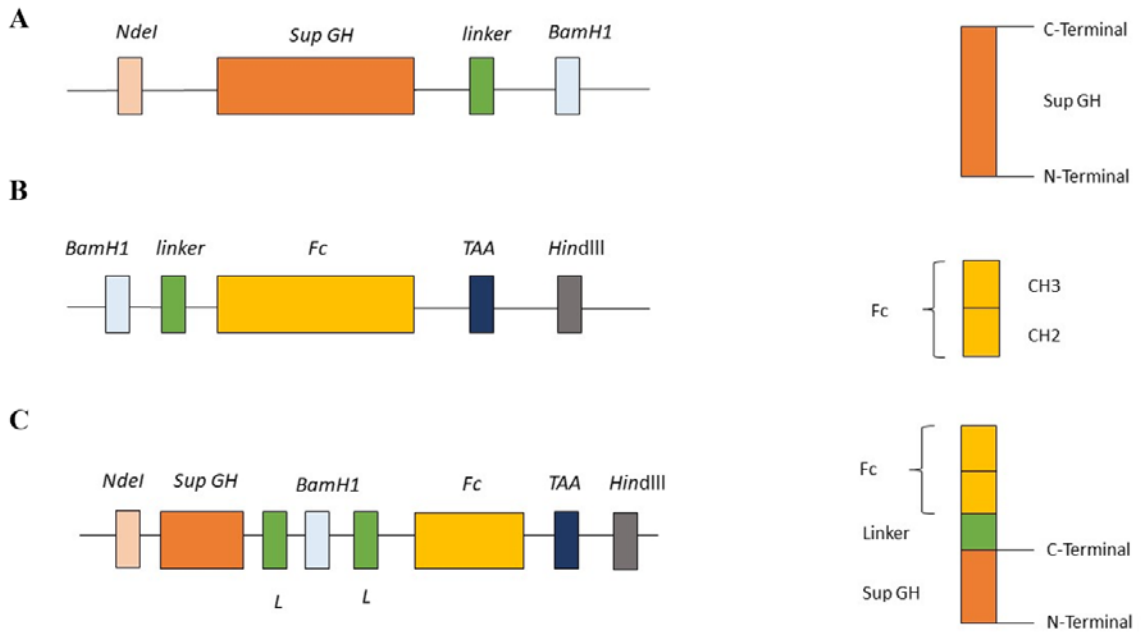

Fig. 1. Schematic diagram of the expression vector and recombinant proteins. (A) Gene sequence of SupGH with NdeI and BamH1 restriction site; (B) gene sequence of Fc with BamH1 and HindIII restriction site; (C) final gene construct of SupGH-Fc and final structure of the recombinant protein. SupGH, Growth hormone superagonist.

E. coli transformation, recombinant protein expression

To produce recombinant protein, *E. coli* Bl21 strain DE3 (Novagen) was transformed with pCold-I plasmid containing SupGH-Fc by heat shock transformation method. After transformation, a single colony from overnight culture was picked and inoculated into Luria-Bertani (LB) medium containing 100 mg/mL ampicillin and allowed to grow overnight in a shaking incubator at 220 rpm, 37 °C. This culture was used for the inoculation of new media for screening of protein expression. For SupGH-Fc expression, the maintained stock was inoculated in new LB media containing 100 mg/mL ampicillin. Then, the culture was grown until OD600 reached 0.7. At this point, the cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for 20 h at 15 °C, 220 rpm. For analysis of SupGH-Fc expression, the cell pellet was formed by centrifugation at 4000 rpm for 10 min. The pellet was sonicated on ice for 4 × 30 cycles and 50% power with 1 min cooling between the cycles to lyse the cells. The lysate extraction was then centrifuged at 12000 rpm, for 20 min at 4 °C and the supernatant was used as a bacterial lysate soluble fraction.

Dot blotting

For primary screening of SupGH-Fc production, the dot-blot technique was used. For this purpose, different concentrations of commercial hGH (10 mg/mL gifted from Professor Richard Ross, Sheffield University, England,) were used as the positive control. Bacterial lysate and medium (2 μL), with different dilutions as the same as a positive control, were transferred on a nitrocellulose membrane. The membrane was dried at room temperature for 10 min. After that, it was blocked with blocking buffer (10% skimmed milk in phosphate buffer saline, PBS) for around 1 h. The membrane was washed with wash buffer (PBS-T 0.1%) three times. Then, it was incubated with 1/1000 dilution of anti-GH antibody (5e1 antibody, gifted from Professor Richard Ross, Sheffield University, England) in blocking buffer for 90 min. After washing (three times, each 5 min), the blot was incubated with secondary horseradish peroxidase (HRP) labeled antibody (goat anti-mouse IgG conjugated HRP, Thermo Scientific, 1/1000 dilution) for 90 min, washed three times. In the end, for detection of the recombinant protein on the membrane, the blot was incubated with 3,3′, 5,5′-tetramethylbenzidine (TMB; Cito Matin Gene, Company, Iran).

SDS-PAGE and western blotting

Total expression levels of SupGH-Fc were analyzed in bacterial culture fraction with SDS-PAGE. Bacterial culture samples were taken before and after IPTG induction. Supernatants of bacterial cell lysates were gained from samples taken by centrifugation at 4000 rpm for 20 min. After centrifugation, the pellet was resuspended by PBS (pH: 8.0) to gain cell lysate. The profile of recombinant SupGH-Fc expression was checked on denaturing SDS-PAGE. For this purpose, all of them were resolved by 12.5% SDS-PAGE.

For the analysis of western blotting, the separated protein on the gel was transferred to a nitrocellulose membrane for 180 min at 40 V. The nitrocellulose membrane was blocked with 10% skim milk in PBS overnight. The rest of the processes were done as stated for dot blotting. In this western blotting, anti-poly-histidine HRP antibody (Sigma cat #A7058) dilution 1:1000 was used as the secondary antibody. Purified SupGH-Fc protein on the membrane was visualized using TMB.

Purification of recombinant SupGH-Fc

To purify the SupGH-Fc, all supernatants after sonication was applied to the nickel-nitriloacetic acid (Ni-NTA) column. At the first, the column was washed with 10 mL of ethanol 20%, 10 mL water, 15 mL of binding buffer (300 mM NaCl, 50 mM tris-base, 10 mM imidazole, pH: 8). At this time, the supernatant was added to the column. Then, 15 mL washing buffer (300 mM NaCl, 50 mM tris-base, 30 mM imidazole, pH: 8) was added to remove non-specific proteins. Finally, 5 mL of elution buffer (300 mM NaCl, 50 mM tris-base, 250 mM imidazole, pH: 8) was used to collect all purified proteins. After collection of the purified proteins, the purity and concentration of the purified samples were evaluated by the SDS-PAGE.

Determining of SupGH-Fc concentration

For accurate assessment of the presence and the concentration evaluation of SupGH-Fc in purified fractions, we used the chemiluminescence technique. The target concentration was measured with LIAISON hGH kit, which is a type of laboratory test to check the presence of hGH in human serum. A multimode reader (Bio Tech, USA) was used to determine the concentration of recombinant SupGH-Fc

Sandwich ELISA

Sandwich ELISA was used to survey the binding affinity of SupGH-Fc compared to commercial native hGH to its receptor. For this purpose, purified recombinant hGH receptors (0.5 mg/mL), gifted from Professor Richard Ross, Sheffield University, England, were diluted in sodium carbonate buffer (0.1 M, pH: 9.5) to reach 1 µg/mL. Then, 100 µL of the above solution was added to a 96-well ELISA plate for coating the hGH receptor and incubated at 4 °C for overnight. The next day, all contents were removed and 250 µL of blocking buffer (bovine serum albumin (BSA) 3% in PBS) was added to each well to block the unbound sites. (3 h in 37 °C). The washing step was repeated with PBS-T 0.1% (4 times). hGH as positive control and BSA 3% as negative control were used in our test. hGH (10 µg/mL) and SupGH-Fc as a test (20 µg/mL) were diluted in PBS and immediately 100 µL of above solutions were added to each well after washing and incubated at 37 °C for 2 h. The washing step was repeated with PBS-T 0.1% (4 times). Primary anti hGH (5e1 antibody) was diluted in BSA 1% in PBS (1/10000 concentration) and 100 µL was added to each well for 2 h at 37 °C. Subsequently, 100 µL of goat anti-mouse IgG-HRP (Thermo Scientific, USA) with 1/9000 concentration, was added to wells for 2 h at 37 °C. For detection of bounded proteins to their receptors, 100 µL of the TMB substrate was added for 30 min at room temperature. At the end to terminate the reaction, 50 µL of stop solution was added and the plate absorbance was read at 450 nm on the ELISA microtiter plate reader (Stat Fax 2100, USA).

Statistical analysis

The statistical analysis was performed through SPSS software using one-way ANOVA followed by Tukey as post hoc. *P* values less than 0.01% were considered significant.

RESULTS

Generation of SupGH-Fc pCold-I expressing vector

First, PCR analysis of original vectors containing SupGH (600 bp) and FC (681 bp) was performed (Fig. 3). Before ligation, SupGH and FC genes were inserted into pCold-I at *Bam*H1 and *Hind*III restriction sites under the control of the T7 promoter. The final construct was transformed to *E. coli* DH5α by the CaCl2 method. PCR analysis of some random colonies of *E. coli DH5α* grown on LB agar containing 100 mg/mL ampicillin as a selection marker was performed to confirm the presence of chimeric SupGH-Fc construct. Some selected colonies had the expected amplified bands at 1281 bp (Fig. 4 lane 3).

Production of SupGH-Fc in E. coli

For the production of recombinant chimeric GH and reaching the highest yield as the soluble proteins, the induction was performed at 15 °C using 1 mM IPTG.

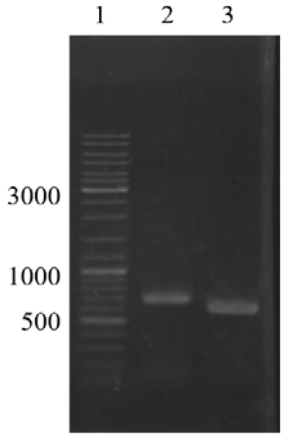

Fig. 3. PCR analysis of original vectors containing SupGH and FC before ligation. Lane 1: DNA ladder, lane 2: PCR product of vector containing Fc at 681 bp, and lane 3: PCR product of vector containing SupGH at 600 bp. SupGH, Growth hormone superagonist; PCR, polymerase chain reaction.

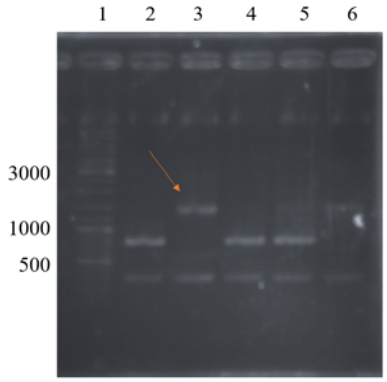

Fig. 4. Colony PCR analysis of *Escherichia coli* DH5α transformed with pCold-I vector containing SupGH-Fc. Lane 1: DNA ladder, lanes 2, 4, and 5 are those colonies which amplification did not occur. Arrow in lane 3 showed an amplified PCR product of SupGH-Fc with 1281 bp length. Although, in lane 6 amplified PCR product of SupGH-Fc was exist. SupGH, Growth hormone superagonist; PCR, polymerase chain reaction.

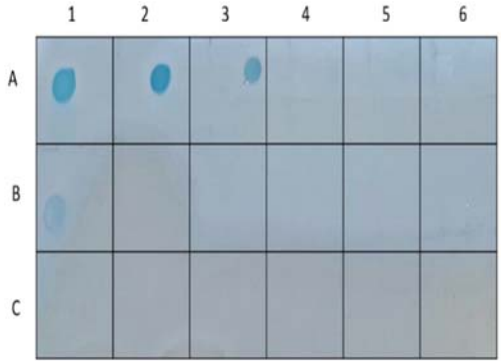

Fig. 5. Dot blot analysis of transformed *Escherichia coli* bl21 expressed SupGH-Fc. A: serial dilution of commercial human GH as a positive control, B: serial dilution of bacterial lysate, and C: serial dilution of culture media. All the digits 1-6 were related to serial dilution respectively: 1/10, 1/100, 1/1000, 1/10000, 1/100000, 1/1000000. SupGH, Growth hormone superagonist.

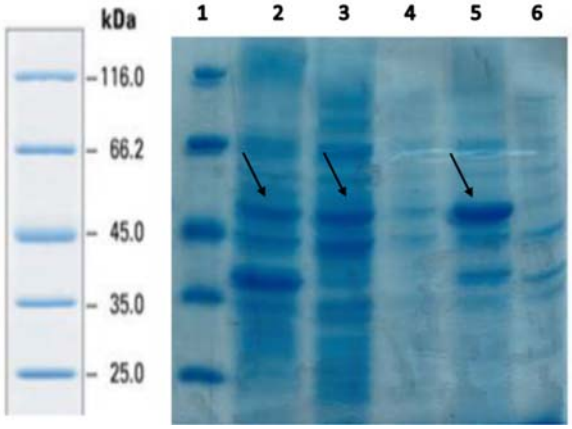

Fig. 6. SDS-PAGE analysis of pellets and supernatant fractions of transformed *Escherichia coli* bl-21. Lane 1: protein molecular weight marker (14-116 kDa), lane 2: supernatants after sonication (soluble fraction), lane3: bacterial pellet after sonication (insoluble fraction), lanes 4 and 5: induced with IPTG with dilution 1/50 and 1/10 respectively. Lane 6: uninduced total cell lysate. Arrow indicates the band representing SupGH-Fc. SupGH, Growth hormone superagonist; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Production of SupGH-Fc in E. coli

For the production of recombinant chimeric GH and reaching the highest yield as the soluble proteins, the induction was performed at 15 °C using 1 mM IPTG. After induction, the cells were collected by centrifugation, then the cells were lysed and aliquots of supernatant and pellet were analyzed for recombinant protein expression. Dot blot analysis as a primary screening showed that recombinant SupGH-Fc protein presented in bacterial cell lysate (Fig. 5, well B1). Bacterial cell fractions were analyzed on 12.5% SDS-PAGE for confirming the SupGH-Fc expression and determining recombinant protein molecular weight. As shown in Fig. 6, the SDS-PAGE result shows the expressed protein was in pellet fraction as soluble and insoluble form (inclusion body) (lanes 2 and 3).

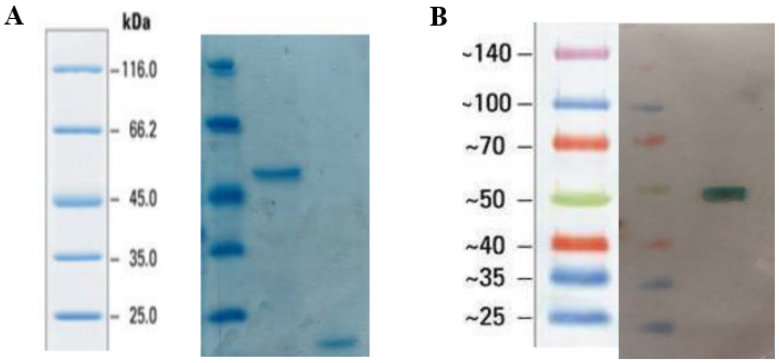

Fig. 7. (A) SDS-PAGE that shows the 99% purification of SupGH-Fc, (B) western blot analysis of SupGH-Fc. In both A and B, purified SupGH-Fc was seen around 50 kDa. SupGH, Growth hormone superagonist; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

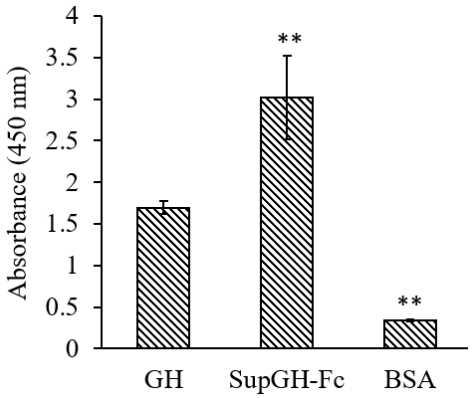

Fig. 8. Sandwich ELISA with SupGH-Fc. ELISA was conducted by plate coated with hGH receptor. Commercial human GH was used as control. The results showed that SupGH-Fc significantly had a high affinity to its receptor compared to human GH as a control group with the same molarity (400 nmol). $^{**}P < 0.001$ Indicates significant differences compared with the control group. BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; SupGH, growth hormone superagonist.

Recombinant SupGH-Fc purification

After protein expression in the *E. coli* Bl21 strain, the soluble protein was purified using Ni-NTA affinity chromatography under natural conditions which led to about 90% purity successfully (Fig. 7). The identity of the purified SupGH-Fc protein was verified by western blotting using an anti-6X His-tag antibody. As shown in Fig. 7, there is a protein band around 50 kDa as theoretically determined by ExPASy in 50.5 kDa.

Survey of SupGH-Fc binding affinity to hGH receptor

Sandwich ELISA was set up to compare the affinity of SupGH-Fc with hGH for hGH receptors. For this purpose, ELISA plate wells coated using the hGH receptor. The concentration of purified SupGH-Fc was determined based on the chemiluminescence technique. The results of the statistical analysis of this experiment (Fig. 8) showed that the new engineered SupGH-Fc protein had a high affinity to its receptor compared to commercial hGH as a control group with the same molarity (400 nM).

DISCUSSION

hGH is a peptide hormone secreted from the anterior pituitary. It has a central function of regulating normal metabolism and exhibits pleiotropic effects on various human tissues such as the immune system and cardiovascular system (15). Despite the common application of rhGH replacement therapy in different diseases, the main complication with the native GH is their short plasma half-life (0.36 h after intravenous injection) (16). This GH instability leads to requiring administration *via* injection at a high dosing frequency. Daily subcutaneous injections result in many difficulties for patients. To address this problem, a variety of long-acting formulations of rhGH have been developed with the hope of achieving comparable efficacy and safety using fewer total injections, including depot or sustained-

release formulations of rhGH (17) or rhGH attached to PEG (18) and fusion proteins (19). Some of these have been withdrawn because of their problems such as difficulties in manufacturing production (20) or local injection-site lipoatrophy (21).

The present study was aimed at producing a new hGH superagonist that can be used to treat various diseases related to hGH deficiency. Our SupGH-Fc is a novel chimeric protein designed not only can keep high-affinity binding to the GHR, but also might have a long half-life. For this purpose, first of all, in the previous study (unpublished data), we designed and produced a mutant rhGH as a novel superagonist that had more affinity to GHR and was able to induce proliferation in cells with more potency compared to wild-type of hGH when used at an equal concentration (data not showed). In the next step, to create a special fusion protein, the Fc sequence of human IgG1 was added to the C-terminal of protein to investigate the growth hormone affinity for its receptor due to its increase in size. This article is a primary report about the creation of the SupGH-Fc.

Some studies attempt to develop long-acting rhGH products focused on either delivery of native rhGH or some modifications such as PEGylation without altering the binding of rhGH to its receptor. These approaches only achieved short-term rhGH exposure and subsequently lower pharmacodynamics responses (22). While recombinant protein technology helps to create a novel GH with improved properties such as more stability and affinity for GHR.

Based on the previous studies, it can be estimated that by designing an antagonist with some changes at the first site of hGH (as maintained in the method), it would be possible to produce an antagonist that compared with native hGH, has a good binding affinity to GHR (8,23,24). Thus, it was possible to draw SupGH according to these 8 changes and it was then tested in cell culture and ELISA for the survey of its capability for binding to GHR and function in comparison with commercial GH, which we did in this study.

Fusion peptide-Fc technologies exploit to prolong the half-life of the biologically active peptide. Currently, several peptides-Fc chimeric proteins have been developed, some of them such as trebananib, romiplostim, and dulaglutide have been approved for clinical treatment (25,26). When a peptide was fused to the Fc-domain of IgG, its plasma half-life was dramatically prolonged, through neonatal Fc receptor mechanism and therefore converts the daily injection to injections with longer time intervals. In addition, clearance by the kidneys is reduced due to the large size of the molecules (27). Moreover, from a technological point of view, the Fc domain attached protein can more easily and efficiently purify by purification of chromatography of G / A protein in the factory (28).

Protein fusion technology has contributed to the development of long-acting rhGH by Kim *et al.* and created fusion protein by fusing the Fc-domain of human IgG1 to rhGH *via* a 16-amino acid GS linker. The pharmacokinetic profiles of rhGH-Fc in plasma have shown an intensely longer half-life, higher C_{max}, and larger area under the curve ($AUC_{0\text{-}14d}$) compared with a single injection of the rhGH in Sprague Dawley rats. The half-life of rhGH-Fc was extended by two days, almost 100 folds longer than that of rhGH in rats (4). In our project, for the first time, we fused an hGH superagonist to the IgG Fc domain and the production and binding affinity to its receptor were confirmed by western blot analysis and ELISA, respectively.

Remarkably, sandwich ELISA with new SupGH-Fc determined that its binding affinity to GHR significantly is more than commercial hGH (2 folds rather hGH). The most important point about this study is that our recombinant chimeric GH, by attachment to the Fc of the IgG1 molecule, did not lose its high affinity to its receptor. On the other hand, based on some studies which show that Fc fusion proteins could have a longer half-life, fusion with Fc and the larger size rather than native form might help to reduce kidney clearance of rhGH after treatment and so might have a long half-life, too.

CONCLUSION

This is a primary report of designing and production of a novel chimeric human SupGH

fused to the human IgG1 Fc domain which could have a much longer half-life in the body than the native form in the GH replacement therapy. Our data in this study clearly demonstrated the expression of the SupGH-Fc in prokaryote expression systems and its binding ability to GH receptor was more efficient than commercial hGH. Final approval of the rhGH needs pharmacokinetic and pharmacodynamic validation in the next step and verifying its proper performance under *in vivo* conditions.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

Authors' contributions

S. Mirbaha performed the experiments and first manuscript writing; M. Rezaei wrote and edited the manuscript; S.H. Zarkesh Esfahani supervised the project and conceptualization; R. Emamzadeh contributed to the work as an advisor of the project.

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