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Research paper

Engineering a pharmacologically superior form of granulocyte-colony-stimulating factor by fusion with gelatin-like-protein polymer ☆

Yan-Shan Huang [a], Xiao-Fang Wen [b], Yi-Liang Wu [b], Ye-Fei Wang [b], Min Fan [b], Zhi-Yu Yang [b], Wei Liu [a,*], Lin-Fu Zhou [b,*]

[a] Department of Cell Biology, Zhejiang University, Hangzhou, China
[b] Medical Biotechnology Laboratory, Zhejiang University, Hangzhou, China

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ABSTRACT

The plasma half-life of therapeutic proteins is a critical factor in many clinical applications. Therefore, new strategies to prolong plasma half-life of long-acting peptides and protein drugs are in high demand. Here, we designed an artificial gelatin-like protein (GLK) and fused this hydrophilic GLK polymer to granulocyte-colony-stimulating factor (G-CSF) to generate a chimeric GLK/G-CSF fusion protein. The genetically engineered recombinant GLK/G-CSF (rGLK/G-CSF) fusion protein was purified from *Pichia pastoris*. *In vitro* studies demonstrated that rGLK/G-CSF possessed an enlarged hydrodynamic radius, improved thermal stability and retained full bioactivity compared to unfused G-CSF. Following a single subcutaneous administration to rats, the rGLK/G-CSF fusion protein displayed a slower plasma clearance rate and stimulated greater and longer lasting increases in circulating white blood cells than G-CSF. Our findings indicate that fusion with this artificial, hydrophilic, GLK polymer provides many advantages in the construction of a potent hematopoietic factor with extended plasma half-life. This approach could be easily applied to other therapeutic proteins and have important clinical applications.

1. Introduction

Peptides and proteins are increasingly being developed as therapeutic drugs. However, the applications of these proteins are often limited due to rapid clearance from the plasma by renal filtration, proteolysis or other undetermined factors [1].

To date, two major strategies have been established to improve the pharmacokinetics of peptide/protein drugs and achieve sustained pharmacological action. One strategy is to conjugate the therapeutic peptide/protein with a highly hydrophilic and physiologically inert polymer such as polyethylene glycol (PEG) or hydroxyethyl starch [1–3]. The conjugation enlarges the hydrodynamic diameter of the therapeutic protein, thereby preventing renal filtration and proteolysis. Unfortunately, the chemical coupling of therapeutic proteins with polymers has shown some drawbacks. For example, high-purity PEG derivatives are expensive, and the conjugation is a multi-step process, which lowers the yield and raises the manufacturing costs. In addition, the conjugated proteins are often heterogeneous, requiring in-depth quality control procedures. Furthermore, depending on the conjugation site, the biological activity of the therapeutic protein might be reduced after conjugation [1].

The second strategy is to utilize long plasma half-life proteins, such as albumin, the Fc fragment of IgGs or transferrin, as carriers for therapeutic proteins. Therapeutic proteins fused with these carriers obtain longer plasma half-lives. For example, Fc fragment has been used in fusions with a variety of active ligands, resulting in longer half-lives [4]. Additionally, cytokines and interferon have been fused to albumin in an attempt to prolong the activities of these two normally short half-lived molecules [5,6]. Therapeutic proteins can also be bound to these carriers through chemical conjugation or affinity binding [7,8]. For example, proteins have been bound to albumin via fusion with a bacterial albumin-binding domain (ABD) from Streptococcal protein G or with a peptide selected against albumin from a phage display library [9]. Although fusion with, or binding to, these carriers improves the pharmacokinetics of therapeutic proteins, at the same time, it often causes a decline of bioactivity due to steric hindrance, unwanted biological functions and additional immunogenicity brought about by the carrier moieties. Recently, some new fusion carriers were also adopted to improve protein drug's half-life but with no substantial progress compared with albumin or Fc fragment [10,11].

Abbreviations: GLK, gelatin-like protein; G-CSF, granulocyte-colony-simulating factor; rGLK/G-CSF, recombinant gelatin-like protein/granulocyte-colony simulating factor; WBC, white blood cells.

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* Corresponding authors. Faculty of Basic Medicine, Medicine School of Zhejiang University, 388 Yuhangtang Road, Post-box 40, Hangzhou 310058, Zhejiang Province, China. Tel./fax: +86 571 88208238.
E-mail addresses: liuwei666@zju.edu.cn (W. Liu), 239zlf@zju.edu.cn (L.-F. Zhou).

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Gelatin, a mixture of high molecular weight peptides consisting of repeating Gly-Xaa-Yaa triplets (Xaa and Yaa are often proline) produced by hydrolysis of collagen, is fairly hydrophilic because its hydrogen bonds are highly exposed and rich in hydrophilic amino acids. Gelatin displays an increased hydrodynamic diameter that is attributable to its open, unfolded conformation and its hydrophilic nature, to some extent like hydrophilic polymers [12]. Gelatins are widely used in the pharmaceutical industry as therapeutic implants, plasma expanders and stabilizers for vaccines and other biopharmaceuticals [13]. Despite the diverse applications of natural gelatin, recombinant gelatins may provide superior features for specific applications because of intentional alteration of the amino acid sequence by gene-engineering technology [14].

Granulocyte-colony-stimulating factor (G-CSF), an 18.8 kDa cytokine that can promote the proliferation and maturation of neutrophil precursors, has been widely applied clinically to accelerate neutrophil recovery after chemotherapy and irradiation-induced myelosuppression [15]. However, G-CSF, like many other therapeutic proteins, possesses a short plasma half-life due to its small molecular size and susceptibility to serum proteases. Pegylated and albumin-fused versions of G-CSFs are long-acting and more effective drugs than non-modified G-CSF [5,16]; however, all of these approaches suffer from the associated problems described above. In this study, we designed a highly hydrophilic gelatin-like protein (GLK) polymer and fused it with G-CSF to construct a new hybrid protein in order to prolong the serum half-life of G-CSF. The genetically engineered recombinant gelatin-like protein/granulocyte-colony stimulating factor fusion protein (rGLK/G-CSF) was expressed in *Pichia pastoris*. We purified the fusion protein and investigated its biochemical properties, pharmacokinetics and pharmacodynamics in rats. Our results suggest that GLK fusion technology is superior to all current technologies to prolong the plasma half-life of long-acting proteins.

2. Materials and methods

2.1. Construction, expression and purification of rGLK and rGLK/G-CSF fusion protein

GLK sequence was composed of four identical artificially designed 116 amino acids monomers (GLK_m) (Fig. 1). The global strategy of GLK cloning is similar to Dan E. Meyer's description [17]. Synthesized GLK_m and human granulocyte-colony-stimulating factor (hG-CSF) gene (Invitrogen Technologies, Shanghai, China) were inserted into pMD18-T (TaKaRa, China) to yield $pGLK_m$-T and pG-CSF-T vectors, respectively. pGLKm-T was first digested by Van91I/DraIII to extract a 328 bp fragment. Then, the $pGLK_m$-T was dephosphorylated after digestion by Van91I. Finally, GLK_m and dephosphorylated $pGLK_m$-T were ligated together to obtain vector $pGLK_d$-T-containing dimeric GLK_m. Sequences digested by endonuclease Van91I and DraIII share the same cohesive end, but cannot be recognized by either of them after ligation. pGLK-T was constructed in the same way by inserting GLK_d into $pGLK_d$-T.

In order to construct expression vector pPIC-GLK/G-CSF, *GLK* gene was excised from pGLK-T by XhoI/Van91I, and pG-CSF-T was digested by DraIII/EcoRI. Extracted *GLK* and *G-CSF* fragments were ligated with pPIC9 (Invitrogen, USA) previously treated with XhoI/EcoRI, followed by transformation into *Escherichia coli* DH5α (Fig. 2). Expression vector pPIC-GLK was constructed in a similar way by digesting pGLK-T with XhoI/EcoRI.

pPIC-GLK and pPIC-GLK/G-CSF were both linearized with SalI. Preparation of competent GS115 (*His*$^-$) (Invitrogen, USA) cells and electroporation were performed according to manufacturer's instruction (*Pichia* Expression Kit, Protein Expression, Invitrogen,

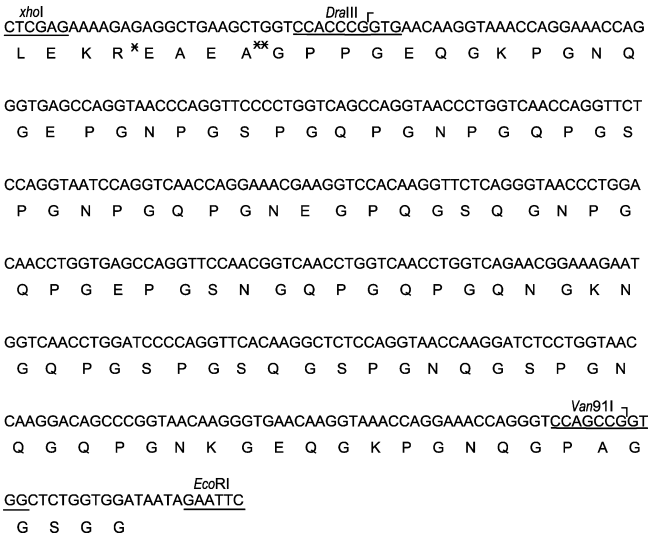

Fig. 1. Artificially designed GLK sequence. Symbols "*" and "**" represent cleavage sites by *STE13* and *KEX2* gene product in *Pichia pastoris*, respectively. Sequence between symbols "⌈" and "⌉" was repeated 4 times to form tetrameric GLK.

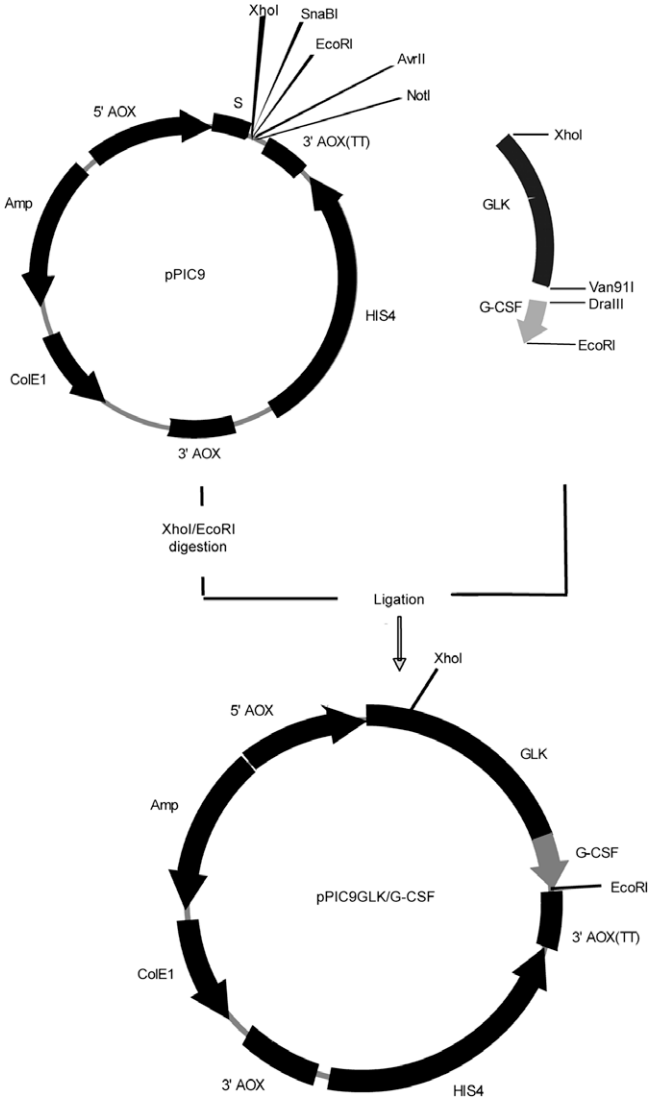

Fig. 2. Construction of the pPIC-GLK/G-CSF expression vector.

USA). After electroporation, cells were spread on RDB plates to screen for *His*$^+$ transformants.

The fermentation procedures are similar to previous reports [12,18]. Briefly, after 50 h of culture, the medium was centrifuged and the supernatant was loaded onto a SP Sepharose FF column, which was pre-equilibrated with buffer A (20 mM NaAc, pH 3.0). The SP resin was washed with 0.3 M NaCl. The target fraction was further purified by Q Sepharose FF (GE Healthcare, USA) and concentrated by ultrafiltration.

2.2. SDS–PAGE

Purified proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 8% acrylamide gel and 5% condensing gel in the Mini-Protein II electrophoresis unit (Bio-Rad), which was stained with 0.25% Coomassie Brilliant Blue R-250 (Aldrich, USA).

2.3. Size exclusion chromatography

Protein in 0.05 mM PB (pH 7.0) was injected on a TSK gel G3000 SWXL column (TOSOH, Japan). Elution was carried out with 0.05 mM PB (pH 7.0) at a flow rate of 1.0 ml/min and monitoring the absorbance at 280 nm.

2.4. Mass spectrometry

Molecular weight was obtained from MALDI-TOF mass spectrometry using a BIFLEX mass spectrometer (Bruker Bremen, Germany). Samples were prepared by mixing a 1 μl aliquot with 2 μl of the matrix solution, a saturated solution of alpha-cyano-4-hydroxycinnamic acid in 50% water/acetonitrile with 0.3% trifluoroacetic acid (TFA). Ionization was accomplished with the 337-nm beam from a nitrogen laser with a repetition rate of 3 Hz with 1.5-m flying tube and 20 kV acceleration voltage was used.

2.5. In vitro bioactivity assay

Bioactivity of the rGLK/G-CSF fusion protein was determined in murine myeloblastic NFS-60 cells by a cell proliferation assay [19]. The cells were then incubated in 50 μl of assay media containing serial dilutions of rGLK/G-CSF or rhG-CSF standard (Filgristim®, 0.7×10^8 IU/mg, Amgen Inc., USA). The cultures were kept at 37 °C in a humidified incubator with 5% CO_2 for two days before 10 μl of 2.5 mg/ml MTT was added. Four hours later, 100 μl of 10% SDS in 0.01 M HCl were added to solubilize the cells and formazan. The plate was then read at 570 nm with the reference beam set at 630 nm. The OD_{570} was plotted against the concentration of the proteins, and the inflection point of the sigmoidal curve represents the concentration at which the ED_{50} is induced.

2.6. Animal immunization and antibody evaluation

Balb/C mice (female, 8–12 weeks), which were purchased from Animal Center of the Medical School, Zhejiang University, were divided into two groups. Mice were administered with subcutaneous injection of rGLK/G-CSF and rGLK proteins (200 μg/mouse and 150 μg/mouse, respectively, about 2.5 nmol/mouse) once a week for four weeks. Animal experiments were compliant with the Principles of Laboratory Animal Care (Zhejiang University). One week after the last administration, the immunological reactivity of GLK or G-CSF in sera were evaluated by enzyme-linked immunosorbent assay. Briefly, polystyrene microplates (Corning, USA) were coated with rGLK (100 ng/well) or rhG-CSF (200 ng/well) in PBS overnight. Plates were blocked with 5% fat-free milk in PBST, and then serial serum dilutions (100 μl/well) were seeded and incubated. Reaction was revealed by addition of peroxidase-conjugated secondary antibodies (Bioss, China), and the color was developed with tetramethylbenzidine (TMB, BBI, China). Sera obtained from naive animals were assayed in the same plate as negative control, and 200 ng/ml mouse anti-human G-CSF polyclonal IgG (Hangzhou Epitomics, China) was used as a positive control.

2.7. Pharmacokinetic and pharmacodynamic studies in rats

SPF grade Sprague–Dawley (SD) rats (7-weeks old) were obtained from the Animal Center of Zhejiang University. rGLK/G-CSF (0.3, 1 or 3 mg/kg) was administered subcutaneously to investigate the pharmacokinetics and pharmacodynamics. rhG-CSF (0.3 mg/kg, approximately the same molar ratio as 1 mg/kg of rGLK/G-CSF) and rGLK (1 mg/kg) were administered in the same way as the positive and negative control, respectively. Blood was collected from the tail vein at the indicated time points after administration. WBCs were counted using a microcell counter CC-180A (Toa Medical Electronics, Japan). rhG-CSF and rGLK/G-CSF concentrations in plasma were analyzed with a sandwich-type ELISA kit (Human G-CSF ELISA Construction Kit, Antigenix, USA) following the manufacturer's instructions. All the pharmacokinetic parameters were calculated using 3P87 software (The Chinese Society of Mathematical Pharmacology).

2.8. Statistical analysis

Statistical analysis of pharmacodynamic studies between the rGLK/G-CSF and rGLK groups (vehicle control) were compared using a Student's *t*-test. Each *p* value reflected a comparison of average responses between the two groups.

3. Results

3.1. Design of GLK polymer sequence

Natural gelatin is composed of Gly-Xaa-Yaa triplets and most of the Xaa and Yaa are proline and hydroxyproline, respectively (the latter being post-translationally modified proline) [20]. The synthetic GLK polymer sequence also consists of Gly-Xaa-Yaa triplets, but all of the hydrophobic amino acids, except proline, were replaced by hydrophilic amino acids.

The artificially designed GLK gene encodes four repeating, reconstructed, gelatin-like monomers. The detailed gene sequence is shown in Fig. 1. The differences in hydrophobicity (grand average of hydropathicity, GRAVY) [21], isoelectric point (pI) and molecular weight (MW) among the reconstructed GLK polymer, cattle bone gelatin and recombinant Col3a1 gelatin are shown in Table 1 [12]. The artificially designed GLK has a lower GRAVY value (−1.808) compared with natural gelatins (−0.75 to −1.09), which means a higher hydrophilic value. The composition of acidic and

Table 1
Physico-chemical parameters of the artificially designed GLK polymer and natural gelatins.

	GLK[a]	Co13al[b]	Cattle bone gelatin[b]
GRAVY[a]	−1.808	−1.08	−0.75 to −1.09
Isoelectric point (pI)	5.06	9.7	4.7–5.4
Molecular weight (kDa)	40.6	20.6	65–300

[a] GRAVY values, isoelectric point and molecular weight were calculated using the ProtParam tool available at the *Expasy WWW server* as described [21].
[b] The data of Co13al and cattle bone gelatin cited from a previous report [12]. Natural cattle bone gelatin is a heterogeneous mixture of molecules of different molecular weights and isoelectric points within the indicated ranges.

basic residues was modulated to give an acidic isoelectric point (pI = 5.06).

3.2. Purification, biochemical characterization and biological activity of rGLK and rGLK/G-CSF

rGLK and rGLK/G-CSF fusion proteins with a purity of at least 96% were obtained after serial purification procedures (determined by SEC-HPLC and SDS–PAGE). About 85 mg rGLK and 92 mg rGLK/G-CSF were obtained from 1 l of cell-free broth, respectively. Under normal electrophoresis conditions, recombinant GLK (rGLK) bands were usually dispersed and could be easily de-colored by destaining solution (methanol–acetic acid). In order to get a sharp band, we used a higher voltage (300 V) for electrophoresis and shortened the destaining time [12]. Nevertheless, compared with rGLK/G-CSF, rGLK still gave a dispersed band (Fig. 3). This is possibly because rGLK could not bind with SDS efficiently due to its hydrophilic nature and coiled linear structure. In fact, some pegylated products also displayed dispersed bands on SDS–PAGE [22]. For the same reasons, rGLK bands were also more easily destained compared with rGLK/G-CSF, which includes the hydrophobic G-CSF moiety. This was consistent with the fact that Coomassie Brilliant Blue R-250 only binds to the hydrophobic regions of proteins.

The apparent MW of rGLK estimated by reduced SDS–PAGE is about 96 kDa, 2.4 times larger than the values deduced from the DNA sequences (40597.5 Da), and the MW of rGLK/G-CSF is about 86 kDa, 1.5 times larger than the values deduced from the DNA sequences (59251.1 Da). We speculated that this was due to incomplete SDS binding caused by the extreme hydrophilic character and coiled linear structure of GLK. Our speculation was also supported by the apparent MW of rGLK/G-CSF which, including hydrophobic G-CSF moieties, is closer to the theoretical value for the fusion protein than that of rGLK. Furthermore, SEC-HPLC analysis gave similar results with the apparent MW of rGLK and rGLK/G-CSF being larger than their theoretical values. The MWs of rGLK and rGLK/G-CSF were approximately 126 kDa and 154 kDa as determined by SEC-HPLC, 3.1 and 2.6 times larger, respectively, than the theoretical MWs. The aberrant higher MWs estimated by SEC-HPLC imply that rGLK and rGLK/G-CSF possess enlarged hydrodynamic diameters. The ultimate molecular weights of rGLK and rGLK/G-CSF were determined by mass spectrometry. The observed molecular weight of rGLK and rGLK/G-CSF were 40598.8 Da and 59238.4 Da, which corresponds well with the theoretical value, respectively. Sequence of the N-terminal of rGLK/G-CSF revealed the expected amino acid sequence (GPPGEQGKP). The sequence of C-terminal was AQP-COOH, completely consistent with that of G-CSF (data not show).

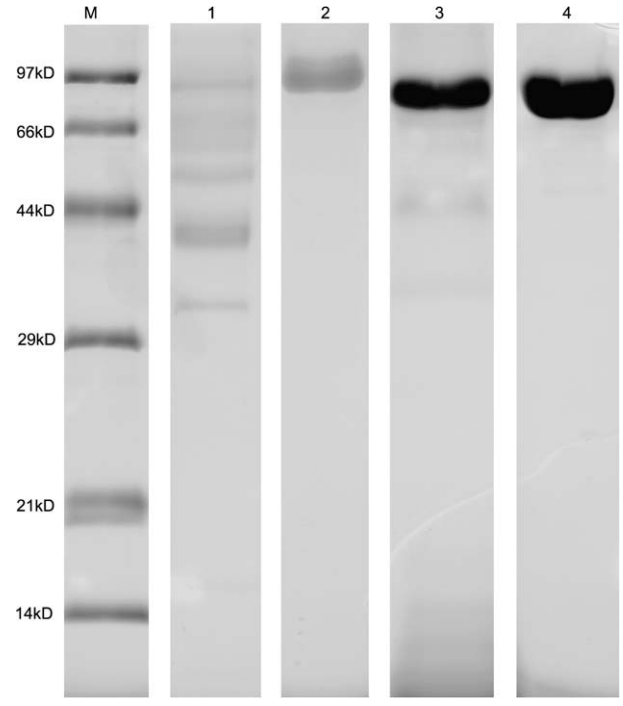

Fig. 3. Expression and purification of rGLK and rGLK/G-CSF. Lane 1, rGLK fermentation supernatant; lane 2, purified rGLK; lane 3, rGLK/G-CSF fermentation supernatant; lane 4, purified rGLK; lane M, molecular weight marker.

Table 2
Thermalstability comparison of rhG-CSF and rGLK/G-CSF (40 °C). Residual protein content (%) = [(monomeric proteins after storage at timed intervals/(monomeric proteins before heating)] × 100. Monomeric proteins were analyzed by SEC-HPLC. The concentration of samples was 1.5 mg/ml (100 mM sodium phosphate, pH 6.0).

	Purity (%)			Residual protein content (%)		
	0 h	24 h	48 h	0 h	24 h	48 h
rhG-CSF	99.8	94.3	85.2	100	91.2	68.9
rGLK/G-CSF	99.5	98.7	97.5	100	97.8	94.7

It has been reported that G-CSF is temperature-sensitive and prone to aggregate at elevated temperatures [23]. We analyzed thermostability and aggregation of G-CSF and rGLK/G-CSF. As shown in Table 2, after incubation at 40 °C, the monomeric form of G-CSF decreased quickly accompanied by the formation of polymeric aggregates. rGLK/G-CSF showed improved thermostability represented by less aggregation formation.

To investigate the *in vitro* bioactivity of rGLK/G-CSF, we used a G-CSF-dependent murine myeloblastic cell line, NFS-60. Representative assay results for rGLK/G-CSF and G-CSF are shown in Fig. 4. The EC_{50} of rGLK/G-CSF is 424 ± 22 pg/ml, while the EC_{50} of rhG-CSF is 202 ± 15 pg/ml, consistent with previous reports [24,25]. Calculated on a molar basis, the EC_{50} of rhG-CSF (10.7 ± 0.8 pM) and rGLK/G-CSF (7.63 ± 0.40 pM) were quite similar, indicating the well-retained *in vitro* biological activity of rGLK/G-CSF. These experiments demonstrate that fusion with GLK did not change the conformation of G-CSF or exhibit steric hindrance in biorecognition.

3.3. Immunogenic analysis

To evaluate the immunogenic properties of the GLK motif, mice were administered subcutaneously either rGLK/G-CSF or rGLK

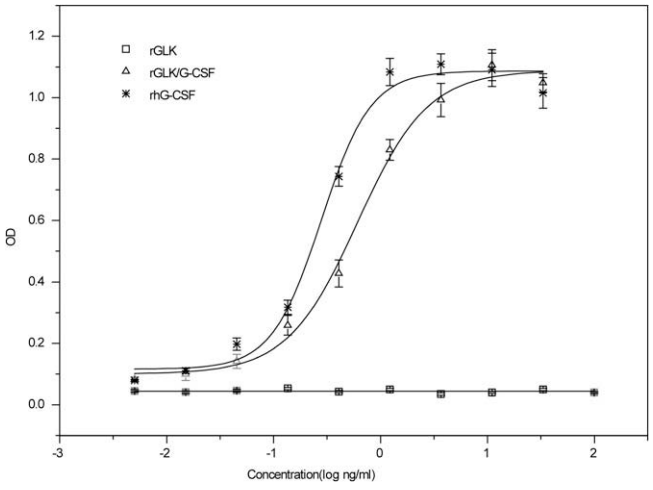

Fig. 4. A typical murine myeloblastic cell line NFS-60-based bioassay of G-CSF (18.8 kDa) and rGLK/G-CSF (59.3 kDa). Proliferation of NFS-60 was measured via MTT assay. Error bars represent SD, $n = 3$.

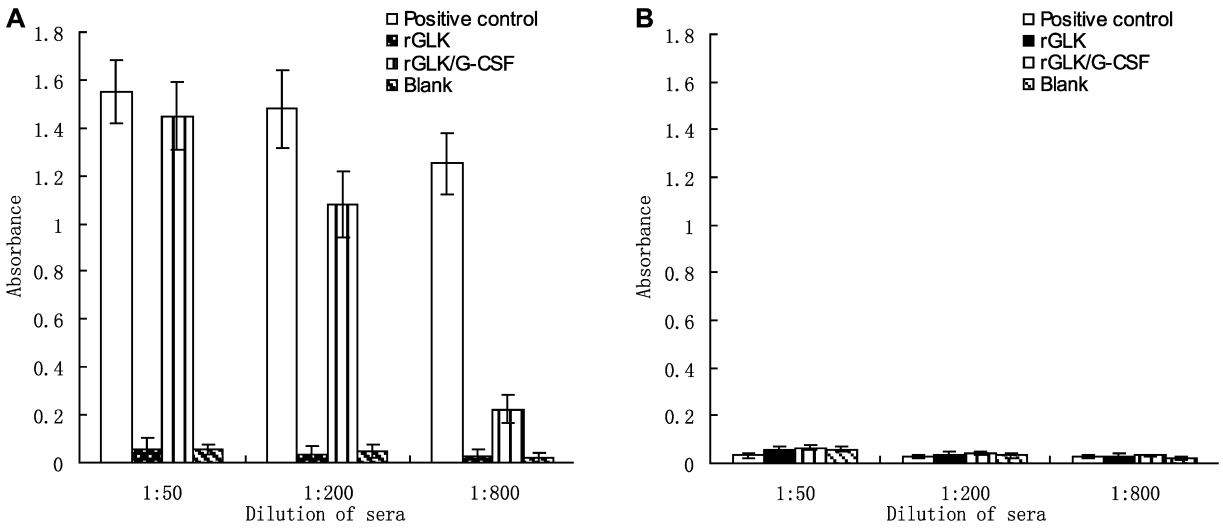

Fig. 5. Immunogenicity of GLK motif. Sera antibody were determined by an enzyme-linked immunosorbent assay at the indicated sera dilutions using rhG-CSF(A) or rGLK (B) as the immobilized antigen. 200 ng/ml mouse anti-human G-CSF polyclonal IgG was added into the negative sera as a positive control.

proteins (2.5 nmol/mouse) every week for 4 times, and the specific antibody response against GLK and G-CSF were assayed by enzyme-linked immunosorbent assay. From our results, we found that repeat administration of rGLK and rGLK/G-CSF did not produce high titer of antibodies against GLK (Fig. 5). Only anti-G-CSF antibody was detected due to sequence heterogeneity of the G-CSF in different species.

3.4. Pharmacokinetics and pharmacodynamics of rGLK/G-CSF

To investigate *in vivo* bioactivity and determine the half-life, rGLK/G-CSF or G-CSF were administered subcutaneously to Sprague–Dawley (SD) rats. Our data demonstrated that after fusion with GLK, rGLK/G-CSF exhibited an improved neutrophilic hematopoiesis effect in rats (Fig. 6A). A single administration of rGLK/G-CSF could increase the leukocyte numbers in rat peripheral blood in a dose dependent manner. The maximum mobilization of peripheral white blood cells (WBCs) occurred at 12 h for rGLK/G-CSF at 0.3 mg/kg and 1.0 mg/kg, with a 2.4-fold ($p < 0.01$) and 2.6-fold ($p < 0.001$) increase, respectively, and at 24 h for rGLK/G-CSF at 3.0 mg/kg (3.4-fold increase) ($p < 0.001$), when compared with mice that received rGLK. The WBC count returned to control levels after 144 h for rGLK/G-CSF in the 0.3 and 1.0 mg/kg group. In the 3.0 mg/kg group, exhibiting a better neutrophilic hematopoiesis effect, the WBC count did not recover to control levels after 144 h ($p < 0.05$). In the positive control group (0.3 mg/kg rhG-CSF, approximately the same molar ratio as 1 mg/kg of rGLK/G-CSF), the maximum mobilization of WBCs occurred at 12 h (1.7-fold increase) compared with the control. WBC count returned to normal levels at 72 h after a single administration of rhG-CSF. There were no significant changes of the WBC count after the same period of time following injection with rGLK. These data demonstrate that rGLK/G-CSF exhibits more effective and sustained hematopoiesis effects *in vivo* compared with rhG-CSF at similar molar amounts.

The serum concentrations of rhG-CSF decreased rapidly after subcutaneous administration (Fig. 6B). Whereas administration of rGLK/G-CSF resulted in prolonged presence in serum. The results of the pharmacokinetic experiment were as follows: the total body

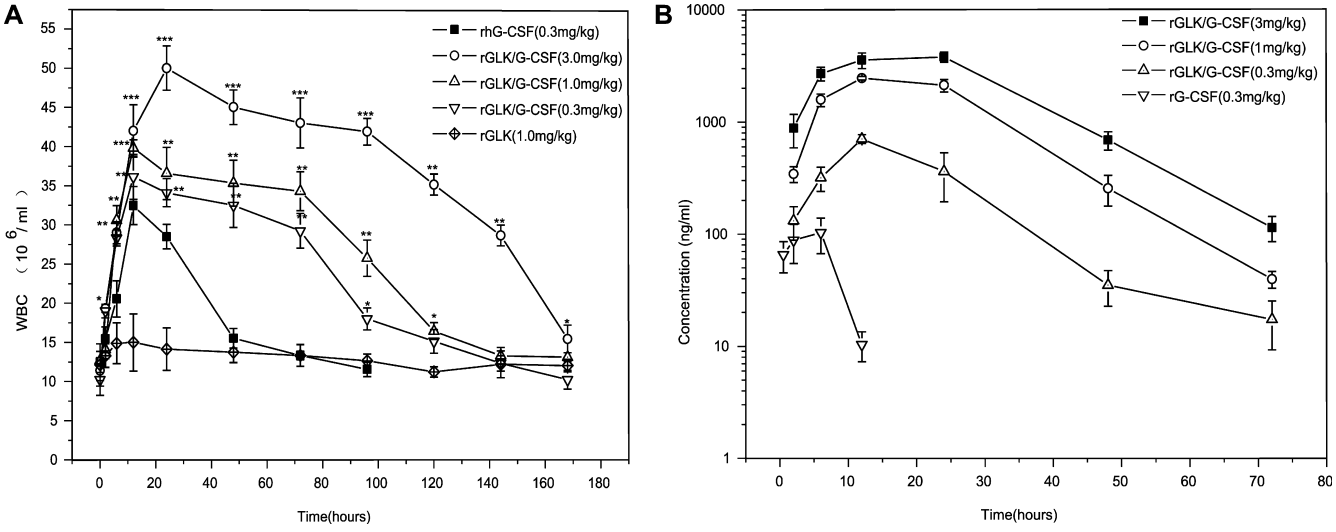

Fig. 6. Pharmacodynamic and pharmacokinetic studies of rGLK/G-CSF fusion proteins. (A) Administration of single dose of rGLK/G-CSF increased peripheral WBC counts greatly compared with rGLK (vehicle control) ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). (B) Changes in plasma protein levels after s.c. administration to rats. Concentration of G-CSF was determined between 0.5 and 12 h and the concentration of rGLK/G-CSF was determined between 0.5 and 72 h. Error bars represent the standard error of the mean, $n = 5$ for all groups.

clearance of rhG-CSF decreased from 157 ml/h/kg to 11.6–38.4 ml/h/kg after fusion with the GLK polymer. The terminal half-life of rhG-CSF was 1.76 h, which was in good agreement with the previously reported value [26]. In contrast, rGLK/G-CSF was absorbed more steadily and cleared much more slowly following a biexponential elimination pattern with a terminal half-life of 7.92–9.96 h. rGLK/G-CSF exhibited a much slower clearance and a longer terminal half-life than rhG-CSF following subcutaneous administration, resulting in much greater exposure (area under concentration–time curve, AUC) following a single dose.

These results suggested that fusion with GLK could effectively prevent G-CSF being eliminated from the serum, thereby prolonging the half-life of G-CSF and improving its *in vivo* bioactivity.

4. Discussion

The short serum half-life is a major drawback of many therapeutic proteins. Fusion to a long-life protein carrier has proven effective in prolonging the half-life of a therapeutic protein. This method provides a rational and economical way of developing homogenous long-acting protein drugs. A suitable fusion carrier should have the following characteristics: long *in vivo* half-life, immunogenicity-free, biological inert, allows the therapeutic activity to be maintained and is easily expressed and manufactured. However, so far, none of the currently used fusion carriers satisfy all of these requirements.

Previous results showed that sequences enriched in hydrophilic and negatively charged residues and inserted into a proline-rich framework, constitute promising candidates to create chimerical proteins with an extended half-life in blood [27]. However, it is difficult to design such a biologically inert and immunogenicity-free sequence without any suitable template. Gelatin is hydrophilic because its proline-rich sequence usually displays an open, unfolded conformation in solution, which results in exposure of a lot of hydrogen bonds. Furthermore, the gelatin sequence is conserved in most species and immunogenicity-free, which had been widely proven in the human body [13]. We therefore chose natural gelatin as our initial template and designed an artificial gelatin-like sequence containing more hydrophilic and negatively charged amino acids. By reserving gelatin's Gly-Xaa-Yaa triplet repeats and replacing all the hydrophobic amino acids, except proline, with hydrophilic ones, we produced a more hydrophilic gelatin-like protein (GLK) polymer.

From a biochemical perspective, a highly hydrophilic polymer, such as GLK, would constitute a more desirable fusion partner than present fusion carriers. First, unlike globular Fc and albumin, GLK is biologically inert and will not elicit any biological activity. Furthermore, the immunogenicity of GLK fusion proteins in animal models is more predictable because gelatin sequences are conserved in most species, unlike other carrier proteins. Second, the stability of therapeutic proteins was greatly improved after fusion with GLK. Under increased temperatures, rGLK/G-CSF showed minimal aggregate formation, compared with rhG-CSF. There is strong evidence that aggregates produced during the process of protein drug manufacture and storage are the primary immunogen eliciting the immune response [28]. Fusion with GLK can inhibit the formation of protein aggregates because of its highly hydrophilic nature, so lower immunogenicity can be expected. Fusion with GLK can also increase the stability of G-CSF *in vitro* because GLK is thought to function as a protein stabilizer. The hydrophilic GLK domain can interact with the exposed groups of partially unfolded proteins, avoiding aggregation of the unfolded proteins [29]. Furthermore, compared with natural gelatin, reconstructed GLK includes more hydrophilic amino acids and therefore has superior protective effects. Third, it has been reported that most of the gelatin in the solution exhibits a random coiled conformation resembling that of a linear polymer, e.g. PEG. Compared with the widely-used carrier proteins (albumin, Fc, etc.), fusion of therapeutic proteins with linear GLK exhibits less steric hindrance during therapeutic protein–ligand biorecognition. *In vitro* biological activity assays showed rGLK/G-CSF is more potent than G-CSF (1.4-fold) on a molar basis. In previous reports, Neulasta® (PEG_{20000} attached in N-terminal of recombinant human G-CSF) retained about 60% biological activity [30] and Albugranin® (a recombinant human G-CSF genetically fused to human albumin) only retained 14.3% bioactivity [31]. Our rGLK/G-CSF retained highest biological activity *in vitro* compared with these long-acting versions of G-CSF. However, it is still unclear why rGLK/G-CSF is more potent *in vitro* than Neulasta®, a pegylated G-CSF that is also modified by a linear molecule. Bioassay method deviation can be excluded because we compared the *in vitro* bioactivity of rGLK/G-CSF and Neulasta® in parallel, and the results were similar to those previously reported (data not shown).

The pharmacokinetics of G-CSF was improved substantially by fusion with GLK. G-CSF's serum half-life increased from 1.76 h to about 10 h in rats, similar to the half-lives of Neulast® and Albugranin® [5,30]. However, the exact mechanism underlying the prolonged serum half-life of the therapeutic proteins by GLK is still to be determined. One possible mechanism is that the outspread structure of GLK can protect the therapeutic protein against proteolysis *in vivo*. Alternatively, the greater hydrodynamic radius of GLK in solution, due to its enriched hydrophilic amino acids and unfolded structure, may to some extent avoid renal filtration. Furthermore, the ionic characteristics of GLK allow GLK fusion proteins to remain stable and reside in serum for longer. The reconstructed GLK sequence contains many negative amino acids, such as Glu and Asp, so the GLK fusion proteins are always negatively charged in physiological conditions. The surface of the vessel wall is covered by negatively charged glycocalyx, which controls substance transport between the vessel and the surrounding matrix [32]. Since both the glycocalyx and the GLK fusion proteins are negatively charged, this reduces the interaction between them, resulting in reduction in the penetration from vessel to tissue. It may be that the integrated effects of all of these mechanisms render the GLK fusion protein's longer serum half-life. However, further investigations are required to substantiate each of these mechanisms.

The approach used in this study presents a more effective strategy to increase the plasma half-life of therapeutic proteins through the use of recombinant fusion proteins that can be readily expressed in microbial systems and potentially scaled-up for production. Compared with pegylated products, GLK fusion proteins are homologous, degradable in the body and more economical to produce. Compared with albumin or Fc fusion proteins, GLK fusion proteins are more potent, less immunogenic and present fewer side effects. This creative approach we described here combined the advantages of pegylation and carrier protein fusion technology while avoiding their disadvantages. Further preclinical, animal experiments are underway to prove the efficacy and safety of this technology.

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References

[1] J.M. Harris, R.B. Chess, Effect of pegylation on pharmaceuticals, Nat. Rev. Drug Discov. 2 (2003) 214–221.

[2] N. Zander, W. Eichner, H. Conradt, F. Hacket, K. Langer, M. Orlando, R. Frank, Conjugates of hydroxyalkyl starch and a protein, prepared by reductive amination, US Patent 0134197, 2007.
[3] S. Jain, D.H. Hreczuk-Hirst, B. McCormack, M. Mital, A. Epenetos, P. Laing, G. Gregoriadis, Polysialylated insulin: synthesis, characterization and biological activity in vivo, Biochim. Biophys. Acta 1622 (2003) 42–49.
[4] S.M. Chamow, A. Ashkenazi, Immunoadhesins: principles and applications, Trends Biotechnol. 14 (1996) 52–60.
[5] W. Halpern et al., Albugranin, a recombinant human granulocyte colony stimulating factor (G-CSF) genetically fused to recombinant human albumin induces prolonged myelopoietic effects in mice and monkeys, Pharm. Res. 19 (2002) 1720–1729.
[6] B.L. Osborn, H.S. Olsen, B. Nardelli, J.H. Murray, et al., Pharmacokinetic and pharmacodynamic studies of a human serum albumin-interferon-α fusion protein in cynomolgus monkeys, J. Pharmacol. Exp. Ther. 303 (2002) 540–548.
[7] L.L. Baggio, Q. Huang, X. Cao, D.J. Drucker, An albumin-exendin-4 conjugate engages central and peripheral circuits regulating murine energy and glucose homeostasis, Gastroenterology 134 (2008) 1137–1147.
[8] S.C. Makrides, P.A. Nygren, B. Andrews, et al., Extended in vivo half-life of human soluble complement receptor type 1 fused to a serum albumin-binding receptor, J. Pharmacol. Exp. Ther. 277 (1996) 534–542.
[9] M.S. Dennis, M. Zhang, Y.G. Meng, Albumin binding as a general strategy for improving the pharmacokinetics of proteins, J. Biol. Chem. 277 (2002) 35035–35043.
[10] J. Xu, L. Tan, K.J. Goodrum, M.J. Kieliszewski, High-yields and extended serum half-life of human interferon alpha2b expressed in tobacco cells as arabinogalactan-protein fusions, Biotechnol. Bioeng. 97 (2007) 997–1008.
[11] I.R. Wilkinson, E. Ferrandis, P.J. Artymiuk, M. Teillot, C. Soulard, C. Touvay, S.L. Pradhananga, S. Justice, Z. Wu, K.C. Leung, C.J. Strasburger, J.R. Sayers, R.J. Ross, A ligand-receptor fusion of growth hormone forms a dimer and is a potent long-acting agonist, Nat. Med. 13 (2007) 1108–1113.
[12] M.W. Werten, W.H. Wisselink, T.J. Jansen-van den Bosch, E.C. de Bruin, F.A. de Wolf, Secreted production of a custom-designed, highly hydrophilic gelatin in *Pichia pastoris*, Protein Eng. 14 (2001) 447–454.
[13] D. Olsen, C. Yang, M. Bodo, et al., Recombinant collagen and gelatin for drug delivery, Adv. Drug Deliv. Rev. 55 (2003) 1547–1567.
[14] D. Olsen, J. Jiang, R. Chang, R. Duffy, M. Sakaguchi, S. Leigh, R. Lundgard, J. Ju, F. Buschman, V. Truong-Le, B. Pham, J.W. Polarek, Expression and characterization of a low molecular weight recombinant human gelatin: development of a substitute for animal-derived gelatin with superior features, Protein Expres. Purif. 40 (2005) 346–357.
[15] K. Welte, J. Gabrilove, M.H. Bronchud, E. Platzer, G. Morstyn, Filgrastim (r-metHuG-CSF): the first 10 years, Blood 88 (1996) 1907–1929.
[16] W.P. Petros, Introduction: pegfilgrastim, a new era in the management of chemotherapy-induced neutropenia, Pharmacotherapy 23 (2003) 1S–2S.
[17] D.E. Meyer, A. Chilkoti, Genetically encoded synthesis of protein-based polymers with precisely specified molecular weight and sequence by recursive directional ligation: examples from the elastin-like polypeptide system, Biomacromolecules 3 (2002) 357–367.
[18] Y.S. Huang, Z. Chen, Z.Y. Yang, T.Y. Wang, L. Zhou, J.B. Wu, L.F. Zhou, Preparation and characterization of a potent, long-lasting recombinant human serum albumin-Interferon-α2b fusion protein expressed in *Pichia pastoris*, Eur. J. Pharm. Biopharm. 67 (2007) 301–308.
[19] N. Shirafuji, S. Asano, S. Matsuda, K. Watari, F. Takaku, S. Nagata, A new bioassay for human granulocyte colony-stimulating factor (hG-CSF) using murine myeloblastic NFS-60 cells as targets and estimation of its levels in sera from normal healthy persons and patients with infectious and hematological disorders, Exp. Hematol. 17 (1989) 116–119.
[20] N. Thyagarajapuram, D. Olsen, C.R. Middaugh, The structure, stability, and complex behavior of recombinant human gelatins, J. Pharm. Sci. 96 (2007) 3363–3378.
[21] J. Kyte, R.F. Doolittle, A simple method for displaying the hydropathic character of a protein, J. Mol. Biol. 157 (1982) 105–132.
[22] Y.Y. Tan, M.X. Xu, Z.L. An, X.Z. Tan, X.Y. Tan, Q.H. Han, D.A. Miljkovic, M. Yang, R.M. Hoffman, X.H. Sun, Polyethylene glycol conjugation of recombinant methioninase for cancer therapy, Protein Expres. Purif. 12 (1998) 45–52.
[23] S. Krishnan, E.Y. Chi, J.N. Webb, B.S. Chang, D. Shan, M. Goldenberg, M.C. Manning, T.W. Randolph, J.F. Carpenter, Aggregation of granulocyte colony stimulating factor under physiological conditions: characterization and thermodynamic inhibition, Biochemistry 41 (2002) 6422–6431.
[24] Y. Bai, D.K. Ann, W.C. Shen, Recombinant granulocyte colony-stimulating factor – transferrin fusion protein as an oral myelopoietic agent, Proc. Natl. Acad. Sci. USA 102 (2005) 7292–7296.
[25] J.E. Layton, N.E. Hall, F. Connell, J. Venhorst, H.R. Treutlein, Identification of ligand-binding site III on the immunoglobulin-like domain of the granulocyte colony-stimulating factor receptor, J. Biol. Chem. 276 (2001) 36779–36787.
[26] H. Tanaka, T. Tokiwa, Pharmacokinetics of recombinant human granulocyte colony-stimulating factor studied in the rat by a sandwich enzymelinked immunosorbent assay, J. Pharmacol. Exp. Ther. 255 (1990) 724–729.
[27] P. Alvarez, C.A. Buscaglia, O. Campetella, Improving protein pharmacokinetics by genetic gusion to simple amino acid sequences, J. Biol. Chem. 279 (2004) 3375–3381.
[28] M.D. Barbosa, E. Celis, Immunogenicity of protein therapeutics and the interplay between tolerance and antibody responses, Drug Discov. Today 12 (2007) 674–681.
[29] N. Thyagarajapuram, D. Olsen, C.R. Middaugh, Stabilization of proteins by recombinant human gelatins, J. Pharm. Sci. 96 (2007) 3304–3315.
[30] O.B. Kinstler, N.E. Gabriel, C.E. Farrar, R.B. Deprince, N-terminally chemically modified protein compositions and methods, US Patent 5985265, 1999.
[31] R. Fleer, A. Fournier, J.D. Guitton, G. Jung, P. Yeh, Fusion polypeptides comprising human serum albumin, nucleic acid encoding same, and recombinant expression thereof, US Patent 5876969, 1997.
[32] M. Simionescu, N. Simionescu, Functions of the endothelial cell surface, Annu. Rev. Physiol. 48 (1986) 279–293.