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GeneOptimizer Program-assisted cDNA Reengineering Enhances sRAGE Autologous Expression in Chinese Hamster Ovary Cells

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Abstract

Soluble receptor for advanced glycation end products (sRAGE) is a secreted mammalian protein that functions as a decoy to counter-react RAGE signaling-resultant pathological conditions, and has high therapeutic potentials. Our prior studies showed that recombinant human sRAGE expressed in Chinese hamster, *C. griseus*, ovary (CHO) cells is modified by specific N-glycosylation, and exhibits higher bioactivity than that expressed in other host systems including insect *S. frugiperda* cells. Here, we show that GeneOptimizer software program-assisted, reengineered sRAGE cDNA enhances the recombinant protein expression in CHO cells. The cDNA sequence encoding human sRAGE was optimized for RNA structure, stability, and codon usages in CHO cells. We found that such optimization augmented sRAGE expression over 2 folds of its wild-type counterpart. We also studied how individual parameter impacted sRAGE autologous expression in CHO cells, and whether sRAGE bioactivity was compromised. We found that the enhanced expression appeared not to affect sRAGE N-glycosylation and bioactivity. Optimization of sRAGE expression provides a basis for future large-scale production of this protein to meet medical needs.

Keywords

GeneOptimizer program; sRAGE; protein expression; CHO

Introduction

The receptor for advanced glycation end products (RAGE) has been considered as a “non-canonical Toll” that transmits endogenous ligand-triggered signals, leading to inflammation and maladaptation in the tissue [1]. RAGE signaling has been implicated in several detrimental human diseases including cardiovascular and Alzheimer's disease, cancers, and diabetes [2-5]. As a natural product, Soluble RAGE (sRAGE) exists in two forms: one is a minor product of an alternative RNA splicing of the *AGER* gene, which is also termed endogenous sRAGE (esRAGE) [6]; and the other is a product from a protease cleavage or “shedding” of membrane-anchored RAGE [7]. The two forms of sRAGE differ at a small

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portion of their C-termini, but both exhibit the decoy function that scavenges various RAGE ligands and dampens RAGE signaling [8].

Although the regulation of sRAGE generation *in vivo* is currently unclear, the potential clinical value of sRAGE has been well recognized [9-11]. Recombinant sRAGE expressed in insect *S. frugiperda* host cells (Sf9) has been used to block various pathological conditions in animal models [12-16]. Our recent studies found that mammalian cell-specific, complex-type N-glycosylation of sRAGE is critical for its bioactivity, and that sRAGE expressed in CHO cells exhibits remarkably higher efficacy than that of other host systems including Sf9 cells to block injury-triggered arterial inflammation and neointimal growth [17]. In addition, glycans originated from insect cells are immunogenic in mammalian system, and therapeutic proteins currently approved by FDA must be produced in mammalian sources [18]. CHO cell system has been widely used by pharmaceutical industries to express therapeutic glycoproteins owing to its efficient glycosylation capacity, and the easy scaled-up for mass production [19, 20]. Thus, in addition to achieving high bioactivity, optimization of recombinant sRAGE expression in CHO cell system should help to meet technical challenges in industrial-scale production of this therapeutic protein for clinical applications.

Multiple parameters may influence recombinant protein expression in a host cell system. Such parameters include the preference of codon usage by specific host cells or tissues [21], the secondary structure of the encoding mRNA that potentially affects ribosomal translation [22], and the GC-content and distribution that may influence mRNA stability as well as transcription efficiency [23]. GeneOptimizer program integrates these multi-parameters that impact gene expression [24]. Because of the rapid progress in synthetic biology that renders large-scale and fast synthesis of long nucleotide sequences, generation of a synonymously mutated gene has become a reality. Recently, a large-scale study using a broad range of target genes has demonstrated that such optimization is a reliable tool for boosting gene expression in different host systems [25].

Here, we use GeneOptimizer software program to reengineer human sRAGE cDNA sequence for its expression in CHO cells. Our studies showed that sRAGE expression is indeed augmented after optimization, while the critical post-translational modifications and bioactivity of the protein are maintained. In addition, we also evaluated the critical parameters that influence sRAGE autologous expression in CHO cells. Our work provides a technical basis for future large-scale production of sRAGE for therapeutic purposes.

Materials and methods

The sRAGE expression vector

The original sRAGE expression vector was constructed with PCR amplification of the coding sequence of human RAGE (reference sequence: NM_001136) from residue 23 to 340 (*i. e.* the ectodomain of RAGE) followed with a stop codon. The amplified fragment was then cloned to the pcDNA 3.1 (zeo+)-based pJP008 membrane-targeting vector, which contains the RAGE signal peptide sequence and renders the expressed membrane protein to be tagged with a T7 epitope tag at its N-terminus [26]. The diagram of T7 tagged sRAGE is illustrated in Fig. 1.

Gene optimization and cDNA synthesis

T7-sRAGE cDNA optimization was performed with GeneOptimizer® expert software from Life Technologies-Invitrogen, using the composite sequence provided by the authors. The sequence-optimized composite T7-sRAGE sequence was then synthesized and subcloned to a pcDNA 3.1(zeo+) vector between KpnI and XbaI sites. Both the wild-type (WT) and the

sequence-optimized sRAGE (OP) were nucleotide-sequenced to ensure their correctness. A PDF file containing both WT and OP T7-sRAGE is provided in Supplemental Material (SF1).

Cell culture and protein expression analyses

Lipofectamine 2000 (Invitrogen)-mediated transfection of constructs harboring sRAGE sequences to CHO-K1-based CHO-CD14 cell line [27] was performed as previously described [28]. Briefly, 1 μ g sRAGE plasmids was transfected to 10^6 cells pre-seeded in a 35 mm plate. The overnight cell culture medium containing secreted sRAGE was collected for analyses. A CMV promotor-driven GFP was co-transfected with sRAGE plasmids as an internal control, and the transfected cells were analyzed with flow cytometry to obtain the transfection efficiency.

The expression levels of sRAGE from wild-type (WT) and optimized (OP) constructs were assessed with two approaches: Western blotting and ELISA. Western blotting was performed as previously described [26], and 26 μ g of total protein in cell culture medium was resolved with NuPAGE SDS 4–12% Bis-Tris gel (Invitrogen). Rabbit anti-T7 antibodies (Millipore) were used to detect the expressed sRAGE protein, and the blot intensity was measured with a Kodak Gel Logic 2200 Imaging System and processed with molecular imaging software.

For ELISA, a Quantikine human RAGE ELISA kit (R & D System) was used and the analysis was performed according to the manufacturer's instruction. Briefly, overnight cell culture medium containing sRAGE was diluted 500 $\times$ (for codon preference studies), or 20 $\times$ (for production rate studies) with the diluent from the kit, and 50 μ l of diluted sample was used for the assay. The concentration of sRAGE was calculated from the standard curve, using the sRAGE standard provided by the manufacturer.

To measure the sRAGE production rate, after over night incubation, GFP co-transfected cells were washed multiple times with 1 $\times$ PBS, and fresh cell culture medium was added to the cell. At various time points, a small volume of medium was removed, and sRAGE concentration was measured in each sample with ELISA. The sRAGE production rate was then calculated using GFP-positive cell numbers determined by flow cytometry analyses.

Real-time RT-PCR measurement of mRNA stability

Total RNA was isolated at various time points from transfected CHO-CD14 cells treated with 6 μ M actinomycin D (Sigma)[29], using TRIZOL reagent (Invitrogen) according to manufacturer's instruction. The obtained RNA pellets were dissolved in DEPC-treated water, and then further treated with DNase I (4 units/sample) at 37 $^{\circ}$ C for 30 min to obtain DNA-free samples. The concentration of RNA was determined by measuring A_{260} .

cDNA was synthesized from 0.5 μ g extracted DNA-free RNA with M-MLV reverse transcriptase (Promega) and random hexamer. cDNA (2 μ l) was then quantified with QuantiFast SYBR Green PCR kits (Qiagen) in an ABI7300 real time PCR system. Serial 10 fold dilution of sRAGE (WT) or sRAGE(OP) plasmid from 10^{-1} to 10^{-5} ng/ml were used as templates to establish a standard curve to calculate the half-life of mRNA, as described [30]. Primers sequence specific to sRAGE(WT) are: forward: 5'-AGCCACTGGTGCTGAAGTGT-3', reverse: 5'-GAATCTGGTAGACACGGACTC-3'; primers sequence specific to sRAGE(OP) are: forward: 5'-CCGAGTTCATGGCCTCTATG-3', reverse: 5'-GAGGACTCAGCACCTTCCAG-3'.

PNGase F digestion

PNGase F (New England Biolabs) digestion of sRAGE from sRAGE(WT) and sRAGE(OP)-transfected cells were performed as described [26]. Briefly, 40 µg of cell culture media from transfected cells were first incubated with the denaturing buffer at 100 °C for 10 min and supplemented with 10 × G7 reaction buffer from the same kit. The mixture was then divided to two portions. PNGase F (500 units/reaction) was added to one portion and the same volume of H₂O was added to another portion as the uncut control. The reaction mixtures were incubated at 37 °C for 1 h and resolved with 4-12% gradient SDS-PAGE, followed with western blot using anti-T7 antibodies.

Purification of sRAGE

Affinity purification of sRAGE from CHO-CD14 cells has been described[17]. Briefly, CHO-CD14 cells stably or transiently transfected with T7-sRAGE construct (WT or OP plasmid) were grown in RPMI1640 cell culture medium supplemented with 10% fetal bovine serum and antibiotics. Cell culture medium was collected daily and centrifuged at low speed to remove the dead cells. A Novagen T7 tag affinity purification kit was used to purify sRAGE, according to the manufacturer's instructions. A small volume of collected fractions were resolved with SDS-PAGE followed with silver staining and western blot to determine the purity. The fractions containing pure T7-sRAGE were then pooled and the protein concentration was determined using a RAGE ELISA kit from R & D Systems, and the protein was stored in aliquot at -80°C.

NF-κB activity assays

NF-κB activity was assayed using a dual-luciferase reporter assay system (Promega) according to manufacturer's instruction, and has been described in our previous publication [17]. RAGE ligands HMGB1(Sigma),S100B (Sigma), and AGE-BSA (Biovision), all in 100 nM were used to stimulate NF-κB activity on HEK293 cells stably transfected with RAGE. A 1:1 molar ratio of sRAGE: ligand was used in the inhibition experiment, and samples were measured in triplicate.

Statistic analyses

Variances of the samples were determined using F-test followed with 2-sample T-test for equal/unequal variances. Error bars are standard error of mean.

Results and discussion

Optimization of T7-tagged human sRAGE cDNA sequence

The composite sequence encompassing the coding sequences of RAGE signal peptide, the inserted T7 epitope tag, and sRAGE with a stop codon was optimized using the Geneoptimizer® expert software (Life technologies-Invitrogen Inc.). The optimization was performed according to the general rationale of gene design [31] to remove putative RNA secondary structures that may potentially obstruct ribosomal translation, to eliminate cryptic splice sites, direct repeats, and RNA destabilizing sequence elements. The program also optimizes the GC content and distribution, and adjusts the codon preference to Chinese hamster (*C. griseus*). In addition, a Kozak consensus sequence was integrated into sRAGE cDNA to facilitate translational initiation in mammalian cells [32].

Augmentation of sRAGE expression after coding sequence optimization

To test whether global optimization of sRAGE coding sequence will enhance its expression, we transiently transfected plasmids harboring sRAGE(WT) and sRAGE(OP) to CHO-CD14 cells. Equal amounts of protein samples from 3 independent transfections were analyzed

with Western blotting, using anti-T7 antibodies. The expression level from sRAGE(OP) is about 2.6 folds of that of sRAGE(WT) (Fig. 2), suggesting that optimization indeed impacts the overall sRAGE autologous expression.

Assessment of parameters that influence sRAGE autologous expression

Global and rational optimization by GeneOptimizer program integrates multi-parameters that potentially influence gene expression. The optimization represents the best theoretical compromise of these parameters that may impact individual gene expression, although each parameter may influence the target gene differently [25]. Geneoptimizer program changed 186 codons out of total 355 codons of T7-sRAGE(WT) coding sequence (52% changes) (SF1), and that codon adaptation index was changed from 79% (WT) to 97% (OP) (SF2), but not all changes were made based on the consideration of a single parameter [31]. To assess how an individual parameter impacts sRAGE autologous expression, we first tested whether codon optimization for *C. griseus* significantly influences sRAGE yield. Plasmids containing sRAGE(OP) and GFP were co-transfected to human embryonic kidney (HEK) 293 and CHO-CD14 cell respectively, and the overnight cell culture medium was analyzed with ELISA. The green fluorescence-positive cells adjusted results showed that the expression level of sRAGE(OP) in CHO cell was 12% higher than that in HEK293 cells (Fig. 3), suggesting that optimization of codon preference does influence sRAGE yield among different mammalian cells. It is possible that optimization of codon preference may have a greater impact on sRAGE expression among cells from different kingdoms where specific tRNA species and their abundance are a critical element for gene expression.

To examine whether such optimization stabilized sRAGE mRNA and prolonged its half-life in the cell, we extracted total RNA from actinomycin D-treated sRAGE(WT)-, and sRAGE(OP)-transfected CHO cells and performed real time PCR. Although the half-life of sRAGE(WT) mRNA is similar, or even longer than that of sRAGE(OP) (Fig. 4A), the steady-state sRAGE(OP) mRNA level (*i. e.* sRAGE-specific transcript/total RNA) is 92 % higher than that of sRAGE(WT) (Fig. 4B). This result suggests that although the optimization compromised sRAGE mRNA stability, a likely higher transcription rate generated more sRAGE transcripts. The coding sequence of sRAGE(OP) is 66 % GC, and WT is 59 % (SF2), suggesting that a small increase (7%) of GC content does not impact sRAGE mRNA stability. Rather, redistribution of GC content to avoid local GC spikes (See SF2), which represent pseudo transcription start and stop sites [33], in the coding sequence may facilitate a more efficient transcription that generate more sRAGE transcripts.

We next measured sRAGE production rate from sRAGE (WT) and sRAGE(OP)-transfected cells, using co-transfected CMV-GFP as the transfection efficiency control. As shown in Fig. 5, the production rate of sRAGE in OP-transfected cells was 58% higher than its WT counterpart, suggesting that optimization may remove potential “roadblocks” on sRAGE mRNA to facilitate overall production of the protein.

Assessment N-glycosylation and bioactivity of sRAGE produced from optimized construct

Although GeneOptimizer program generates a synonymously mutated gene that does not alter the amino acid sequence, a change of overall production rate may impact protein folding and potentially affect post-translational modifications [34]. We therefore examined whether sRAGE from OP plasmid transfected cells is still N-glycosylated and maintains bioactivity. PNGase F treatment of sRAGE from OP and WT plasmid transfected cells showed a similar migration pattern in SDS-PAGE (Fig. 6), suggesting that sRAGE produced from OP-transfected cells is similarly modified.

Because sRAGE scavenges RAGE ligands and reduces RAGE signaling to NF- κ B transcription program [35], we next tested whether sRAGE produced from OP-transfected cells blocks NF- κ B activity as efficient as that produced in WT-transfected cells. We found that sRAGE produced in OP-transfected cells blocked RAGE ligands, HMGB1-, S100B-, and AGE-induced NF- κ B activation, and that the blocking efficiency is similar to that of sRAGE produced in WT-transfected cells (Fig. 7). Given that our recent studies demonstrating the key role of N-glycosylation on sRAGE bioactivity and *in vivo* efficacy [17], and that the specific N-glycoform modification may not be sufficiently resolved in SDS-PAGE (Fig. 6), the current bioactivity assessment reaffirms that sRAGE produced from OP-transfected cells is likely to be correctly modified, and therefore is fully active. These assessments confirmed that sRAGE(OP) construct boosts sRAGE production and can be used for future scale-up production.

Conclusion

In this study we used GeneOptimizer technology to reengineer human sRAGE cDNA. The optimization led to a higher level of expression of a fully-active recombinant human sRAGE in CHO cells. Assessment of critical parameters that impact autologous sRAGE expression provides a technical information for future large-scale production of this therapeutic protein for clinical trials and medical needs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We use GeneOptimizer software program to redesign the cDNA sequence of sRAGE.
- The expression of sRAGE in CHO cell increased > 2-fold after the reengineering.
- Bioactivity and post-translational modifications of sRAGE remains unchanged.
- Parameters that influence protein expression have been analyzed.
- The work provides a basis for large-scale production of this therapeutic protein.

Fig. 1.

Diagram of T7 tagged sRAGE. The upper portion of the diagram illustrates T7-sRAGE cDNA construct, and the lower portion of the diagram shows the expressed T7-sRAGE protein.

Fig. 2.

Augmentation of sRAGE expression in CHO-CD14 cells after reengineering sRAGE cDNA using GeneOptimizer program. (A) Western blot analyses of sRAGE expression. Cell culture medium from 3 independently transfected CHO-CD14 cells in 35 mm plate was collected for the analyses. (B) Quantification of the Western blot. The sRAGE yield in transfected cells $\text{sRAGE(OP)}/\text{sRAGE(WT)} = 2.6 \pm 0.2$.

Fig. 3.

Assessment of codon preference on sRAGE expression. A plasmid harboring sRAGE(OP) was co-transfected with a GFP expression vector to CHO-CD14 and HEK293 cells and ELISA was performed with the overnight cell culture medium. The green fluorescence-adjusted expression levels were shown. The data was from 4 independent transfections, and each sample was assayed in triplicate. sRAGE(OP)-transfected cells showed 12% increase of sRAGE expression than that of sRAGE(WT)-transfected cells.

Fig. 4. Real-time PCR comparison of mRNA stability and steady state yield of sRAGE(WT) and sRAGE(OP) transcripts. After overnight incubation, actinomycin D (Sigma, final concentration= 6 μ M) was added to cell culture medium, and cells at 0, 2, 4, 7, 10 and 24 h post-treatment were harvested for total RNA extraction and real time PCR. The data was from 3 independent transfections. (A) The half-life of sRAGE(WT) and sRAGE(OP), NS: not significant; (B) The steady state mRNA yield of sRAGE(WT) and sRAGE(OP).

Fig. 5.

Assessment of sRAGE production rate of sRAGE(WT) and sRAGE(OP) plasmid transfected CHO-CD14 cells. After overnight incubation, GFP co-transfected cells were washed multiple times with $1 \times$ PBS, and fresh cell culture medium was added. At various time points (0, 5, 10, 20, 40, and 60 min), a small volume of cell culture medium were collected and subjected to ELISA analyses. Samples were assayed in triplicate for each time point. The data were plotted with the Microsoft Excel program to calculate the production rate of sRAGE.

Fig. 6. sRAGE expressed from the OP plasmid is N-glycosylated. The cell culture medium containing sRAGE from OP and WT plasmids-transfected cells were treated with PNGase F and resolved in SDS-PAGE, followed with western blotting with anti-T7 antibodies.

Fig.7.

sRAGE expressed from the OP plasmid exhibits full bioactivity to inhibit RAGE ligand-elicited NF-κB activation. HEK293 cells stably expressing RAGE were transfected with NF-κB-driven firefly luciferase reporter construct and internal control *Renilla* expression vector, and then treated with indicated RAGE ligand (all in 100 nM). sRAGE purified from WT or OP-transfected CHO-CD14 cells were added in 1:1 molar ratio to the ligand for 3 h, the cells were then lysed for luciferase activity assay. The percentages of inhibition relative to uninhibited controls were indicated.