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Enhanced biological effects of Phe140Asn, a novel human granulocyte colony-stimulating factor mutant, on HL60 cells

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Granulocyte colony-stimulating factor (G-CSF) is a cytokine secreted by stromal cells and plays a role in the differentiation of bone marrow stem cells and proliferation of neutrophils. Therefore, G-CSF is widely used to reduce the risk of serious infection in immunocompromised patients; however, its use in such patients is limited because of its non-persistent biological activity. We created an *N*-linked glycosylated form of this cytokine, hG-CSF (Phe140Asn), to assess its biological activity in the promyelocyte cell line HL60. Enhanced biological effects were identified by analyzing the JAK2/STAT3/survivin pathway in HL60 cells. In addition, mutant hG-CSF (Phe140Asn) was observed to have enhanced chemoattractant effects and improved differentiation efficiency in HL60 cells. These results suggest that the addition of *N*-linked glycosylation was successful in improving the biological activity of hG-CSF. Furthermore, the mutated product appears to be a feasible therapy for patients with neutropenia. [BMB reports 2011; 44(10): 686-691]

INTRODUCTION

Cytokines and growth factors are potential therapeutic agents because of their key roles in regulating the production, maturation, and activity of blood, muscle, and bone cells (1). A number of cytokines, such as erythropoietin (EPO) and colony-stimulating factors (CSFs), are currently used for the treatment of diseases, such as cancer and blood deficiencies (1, 2). Among them, human granulocyte CSF (hG-CSF) is a hematopoietic factor that plays an important role in stimulating the proliferation, differentiation, and functional activation of blood cells (3).

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G-CSF is made up of 4 anti-parallel α-helices (4), and the G-CSF : G-CSF-receptor (G-CSFR) complex forms a 2 : 2 stoichiometry by means of a cross-over interaction between the Ig-like domains of G-CSFR and G-CSF (5). G-CSF stimulates the JAK2/STAT3 pathway in the human promyelocyte cell line HL60 (6). Survivin is stimulated by granulocyte-macrophage (GM) CSF in $CD34^+$ bone marrow and HL60 cells (7) and is activated by STAT3, which is induced by G-CSF, in a bladder carcinoma cell line (8). Survivin plays a role in the proliferation of leukemic cell lines and has an anti-apoptotic function (9). In addition, G-CSF also has a chemoattractant activity and induces the differentiation of HL60 and bone marrow stem cells into neutrophils (2).

In general, glycosylation is critical for the biological activity of numerous molecules (10). *N*-Linked glycosylation enhances the stability, extends the half-life, and increases the solubility of enzymes (11). Therefore, we generated an *N*-linked glycosylated hG-CSF in this investigation. Following observations in previous reports (3, 10), we speculated that mutant hG-CSF (Phe140Asn) would be more efficient than a native molecule for promoting the proliferation and differentiation of hematopoietic precursors.

hG-CSF has 5 cysteines (Cys, C) and 2 disulfide bonds (Cys^{36}-Cys^{42} and Cys^{64}-Cys^{74}) (12). hG-CSF, in which the free cysteine (Cys^{17}) is mutated to alanine (Ala, A), is more stable than the wild-type (WT) molecule (13). A 7-point mutation, in which the glycines (Gly, G) were changed to Ala (1), yielded 4 versions of hG-CSF that were significantly more thermodynamically stable than the WT molecule. However, no study has determined whether these mutants have any enhanced biological effects on myelocytes.

In this study, we used site-directed mutagenesis to create a novel hG-CSF mutant with *N*-linked glycosylation. The enhanced biological activities of the mutant hG-CSF were analyzed using a cell viability test, with additional investigations into the molecular mechanism as well as its chemoattractant and differentiation abilities. Our findings suggest that the novel mutant can be used as a therapeutic agent to treat immun-

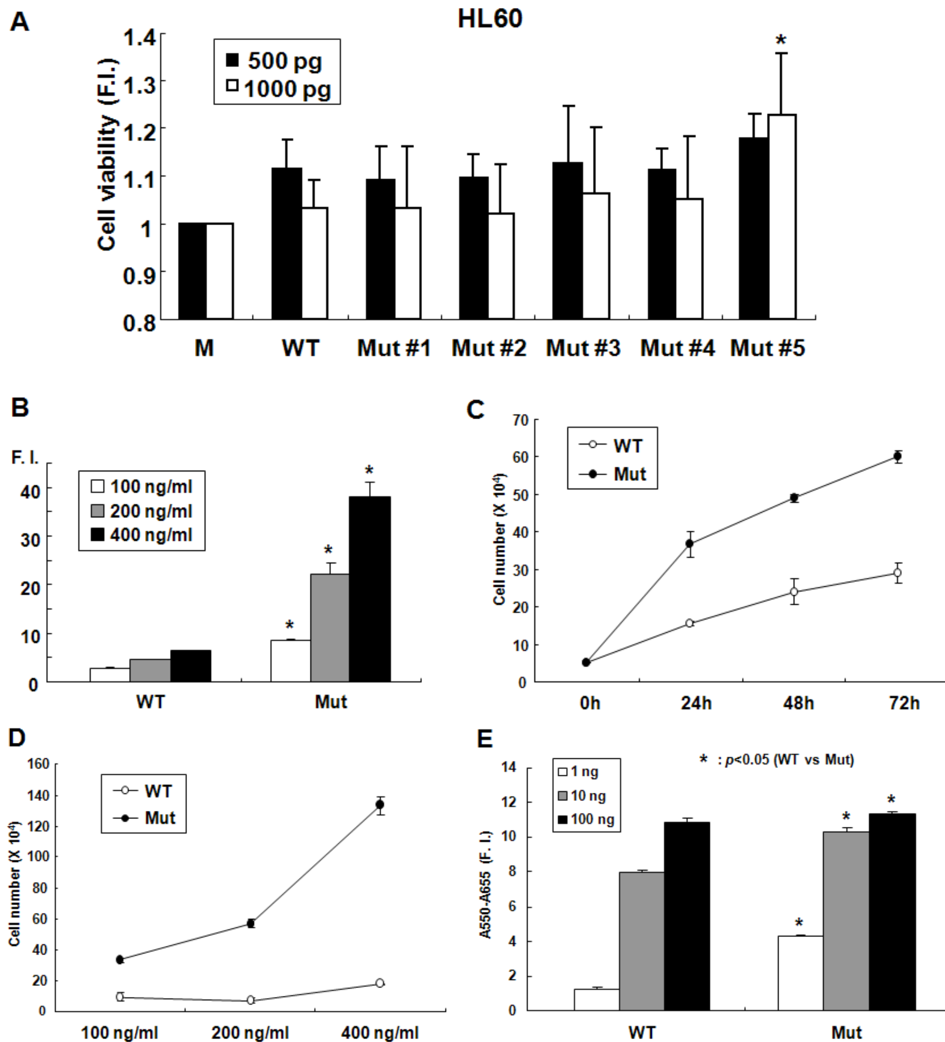

Fig. 1. Cell viability of Mut in M-NFS60 and HL60 cells. (A) HL60 cells were treated with WT and mutant hG-CSFs for 2 days (white bar, 500 pg; black bar, 1,000 pg). Absorbance for the MTT assay was evaluated at 550 nm with the reference filter set at 655 nm. The data represent the means from 3 sets of experiments performed in triplicate; error bars indicate standard deviation. The asterisk (*) indicates values that were significantly different ($P < 0.05$) from those observed for the WT. M: control (culture media from the CHO cells transfected with an empty vector, pCMV-Tag4); F. I. means fold increase compared to M. (B) MTT assay in M-NFS60 cells (white bar, 100 ng/ml; gray bar, 200 ng/ml; black bar, 400 ng/ml WT and Mut). F. I. indicates the fold increase for untreated cells. (C) M-NFS60 cells (5.0×10^4) were plated on 12-well plates and counted at the indicated time on addition of 400 ng/ml WT or Mut. (D) M-NFS60 cells were plated at a density of 5.0 $\times$ 10^4 cells per well on 12-well plates and grown for 4 days on treatment with hG-CSF at the indicated concentrations. (E) MTT assay in HL60 cells. F. I. indicates the fold increase for untreated cells. Asterisks (*) indicate $P < 0.05$ in a comparison between WT and Mut.

ocompromised patients for conditions such as neutropenia.

RESULTS

Viability of Mut in HL60 and M-NFS60 cells

To examine the biological activities of the generated mutants, the culture media of CHO cells that had been transfected with mutant hG-CSFs were used to assess the viability of HL60 cells using the MTT assay. The results revealed that only mutant #5 (Phe140Asn; Mut) had a statistically higher biological activity than that of the WT molecule (Fig. 1A). Therefore, Mut was chosen for subsequent experiments. The *N*-linked glycosylation of Mut was confirmed by Western blotting (data not shown). The M-NFS60 cell line, which is a representative CSF-dependent cell line, was first tested for cell viability using Mut. In Fig. 1B, Mut produced higher cell viabilities than WT when applied in a dose-dependent manner. To determine the efficiency of WT and Mut for promoting cell proliferation, M-NFS60 cells were counted in a time- (Fig. 1C) and dose-dependent manner (Fig. 1D). In addition, the proliferating effect of Mut was assessed in HL60 cells (Fig. 1E). Consequently, Mut was seen to have a higher proliferating effect than that of WT hG-CSF when used to treat both M-NFS60 and HL60 cells in both a time- and dose-dependent manner.

Molecular mechanism of Mut in HL60 cells

To investigate the molecular mechanism underlying the enhanced proliferating activity of Mut, a signaling assay was performed using HL60 cells. In general, G-CSF induces the JAK2/STAT3 pathway (14). To identify the signal transduction pathway upon treatment with Mut, HL60 cells were prepared as shown in Fig. 2A. Phospho-JAK2 (pJAK2) and STAT3 (pSTAT3) were expressed at a higher level upon treatment with Mut than with WT hG-CSF at 5 min and 15 min, respectively (Fig. 2B

and 2C). Furthermore, the bands for pJAK2 and pSTAT3 were more sustained following treatment with Mut rather than WT. In addition, the expression of survivin, a target for JAK2/STAT3 and a proliferating molecule, was determined by real-time RT-PCR (Fig. 2D). Mut promoted a 2-fold increase in human survivin expression compared with that produced by the equivalent WT dose at 15 min (Fig. 2D).

Differentiation and chemoattractant effects of Mut in HL60 cells

To examine the differentiation potency of Mut, promyelocytes (HL60) were differentiated to monocytes with ATRA. Fig. 3A displays the overall scheme for the differentiation and chemoattractant tests. Fig. 3B shows the differentiation potency of WT and Mut using various concentrations of ATRA in HL60 cells. Mut was seen to have a higher differentiation capacity than WT hG-CSF at concentrations of 500 and 1,000 nM ATRA (Fig. 3B).

In general, ATRA and DMSO are inducing agents used to differentiate myelocytes (15). G-CSF has a synergistic effect in this differentiation process (2). This effect was confirmed in our experiments and was more apparent when using Mut. In the inflammatory response, neutrophils are recruited to sites of inflammation by chemoattractants (16). Therefore, the chemoattractive effect of this cytokine is critical to mount an effective inflammatory response. To investigate the chemoattractant efficiency of Mut, migrated HL60 cells, which had not undergone differentiation, were assayed upon treatment with WT and Mut for 1 day (Fig. 3C). hG-CSF-treated cells were much more chemoattractive than M (control), and this effect was even more

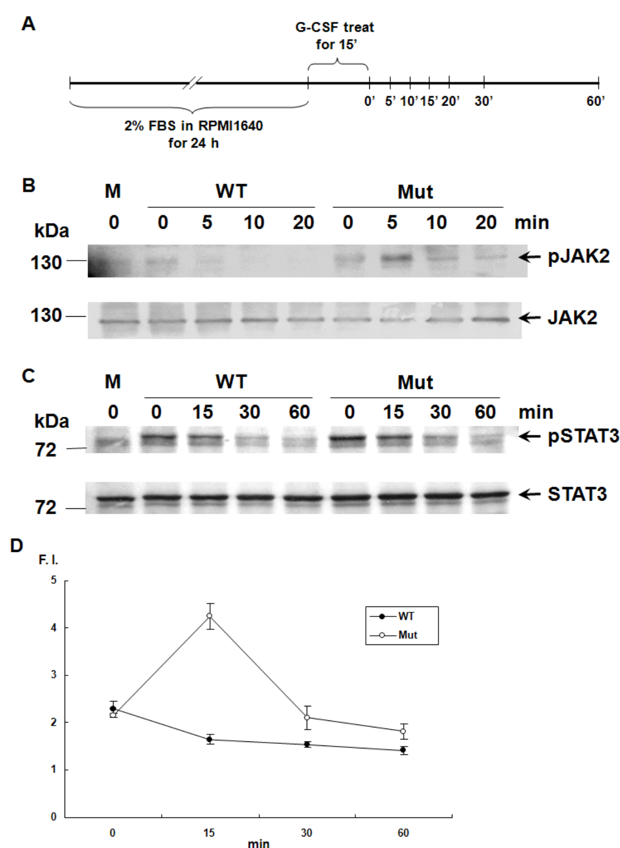

Fig. 2. Molecular mechanism underlying Mut action in HL60 cells. (A) HL60 cells were first incubated in 2% FBS-containing media for 24 h and then treated with 400 ng/ml WT or Mut for 15 min. Cells were collected at the indicated time for Western blot. (B) Activation of pJAK2 was visualized with anti-pY from the collected cell lysates immunoprecipitated with anti-JAK2. JAK2 was used as a loading control. (C) Phosphorylation of STAT3 was detected with anti-phospho STAT3. STAT3 was used as a loading control. M indicates the cultured media from CHO cells transfected with empty vector (pCMV-Tag4). (D) Real-time RT-PCR for human survivin upon hG-CSF treatment to HL60 cells. F. I. indicates the fold increase for cells treated with M.

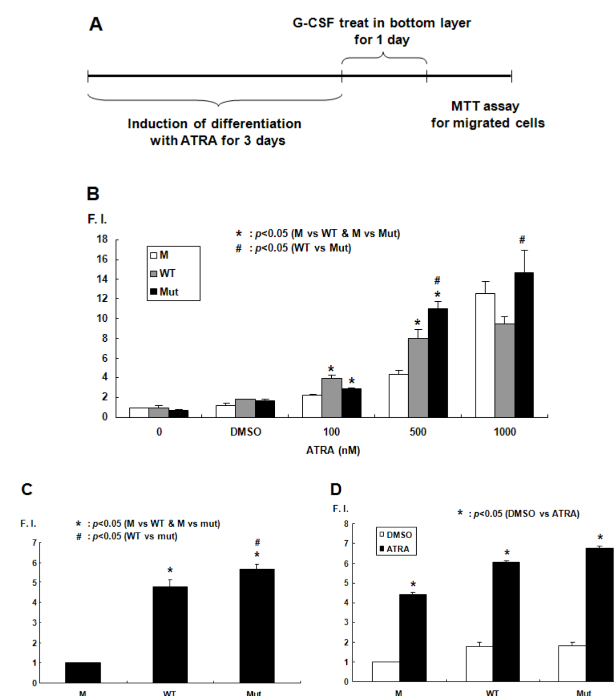

Fig. 3. Differentiation and chemoattractant test for Mut in HL60 cells. (A) Time schedule for the differentiation and chemoattractant test for Mut. (B) Real-time RT-PCR for hCD11b upon treatment with 400 ng/ml hG-CSF. Differentiation was induced by ATRA. M indicates the cultured media from CHO cells transfected with empty vector. F. I. indicates the fold increase for undifferentiated cells (0 nM ATRA) treated with M. (C) Chemoattractant test upon treatment with 400 ng/ml WT and Mut. F. I. indicates the fold increase for cells treated with M. Asterisks (*) indicate $P < 0.05$ in comparison with M, and hashes (#) indicate $P < 0.05$ for the comparison between WT and Mut. (D) Migration test of HL60 upon treatment with 400 ng/ml WT and Mut. F. I. represents the fold increase for cells treated with M and DMSO. Asterisks (*) indicate $P < 0.05$ compared with DMSO.

apparent with Mut treatment (Fig. 3C). Finally, to study the migration efficiency of differentiated cells, the chemoattractive activity of the differentiated cells was assessed (Fig. 3D). Consequently, the differentiated cells were not found to have any chemoattractive activity upon hG-CSF treatment (Fig. 3D).

DISCUSSION

G-CSF is widely used to increase the levels of white blood cells in patients with congenital neutropenia and myeloid leukemia, after high-dose chemotherapy with stem cell rescue, and for treating other non-myeloid malignancies (17). However, G-CSF has a short life in serum, requiring patients to receive multiple, and often expensive injections. Therefore, we used *N*-linked glycosylation to develop a novel hG-CSF that would have more potent biological activities towards myelocytes.

In general, G-CSF promotes the proliferation of leukemic cell lines, such as HL60, AML193, and M-NFS60 cells (18), and is regulated by the phosphorylation of JAK2 and STAT3 (19). This signaling activation was confirmed through our results (Fig. 2B and C). The phosphorylated JAK2 and STAT3 signals were sustained for a longer time in Mut. This prolonged signal results in the higher expression of survivin (Fig. 2D). These results indicate that our novel Mut has an enhanced stability, or binds more tightly to G-CSFR. Both G-CSF and G-CSFR are glycoproteins (20); therefore, additional glycosylation in the hG-CSF ligand could potentially increase the receptor binding affinity because of glyco-glyco interactions (21).

G-CSF also plays a critical role in chemotaxis and differentiation in hematopoietic stem cells (2). G-CSF induces the migration of hematopoietic stem cells to injured tissues where they participate in the immune response (22). This phenomenon was also observed with the Mut (Fig. 3C). GM-CSF induces chemotaxis by regulating the ribosomal p70 S6 kinase (p70S6K) signaling pathway (23). In general, G-CSF shares the same signaling pathway with GM-CSF – the JAK2/STAT3 and PI3K pathway (24) – in which PI3K activates mTOR and p70S6K (25). Therefore, Mut would also regulate chemotaxis through this signaling pathway. However, the advanced chemoattractant effect was not seen in differentiated HL60 cells (Fig. 3D). In contrast, Mut, together with ATRA, induced the differentiation of HL60 cells (Fig. 3B). These results indicate that our Mut promotes the mobilization of promyelocytes to the injured tissues site; however, once the cells were differentiated, they no longer moved to the inflammation site. The Mut may act as a modulator between stem cells and differentiated cells. Taken together, our results demonstrate that the Mut plays a role in proliferation and chemotaxis ahead of differentiating signals such as those promoted by ATRA; following this signal, its role is changed to support the differentiation of stem cells.

In this study, we have shown that, compared with WT hG-CSF, an *N*-glycosylated Mut exerts enhanced biological activities in HL60 cells. *N*-Linked glycosylation of cytokines and glycoproteins is pivotal to their biological activity. In the future, we hope to provide a more detailed characterization of the glycans present in Mut. For now, however, we conclude that Mut can be used to effectively and economically treat patients with neutropenia.

MATERIALS AND METHODS

Cloning of Mut cDNA

Human G-CSF cDNA was obtained from the National Institute of Animal Science (NIAS), Rural Development Administration (Suwon, Republic of Korea). hG-CSF cDNA was amplified by polymerase chain reaction (PCR) using the primers G-CSF F (5′-GGAATTCGCGATATCGCCACCATGGC-3′, underlined: *Eco*RV) and G-CSF R (5′-TTTCCTCCTCGAGGGGCTGGGCA A-3′, underlined: *Xho*I). The PCR product was digested with *Eco*RV and *Xho*I and cloned into the pCMV-Tag4A vector (Promega). Human G-CSF mutants were created using the QuikChange II XL site-directed mutagenesis kit (Stratagene), using the primers (hG-CSF mutant #5 F, 5′-GGTGCCATGCCG GCCAATGCCTCTGCTTTCCA-3′; hG-CSF mutant #5, 5′-TGG AAAGCAGAGGCATTGGCCGGCATGGCACC-3′, underlined: mutated sequences), according to the manufacturer's protocol.

Transfection

The day before transient transfection, CHO cells were plated onto 100-mm dishes at a density of 3.0×10^6 cells. The cells were transfected with plasmids (20 μg) and the culture medium was replaced with 4 ml Opti-MEM (Invitrogen) after 4 h; subsequently, the cells were incubated for a further 42 h. Transfected medium was harvested and concentrated using centri-prep (Amicon® Ultra, Ultracel 3k; Millipore) and retained for use in the following experiments.

Cell cultures

HL60 and M-NFS 60 cells were maintained in RPMI-1640 (Invitrogen) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Invitrogen), at 37°C with 5% CO_2. In the medium for M-NFS60 cells, 0.05 mM 2-mercaptoethanol and 62 ng/ml human recombinant macrophage colony-stimulating factor (M-CSF) were added.

Cell viability and proliferation test

An MTT assay was performed using a Cell Proliferation Kit I (Roche). HL60 and M-NFS60 cells were plated on a 96-well microplate at a density of 5.0×10^3 cells per well. Each well was treated with WT and mutant hG-CSFs for 72 h. Subsequent experiments were conducted following the manufacturer's protocol. For the proliferation test, cells were plated on a 12-well plate at a density of 5.0×10^4 cells per well. Cells were counted by Countess™ (Invitrogen).

Immunoprecipitation (IP) and western blotting (WB)

On the day before hG-CSF treatment, HL60 cells were plated

onto 100-mm dishes at a density of 3.0×10^6 cells. The cells were grown in RPMI-1640 media with 2% fetal bovine serum (FBS) for 24 h. Cells were harvested after the treatment with WT hG-CSF and Mut for 15 min. Total proteins were extracted using M-PER buffer (PIERCE) containing a protease inhibitor cocktail (Santacruz). For detection of pJAK2, protein (2 mg) was immunoprecipitated with 2 μg pY antibody (Upstate) at 4°C overnight. Blots were probed with a primary antibody against JAK2 (Cell Signaling) at 4°C overnight, followed by 1 h incubation with an anti-rabbit secondary antibody (Zymed). For identification of pSTAT3 (Cell signaling) and STAT3 (BD Transduction laboratories™), 100 μg protein was used for Western blotting.

Real-time reverse transcriptase PCR (RT-PCR)

Total RNA was isolated from HL60 cells treated with hG-CSF using TRIZOL® (Invitrogen). Total RNA (5 μg) was reverse-transcribed to cDNA with reverse transcriptase (First Strand cDNA Synthesis Kit, Roche) and analyzed by real-time PCR using a SYBR Green® Realtime PCR Master Mix Plus (Toyobo). Real-time PCR was used to amplify hsurvivin (forward primer, 5′-GTACCTGAACCGGCATCTG-3′; reverse primer, 5′-GGGGCCATATAGTTCCACAA-3′) and hCD11b (forward primer, 5′-GGCATCCGCAAAGTGGTA-3′; reverse primer, 5′-GGATCTTAAAGGCATTCTTTCG-3′). β-Actin was used as an internal control, using the primers (forward primer, 5′-CCCGCGAGTA CAACCTTCT-3′; reverse primer, 5′-CGTCATCCATGGCGAAC T-3′). The PCR ingredients included 3.6 μl distilled water, 2 μl Plus reagent, 1.2 μl 10 pmol each of sense and antisense primers, 2 μl template, and 10 μl SYBR® Green Realtime PCR Master Mix, for a total volume of 20 μl.

Differentiation and migration assay

Differentiation of HL60 cells was induced by all-trans retinoic acid (ATRA, Sigma) for 3 days. For the migration assay, 0.5×10^6 cells were added to each insert of a 5.0-μm transwell (12-well, Corning), and 400 ng WT or Mut with Opti-MEM (total volume, 600 μl) were placed in the bottom layer. After incubation for 24 hr, migrated cells were analyzed by MTT assay.

Statistical analysis

Statistical analyses were conducted using SAS Enterprise Guide 4.2 software (SAS Institute). Means were calculated for each treatment and Duncan's multiple-range tests, Tukey's studentized range tests, and t-tests were used to examine whether the results were significantly different. Significance was defined as $P < 0.05$.

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