Antiapoptotic effects of Phe140Asn, a novel human granulocyte colony-stimulating factor mutant in H9c2 rat cardiomyocytes

Hee Kyoung Chung1,4, Eun Mi Ko3, Sung Woo Kim3, Sung-June Byun1, Hak-Jae Chung1, Moosik Kwon4, Hwi-Cheul Lee5, Byoung-Chul Yang1, Deug-Woo Han1, Jin-Ki Park1, Sung-Gu Hong1, Won-Kyong Chang6,7† & Kyung-Woon Kim1,7*,†

1Animal Biotechnology Division, National Institute of Animal Science, Rural Development Administration, 2Post-harvest & Food Engineering Division, Department of Agricultural Engineering, National Academy of Agricultural Science, Suwon 441-707, 3Animal Genetic Resources Station, National Institute of Animal Science, Rural Development Administration, Namwon 590-832, 4Department of Genetic Engineering, Sungkyunkwan University, Suwon 440-746, 5Planning & Coordination Division, National Institute of Animal Science, Rural Development Administration, 6National Institute of Animal Science, Rural Development Administration, Suwon 441-706, Korea

INTRODUCTION

Granulocyte colony-stimulating factor (G-CSF) is used for heart failure therapy and promotes myocardial regeneration by inducing mobilization of bone marrow stem cells to the injured heart after myocardial infarction; however, this treatment has one weakness in that its biological effect is transient. In our previous report, we generated 5 mutants harboring N-linked glycosylation to improve its antiapoptotic activities. Among them, one mutant (Phe140Asn) had higher cell viability than wild-type hG-CSF in rat cardiomyocytes, even after treatment with an apoptotic agent (H2O2). Cells treated with this mutant significantly upregulated the antiapoptotic proteins, and experienced reductions in caspase 3 activity and PARP cleavage. Moreover, the total number of apoptotic cells was dramatically lower in cultures treated with mutant hG-CSF. Taken together, these results suggest that the addition of an N-linked glycosylation was successful in improving the antiapoptotic activity of hG-CSF, and that this mutated product will be a feasible therapy for patients who have experienced heart failure. [BMB Reports 2012; 45(12): 742-747]

Keywords: Antiapoptotic activity, Heart failure, hG-CSF (Phe140Asn)

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these mutants had antiapoptotic effects in cardiomyocytes.

Here, we compared the antiapoptotic activity of previously generated mutant and WT hG-CSF by evaluating their abilities to upregulate antiapoptotic molecules, downregulate factors involved in the apoptotic process, and decrease the total number of apoptotic cells in cultures treated with H2O2. Our findings suggest that the novel mutant can be used to treat heart failure patients, including those who have experienced myocardial infarction.

RESULTS

Generation of mutant hG-CSFs via site-directed mutagenesis
Mutant candidates were selected as target products of site-directed mutagenesis if they exceeded the threshold for N-glycosylation potential (Supplemental Fig. S2). To develop hG-CSF with enhanced biological activity, 5 mutants for hG-CSF were produced by exchanging the target residue with Asn (N). Human G-CSF has 14 Ser and 7 Thr residues. Of these, residues within the 4 α-helices (Ser12, Ser76, Ser80, Ser155, Ser159, Ser164, Thr38, Thr102, Thr105, Thr115, and Thr116) were eliminated as possible mutagenesis candidates because exchanging them with Asn could have disrupted the structure of hG-CSF. This left 5 serines that were potential candidates for mutagenesis (Ser7, Ser8, Ser53, Ser62, and Ser142). The residues positioned prior to these amino acids were replaced with Asn, generating 5 mutants: Pro5Asn (Mut #1), Ala6Asn (Mut #2), Gly51Asn (Mut #3), Pro60Asn (Mut #4) and Phe140Asn (Mut #5) (Fig. 1A).

Expression and N-linked glycosylation of hG-CSF mutants in CHO cells
To determine the expression of the generated hG-CSF, WT and mutant hG-CSFs cloned to pCMV-Tag4A were transiently transfected into CHO cells. WT hG-CSF was expressed at a higher level than mutant hG-CSFs (approximately 0.4 ng per 0.05 μg of total protein) (Fig. 1B). Western blot analysis showed that N-linked glycosylation had successfully been introduced to 3 of the mutants (Mut #3, #4, and #5); this element can be visualized as an additional (upper) band (Fig. 1C). The presence of the glycosylation was confirmed by treatment with N-glycosidase F (Fig. 1D). N-glycosidase F cleaves all types of Asn bound N-glycans provided that the amino-group as well as the carboxyl group are present in a peptide linkage, and that the oligosaccharide has the minimum length of the chitobiose core unit (18, 19). As a result, N-glycosylated bands of mutant hG-CSFs were identified by detecting the down-developed bands. As a positive control, O-glycosidase was also applied to the culture media transfected with WT hG-CSF. O-glycosidase is used to release the Gal β(1-3)GalNAc unit from O-glycans linked to the hydroxyls of Ser or Thr (20). Consequently, the down bands of non-glycosylated WT were detected (upper panel in Fig. 1D). These data indicate that the mutants (#3, #4, and #5) for hG-CSF have N-linked glycosylation.

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Enhanced viability of H9c2 cells upon Mut #5 treatments
Cell viabilities of N-linked glycosylated mutants were tested using an MTT cell proliferation assay with rat cardiomyocytes (H9c2 cells). The overall scheme for cell viability and apoptosis analysis of the hG-CSFs generated is illustrated in Fig. 2A. Mutants #3, #4, and #5 had N-linked glycosylation (Fig. 1D); however, only cells treated with Mut #5 had statistically higher proliferation rates than cells treated with WT hG-CSF (P < 0.05, Fig. 2B). H2O2 treatment induced apoptosis in H9c2 cells (8, 21). Consistent with the cell proliferation results, Mut #5 maintained a statistically higher level of cell proliferation after addition of H2O2 (P < 0.05) (Fig. 2B). To verify the dosage effects of WT and Mut #5 hG-CSF, they were treated with three different doses. As a result, dosage effects for WT and Mut #5 hG-CSF were seen upon H2O2 treatment (Fig. 2D); however, the effects were not seen in normal condition (Fig. 2C). This suggests that Mut #5 is better than WT hG-CSF at protecting cardiac myocytes from H2O2-induced apoptosis. In this regard, we further analyzed the antiapoptotic effects for Mut #5 hG-CSF in H9c2 cells.

Anti-apoptotic effects of Mut #5 in H9c2 cells
To explore the enhanced anti-apoptotic effects of Mut #5, the expression of antiapoptotic proteins Bcl-xL and Bcl-2 upon Mut #5 was examined. Bcl-xL and Bcl-2 expression in H9c2 cells was upregulated in a time-dependent manner after treatment with hG-CSF (Fig. 3A and 3B). Additionally, these effects were more presented in Mut #5 than WT hG-CSF (P < 0.05 at both time points for Bcl-xL and Bcl-2). These same patterns were also found in cells treated with H2O2 (Fig. 3C). In H2O2-treated cells, the up-regulation of Bcl-2 by Mut #5 was particularly strong (Fig. 3C), suggesting that this mutant is more efficient for stimulating anti-apoptotic defense mechanisms.

To determine whether these mechanisms actually reduced apoptosis in rat cardiomyocytes, caspase 3 activity, an indicator of apoptosis, was evaluated in cells treated with H2O2 (Fig. 3D). Both WT and Mut #5 treatments significantly decreased apoptotic activity (P < 0.05) in comparison to the control treatment, but Mut #5 had a significantly higher antiapoptotic effect than WT hG-CSF (P < 0.05) (Fig. 3D). Furthermore, a Western blot analysis demonstrated that active caspase 3 induced PARP cleavage (Fig. 3E). Control cells (M) contained the highest levels of cleaved PARP (whole/cleaved PARP ratio: 0.58), while cells treated with WT hG-CSF had intermediate levels of PARP cleavage (ratio: 0.95), and PARP cleavage was lowest in cells treated with Mut #5 (ratio: 1.14) (Fig. 3E).

The anti-apoptotic effect of Mut #5 in H2O2-treated H9c2 cells was further demonstrated by FACS analysis, which showed that cell death (as demonstrated by testing positive for Annexin V and PI) occurred at a lower rate in Mut #5- than WT hG-CSF-treated cells (33.22% vs. 35.81%); conversely, survival was higher in Mut #5-treated than WT hG-CSF-treated cells (58.87% vs. 49.70%, respectively) (Fig. 4A). Additionally, the histogram data showed that apoptotic cells (PI positive; M2)
were more common in the WT group (48.35%) than the Mut #5 group (39.72%) (Fig. 4B). Taken together, these data suggest that Mut #5 has potential to act as a therapeutic drug for patients who have experienced a myocardial infarction.

DISCUSSION

The number of heart failure patients has risen over the past several decades (22), over which time a variety of promising therapies has been developed to reduce the damage caused by myocardial infarction. Among them, treatment with G-CSF, which in combination with stem cell factor (SCF), significantly enhances cardiac function and decreases fatalities among mice that have experienced acute myocardial infarction (3). However, G-CSF has a short life in serum, requiring patients to receive multiple, and often expensive injections. Thus, we used N-linked glycosylation to develop a novel hG-CSF that would have stronger anti-apoptotic activities in injured cardiomyocytes.

The mutagenesis process produced 2 types of mutant hG-CSFs (those with only 1 band, like WT, and those with 2 bands, indicating the presence of N-linked glycosylation) (Fig. 1C). This suggests that the N-linked glycosylation event for the single-band mutants, #1 and #2, was not complete. Processing of N-glycans occurs mainly in the endoplasmic reticulum (ER), whereas O-linked glycosylation is initiated in the Golgi apparatus (23-26). Therefore, it is possible that there may be some polypeptides that have not undergone ER processing. Moreover, the bands without N-glycosidase treatment were 2 different sizes (Fig. 1D, lower panel). Lenograstim, expressed in CHO cells, is a 50:50 mixture of 2 glycosylated G-CSF forms containing sialic acid, galactose (Gal), and galactosamine (27, 28). Additionally, CHO cells contain various glycans, including sialic acids. The size of glycoprotein molecules varies depending on how many sialic acids they contain. It would appear that most glycoproteins, including hG-CSF, can achieve glycosylation through multiple pathways. Therefore, it is not surprising that Mut #5 was so different from the other mutant hG-CSFs.

Despite the fact that N-linked glycosylation plays a pivotal role in enhancing enzymatic activity (13), Mut #3 and #4 hG-CSFs with this feature did not perform significantly different from WT hG-CSF (Fig. 1C and 2B). Human G-CSF has another short helix structure, but with 4 anti-parallel helices. The short helix is involved in major binding with G-CSF receptor (G-CSFR) (29); therefore, a mutation in this region disrupts the interaction between G-CSF and G-CSFR. Mut #3 (Gly51Asn) may have failed to promote proliferation in H9c2 cells (Fig. 2) because it was positioned within this short helix structure. Mut #4 (Pro60Asn), on the other hand, appears to have changed the structure of hG-CSF by removing a proline (Pro, P), which has an aliphatic side chain that is vital for determining the proper folding structure of the protein. Therefore, a mutation in this residue (Mut #4; Pro60Asn) may abolish the original structure of hG-CSF such that the changed hG-CSF does not bind to G-CSFR. This would explain why Mut #4 did not significantly increase the via-

![Fig. 3. Regulation of apoptotic proteins in H9c2 cells treated with Mut #5. (A) Comparison of Bcl-xL expression at 24 and 48 h upon 400 ng/ml WT and Mut #5 hG-CSF; histogram showed the band intensities in the lower panel. M indicates H9c2 cells treated with CHO cell culture media transfected with empty vector. F.I. means fold increase with M, and double asterisks (**) indicate P < 0.05 in a comparison between WT and Mut #5. (B) Results from real-time RT-PCR investigating expression of Bcl-2 and Bcl-xL in cells that were (black) or were not (white) treated with H2O2. Single symbols (# for 24 h and * for 48 h) indicate P < 0.05 in a comparison with WT and Mut #5. (C) Results from real-time RT-PCR for Bcl-2 and Bcl-xL expression in cells that were (black) or were not (white) treated with H2O2. Single symbols (#untreatment; *, **) indicate P < 0.05 in a comparison with WT and Mut #5. (D) Results from real-time RT-PCR for Bcl-2 and Bcl-xL expression in cells that were (black) or were not (white) treated with H2O2. Single symbols (#untreatment; *, **) indicate P < 0.05 in a comparison with WT and Mut #5. GAPDH and β-actin were used as internal controls. (E) Results from real-time RT-PCR investigating caspase 3 activity in the control (M) and in cells treated with either 400 ng/ml WT or Mut #5 hG-CSF. M means H9c2 cells treated with the culture media transfected with empty vector. F.I. means fold increase compared to M. Single asterisks (*) indicate P < 0.05 in a comparison with WT and Mut #5. GAPDH and β-actin were used as internal controls. (F) Results from PARP cleavage (cleaved fragment: 89 kDa) upon 400 ng/ml WT and Mut #5 hG-CSF. Numbers below the bands are ratios indicating the intensities between upper and lower bands. Actin was used as the loading control.

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Fig. 4. Visualization of apoptotic activity in cells treated with Mut #5. (A) Results from flow cytometry analysis used to visualize the amount of apoptotic activity in the control (M), WT, and Mut #5 hG-CSF cells stained with Annexin V and PI. The histogram in the lower panel provides a comparison between the total number of apoptotic cells (black and gray bars) and surviving cells (white bars). (B) Results from the FACS analysis comparing the number of live (M1, white bars) and dead (M2, black bars) cells among the 3 treatments.

Mut #5 was associated with higher levels of Bcl-xL and Bcl-2 in both the presence and absence of H2O2 (Fig. 3). Bcl-xL is a downstream molecule in the ERK and AKT pathway activated by the hematopoietic growth factor macrophage-CSF (M-CSF) (30). Upregulation of this molecule is associated with the inhibition of apoptosis. Similarly, Bcl-2 also has anti-apoptotic activities and, like Bcl-xL, can be activated via the JAK/STAT3 pathway (4). Although the current study did not explicitly show activation of JAK/STAT3 pathways, the upregulation of both Bcl-xL and Bcl-2 in the presence of Mut #5 suggests that this mutant is capable of inhibiting myocardial cell death.

Reperfusion is a reliable treatment to diminish heart injury caused by myocardial ischemia (31). However, this treatment induces side effects mediated by reactive oxygen species (ROS) such as hydrogen peroxide, and activates the transcription of proinflammatory genes (e.g., PARP-1) that are cleaved by caspase 3 (32). The PARP inhibitor PJ-34 can alleviate problems associated with heart disease, and reduces doxorubicin-induced apoptosis of cardiomyocytes (33). Because of this, PARP-1 null mice have been used as an animal model in numerous experiments investigating heart failure (31). We found that Mut #5 also down-regulates caspase 3 activity and PARP cleavage in response to H2O2 treatment (Fig. 3). Therefore, a newly developed hG-CSF (Phe140Asn) will be further employed for studying heart failure using this animal model.

In this study, we have shown that Mut #5 exerts higher anti-apoptotic effect than WT hG-CSF against H2O2-induced apoptosis in H9c2 cardiomyocytes. This illustrates the significance of N-glycosylation in determining the functionality of cytokines and glycoproteins. In the future, we hope to provide a more detailed characterization of the glycans present in Phe140Asn. For now, however, we conclude that Mut #3 can be used to effectively and economically treat patients with heart failure.

MATERIALS AND METHODS

Detailed information is described in Supplementary Material.

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REFERENCES

1. Takano, H., Qin, Y., Hasegawa, H., Ueda, K., Niitsuma, Y., Ohtsuka, M. and Komuro, I. (2006) Effects of G-CSF on left ventricular remodeling and heart failure after acute myocardial infarction. J. Mol. Med. 84, 185-193.
2. Minatoguchi, S., Takemura, G., Chen, X. H., Wang, N., Uno, Y., Koda, M., Arai, M., Misao, Y., Lu, C., Suzuki, K., Goto, K., Komada, A., Takahashi, T., Kosai, K., Fujiwara, T. and Fujihara, H. (2004) Acceleration of the healing process and myocardial regeneration may be important as a mechanism of improvement of cardiac function and remodeling by post-infarction granulocyte colony-stimulating factor treatment. Circulation 109, 2572-2580.
3. Orlic, D., Kajstura, J., Chimenti, S., Limana, F., Jakoniuk, I., Quaini, F., Nadal-Ginard, B., Bodine, D. M., Leri, A. and Anversa, P. (2001) Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc. Natl. Acad. Sci. U.S.A. 98, 10344-10349.
4. Harada, M., Qin, Y., Takano, H., Minamino, T., Zou, Y., Toko, H., Ohtsuka, M., Matsuura, K., Sano, M., Nishi, J., Iwana, K., Akazawa, H., Kunieda, T., Zhu, W., Hasegawa, H., Kunisada, K., Nagai, T., Nakaya, H., Yamauchi-Takihara, K. and Komuro, I. (2005) G-CSF prevents cardiac remodeling after myocardial infarction by activating the Jak-Stat pathway in cardiomyocytes. Nat. Med. 11, 303-311.
5. Martin-Christin, F. (2001) Granulocyte colony stimulating factors: how different are they? How to make a decision? Anticancer. Drugs. 12, 185-191.
6. Mallat, Z., Philip, I., Lebret, M., Chatel, D., Maclouf, J. and Tedgui, A. (1998) Elevated levels of 8-iso-prostaglandin F2α in pericardial fluid of patients with heart failure: a potential role for in vivo oxidant stress in ventricular dilatation and progression to heart failure. Circulation 97, 1536-1539.

7. Hill, M. F. and Singal, P. K. (1996) Antioxidant and oxidative stress changes during heart failure subsequent to myocardial infarction in rats. Am. J. Pathol. 148, 291-300.

8. Eguchi, M., Liu, Y., Shin, E. J. and Sweeney, G. (2008) Leptin protects H9c2 rat cardiomyocytes from H2O2-induced apoptosis. FASEB J. 22, 3136-3144.

9. Liu, J., Mao, W., Ding, B. and Liang, C. S. (2008) ERKs/p53 enhanced stability. J. Biol. Chem. 283, 172-177.

10. Lu, H. S., Boone, T. C., Gabrilove, J., Lai, P. H., Zsebo, K. M., Murdock, D. C., Chazin, V. R., Bruszewski, J., Lu, H., Chen, K. K., Barendt, J., Platzer, E., Moore, M. A. S., Mertelsmann, R. and Welte, K. (1986) Recombinant human granulocyte colony-stimulating factor mutant, on HL60 cells. J. Biol. Chem. 261, 61-65.

11. Kubota, N., Orita, T., Hattori, K., Ohveda, M., Ochi, N. and Yamazaki, T. (1990) Structural characterization of natural and recombinant human granulocyte colony-stimulating factors. J. Biochem. 107, 486-492.

12. Gervais, V., Zerial, A. and Oschkinat, H. (1997) NMR investigation of the role of the sugar moieties in glycosylated recombinant human granulocyte-colony-stimulating factor. Embo J. 16, 386-395.

13. Cumming, D. A. (1991) Glycosylation of recombinant protein therapeutics: control and functional implications. Glycobiology 1, 115-130.

14. Chung, H. K., Kim, S. W., Byun, S. J., Ko, E. M., Chung, H. J., Woo, J. S., Yoo, J. G., Lee, H. C., Yang, B. C., Kwon, M., Park, S. B., Park, J. K. and Kim, K. W. (2011) Enhanced biological effects of Phe140Asn, a novel human granulocyte colony-stimulating factor glycoforms produced in Chinese hamster ovary cells. J. Chromatogr. A. 1277, 55-62.

15. Lu, H. S., Boone, T. C., Souza, L. M. and Lai, P. H. (1989) Disulfide and secondary structures of recombinant human granulocyte colony stimulating factor. Arch. Biochem. Biophys. 268, 81-92.

16. Ishikawa, M., Iijima, H., Satake-Ishikawa, R., Tsumura, H., Iwamatsu, A., Kadoya, T., Shimada, Y., Fukamachi, H., Kobayashi, K., Matsu, S. and Asano, K. (1992) The substitution of cysteine 17 of recombinant human G-CSF with alanine greatly enhanced its stability. Cell Struct. Funct. 17, 61-65.

17. Bishop, B., Koay, D. C., Sartorelli, A. C. and Regan, L. (2001) Reengineering granulocyte colony-stimulating factor for enhanced stability. J. Biol. Chem. 276, 33465-33470.

18. Tarentino, A. L., Gomez, C. M. and Plummer, T. H. Jr. (1985) Deglycosylation of asparagine-linked glycans by peptide N-glycosidase F. Biochemistry 24, 4665-4671.

19. Chu, F. K. (1986) Requirements of cleavage of high mannose oligosaccharides in glycoproteins by peptide N-glycosidase F. J. Biol. Chem. 261, 172-177.

20. Uemoto, J., Bhavanandan, V. P. and Davidson, E. A. (1977) Purification and properties of an endo-alpha-N-acetyl-D-galactosaminidase from Diplodocus pneumoniae. J. Biol. Chem. 252, 8609-8614.

21. Wang, Z., Cui, M., Sun, L., Jia, Z., Bai, Y., Ma, K., Chen, F. and Zhou, C. (2007) Angiopoietin-1 protects H9c2 cells from H2O2-induced apoptosis through AKT signaling. Biochem. Biophys. Res. Commun. 359, 685-690.

22. Takano, H., Ueda, K., Hasegawa, H. and Komuro, I. (2007) G-CSF therapy for acute myocardial infarction. Trends. Pharmacol. Sci. 28, 512-517.

23. Kornfeld, R. and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. 54, 631-664.

24. Van den Steen, P., Rudd, P. M., Dwek, R. A. and Opdenakker, G. (1998) Concepts and principles of O-linked glycosylation. Crit. Rev. Biochem. Mol. Biol. 33, 151-208.

25. Burda, P. and Aebi, M. (1999) The dolichol pathway of N-linked glycosylation. Biochim. Biophys. Acta 1426, 239-257.

26. Munro, S. (2001) What can yeast tell us about N-linked glycosylation in the Golgi apparatus? FEBS Lett. 499, 223-227.

27. Oheda, M., Hase, S., Ono, M. and Ikenaka, T. (1988) Structures of the sugar chains of recombinant human granulocyte-colony-stimulating factor produced by Chinese hamster ovary cells. J. Biochem. 103, 544-546.

28. Clogston, C. L., Hu, S., Boone, T. C. and Lu, H. S. (1993) Glycosidase digestion, electrophoresis and chromatographic analysis of recombinant human granulocyte colony-stimulating factor glycoforms produced in Chinese hamster ovary cells. J. Chromatogr. 637, 55-62.

29. Tamada, T., Honjo, E., Maeda, Y., Okamoto, T., Ishibashi, M., Tokunaga, M. and Kuroki, R. (2006) Homodimeric cross-over structure of the human granulocyte colony-stimulating factor (GCSF) receptor signaling complex. Proc. Natl. Acad. Sci. U.S.A. 103, 3135-3140.

30. Okazaki, T., Ebihara, Y., Asada, M., Yamada, S., Saijo, Y., Shiraishi, Y., Ebihara, T., Niu, K., Mei, H., Arai, H. and Yambe, T. (2007) Macrophage colony-stimulating factor improves cardiac function after ischemic injury by inducing vascular endothelial growth factor production and survival of cardiomyocytes. Am. J. Pathol. 171, 1093-1103.

31. Pacher, P. and Szabo, C. (2007) Role of poly(ADP-ribose) polymerase 1 (PARP-1) in cardiovascular diseases: the therapeutic potential of PARP inhibitors. Cardiovasc. Drug. Rev. 25, 235-260.

32. Ungvari, Z., Gupta, S. A., Recchia, F. A., Batkai, S. and Pacher, P. (2005) Role of oxidative-nitrosative stress and downstream pathways in various forms of cardiomyopathy and heart failure. Curr. Vasc. Pharmacol. 3, 221-229.

33. Pacher, P., Llaudet, L., Bai, P., Virág, L., Mabley, J. G., Hasko, G. and Szabo, C. (2002) Activation of poly(ADP-ribose) polymerase contributes to development of doxorubicin-induced heart failure. J. Pharmacol. Exp. Ther. 300, 862-867.