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The Substitution of Cysteine 17 of Recombinant Human G-CSF with Alanine Greatly Enhanced its Stability

Masaharu Ishikawa, Hiroshi Iijima*, Rika Satake-Ishikawa, Haruhiko Tsumura, Akihiro Iwamatsu*, Toshihiko Kadoya, Yoshihiro Shimada, Hiromi Fukamachi, Keiko Kobayashi, Shigeru Matsuki†, and Katsuhiko Asano

*Pharmaceutical Laboratory, and *Central Laboratories for Key Technology, Kirin Brewery Co., Ltd. Soujamachi, Maebashi, Gunma, 371, Japan*

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ABSTRACT. **Human recombinant granulocyte-colony stimulating factor (rhG-CSF) has one free cysteine at position 17 and has two disulfide bridges (Cys36-Cys42 and Cys64-Cys74). The Cys17 of rhG-CSF was substituted with Gly, Ala, Ser, Ile, Tyr, Arg, and Pro, or deleted using site-directed mutagenesis in order to improve its thermostability. With the exception of Pro17-rhG-CSF, all mutant proteins retained biological activity which promotes the growth of mouse bone marrow cells *in vitro*. Among these mutant proteins, Ala17-rhG-CSF had more than 5 times higher stability than rhG-CSF. But Ser17-rhG-CSF had almost same stability as rhG-CSF and other mutant proteins had only lower stability.**

Human G-CSF (hG-CSF) is a hemopoietic factor which regulates neutrophil proliferation and differentiation (13). The cDNA encoding hG-CSF was cloned (15, 19) and large scale production of the recombinant human G-CSF (rhG-CSF) was obtained using genetically engineered *Escherichia coli* (19). rhG-CSF is also capable of supporting the formation of granulocytic colonies from bone marrow cells and is undergoing clinical testing for cancer patients with drug- or irradiation-induced myelosuppression (14, 22).

rhG-CSF has one free cysteine at position 17 and has two disulfide bridges (Cys36-Cys42 and Cys64-Cys74) (9). It is known that the free cysteines in T4 Lysozyme (16), Interferon-β (11) and Interleukin-2 (IL-2) (2) are involved in intermolecular thiol/disulfide interchange leading to inactive oligomers. Mutagenic replacement of unpaired Cys54 of T4 Lysozyme with Thr or Val generated one exhibiting enhanced stability toward irreversible thermal inactivation. In the case of IL-2, substitution of Cys125 with Ser or Ala yielded one with greater stability. In the case of rhG-CSF, previous studies showed that the substitution of unpaired Cys17 of rhG-CSF with Ser did not change its conformational stability (23) and that the 17th residue of murine G-CSF was serine (18).

In this report, we substituted the unpaired Cys17 of rhG-CSF with several other amino acids or deleted and compared their stabilities. Among those mutant proteins, Ala17-rhG-CSF was found to have more than 5 times greater stability than rhG-CSF.

† To whom correspondence should be addressed.

Abbreviations: G-CSF, granulocyte-colony stimulating factor; CD, circular dichroism.

MATERIALS AND METHODS

Materials. *E. coli* DNA polymerase I Klenow fragment, T4 polynucleotide kinase, T4 DNA polymerase, T4 DNA ligase, T4 gene 32 protein and restriction enzymes were purchased from Boehringer Mannheim, Takara Shuzo or Bio-Rad. Bacteriophage M13mp19 (24) was obtained from Takara Shuzo.

Recombinant DNA methods. Recombinant DNA methods were performed as described by Manniatis *et al.* (10).

Synthesis of oligonucleotide. Oligonucleotides were prepared by the phosphoramidite method (3) using a DNA synthesizer (Applied Biosystems).

Site-directed mutagenesis of human G-CSF gene. An hG-CSF expression plasmid, pCFM1156 hG-CSF, containing a chemically synthesized G-CSF gene (20), was digested with *Xba*I and *Eco*RI. The smaller *Xba*I-*Eco*RI fragment was inserted between the *Xba*I and *Eco*RI sites of bacteriophage M13mp19. The resulting M13 bacteriophage contains the hG-CSF gene. Site-directed mutagenesis of hG-CSF gene using an oligonucleotide was performed as described by Zoller *et al.* (25) or Kunkel *et al.* (7). The nucleotide sequence of the mutated hG-CSF genes were confirmed by the dideoxy sequencing method (12). To insert the mutated hG-CSF genes into the expression plasmid, an *Eco*RI site located at the 3′ end of human G-CSF gene was changed to *Xho*I site by site-directed

mutagenesis. The resulting M13 bacteriophages containing the mutated hG-CSF genes were digested with *Xba*I and *Xho*I. The small *Xba*I and *Xho*I fragments containing the mutated hG-CSF genes were ligated with the large *Xba*I-*Xho*I fragment of the hG-CSF expression plasmid, pCFM536 (19).

Production and purification of rhG-CSF mutant proteins. Expression of the mutated human G-CSF genes in *E. coli* and purification of mutant proteins of rhG-CSF were performed as previously described (21).

Biological activity. Biological activities of rhG-CSF mutant proteins were measured by the uptake of [^{3}H]-thymidine into mouse bone marrow cells following the procedure of Ralph *et al.* (17) with some modifications. In brief, low-density (<1.077 g/cm^{-3}) nonadherent bone marrow cells from female Balb/c mice were incubated for 3 days in dilutions of rhG-CSF mutant proteins in supplemented McCoy's 5a medium (10% fetal calf serum). The cell concentration was 8×10^4 cells per well in a volume of 0.2 ml in 96 well flat-bottom plates. After the incubation, the cells were pulse-labelled with ^{3}H-thymidine for 5 hours (1 μCi per well), harvested on glass fiber filters and assayed for radioactivity using a liquid-scintillation counter. Biological activities of rhG-CSF mutant proteins were calculated by a parallel line assay. A purified preparation of rhG-CSF, quantified by the mouse bone marrow colony assay, was used as a standard.

Amino acid sequence analysis. The N-terminal sequences of rhG-CSF and its mutant proteins were determined using a gas-phase sequencer (Applied Biosystems model 470A) following the procedure of Hewick *et al.* (6). The phenylthiohydantoin derivatives (PTH-amino acids) obtained were analyzed by a reverse phase HPLC chromatography under the conditions described previously (1).

Amino acid composition. Proteins were hydrolyzed for 24 hr in 6 N HCl (constant boiling) containing 0.2% phenol at 110°C in evacuated tubes. Amino acid analysis was carried out with a Hitachi 835 analyzer. Cysteine and half cystine were determined as S-carboxymethyl cysteine after reduction and S-carboxymethylation of the protein according to the method of Crestfield *et al.* (4).

Circular dichroic (CD) spectrometry. The CD spectra of the proteins were measured with a Jasco J600 spectropolarimeter. Spectra were recorded at 15°C using a 0.1 cm-path-length cell with 1 nm band-width. Measurements were carried out using approximately 0.5 mg/ml at pH 4.0. The spectral data were expressed as molar ellipticity ([θ]mrw), calculated using a mean residue weight of 104.4 (5).

Gel filtration HPLC analysis. The quantitative measurements of monomer contents of rhG-CSF mutant proteins were carried out by gel filtration analysis using a HPLC chromatography system. The column was a TSKgel-G3000SW (7.5 mm × 60 cm). The solvent, used at a flow rate of 1.0 ml/min, was 10 mM Sodium acetate buffer (pH 5.4). Absorbance at 280 nm was used for the detection of proteins.

Other methods. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Laemmli (8) using 15% polyacrylamide gels. Coomassie brilliant blue R-250 was used to stain the protein bands. Protein concentration was spectrophotometrically determined by absorbance at 280 nm.

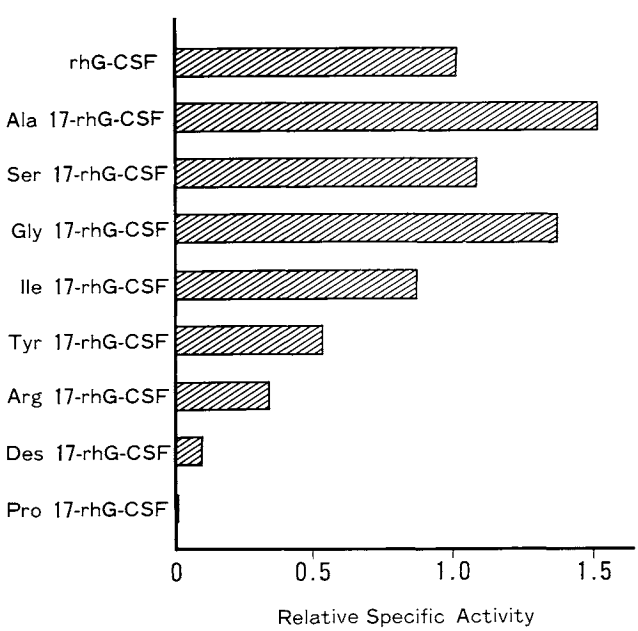

Fig. 1. Comparison of relative specific activities of rhG-CSF mutant proteins.
Relative specific activities are expressed using a value of 1.0 for rhG-CSF.

RESULTS AND DISCUSSION

Preparation of rhG-CSF mutant proteins. The cysteine situated at position 17 of the chemically synthesized hG-CSF gene was replaced by Gly, Ala, Ser, Ile, Tyr, Arg and Pro, or deleted by site-directed mutagenesis using an oligonucleotide. The mutated hG-CSF genes were expressed in *E. coli*. The mutant rhG-CSF proteins were extracted from *E. coli* cells and purified to homogeneity except Pro17-rhG-CSF. Pro17-rhG-CSF could be expressed in the cells, but could not be completely purified because it formed aggregation bodies during the purification process. The molecular weights

Table I. Residual biological activities (%) of rhG-CSF mutant proteins.

sample	Residual Activity (%)		
	6 hr	12 hr	72 hr
rhG-CSF	65	70	1
Ala17-rhG-CSF	99	116	54
Ser17-rhG-CSF	84	57	3

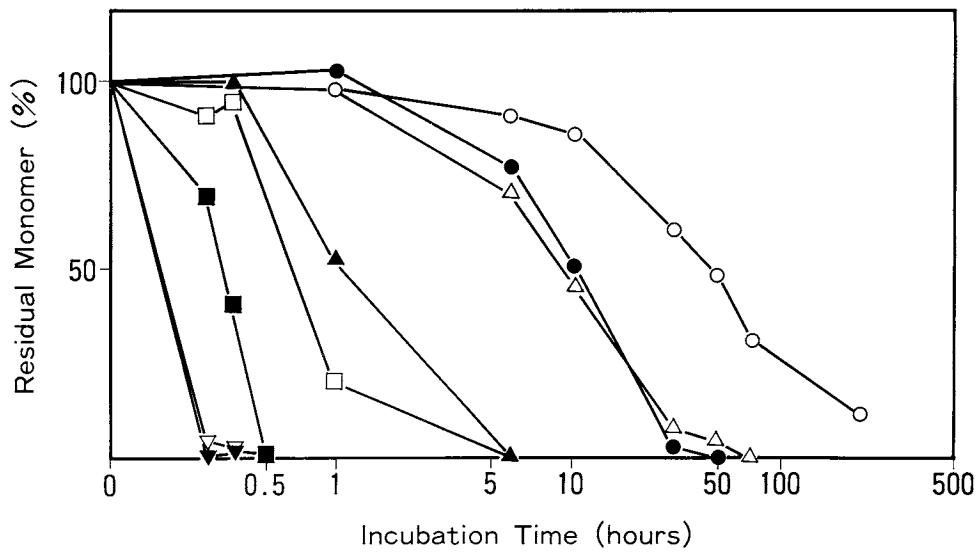

Fig. 2. Residual monomer contents (%) of rhG-CSF mutant proteins.
The residual monomer contents (%) of samples incubated at 53°C for various times were measured by gel filtration HPLC analysis as described in Materials and Methods.
rhG-CSF (●), Ala17-rhG-CSF (○), Ser17-rhG-CSF (△), Gly17-rhG-CSF (▲), Ile17-rhG-CSF (□), Tyr17-rhG-CSF (■), Arg17-rhG-CSF (▽) and Des17-rhG-CSF (▼).

of mutant proteins as estimated by SDS-PAGE were about 19,000. The substitution or deletion of Cys17 of each mutant protein was confirmed by amino acid analysis and by N-terminal sequence analysis (data not shown).

Biological activity of mutant proteins. Figure 1 shows the relative specific activities *in vitro* of mutant proteins. The biological activities *in vitro* of mutant proteins were determined by incorporation of [^{3}H]thymidine into bone marrow cells of mice. With the exception

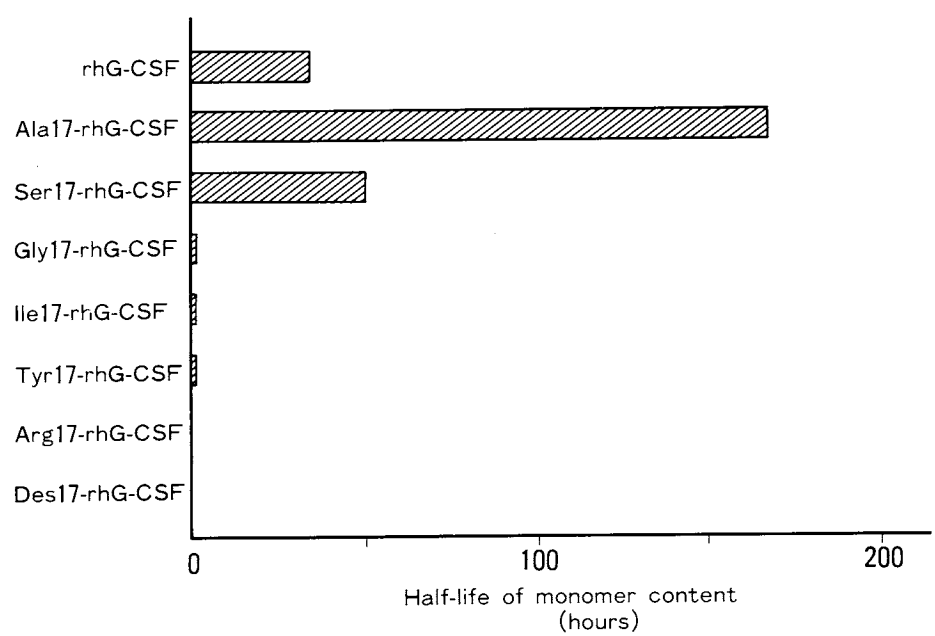

Fig. 3. Half-life times of residual monomer contents of rhG-CSF mutant proteins.
Each half-life time was calculated from the data of Fig. 2.

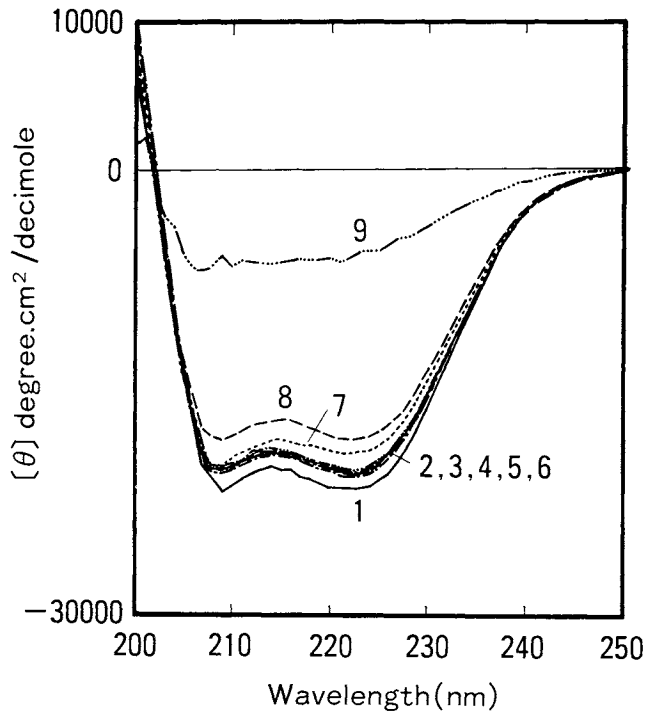

Fig. 4. CD spectra of rhG-CSF mutant proteins.
1: rhG-CSF, 2: Ala17-rhG-CSF, 3: Ser17-rhG-CSF, 4: Gly17-rhG-CSF, 5: Ile17-rhG-CSF, 6: Tyr17-rhG-CSF, 7: Arg17-rhG-CSF, 8: Des17-rhG-CSF, 9: Pro17-rhG-CSF

of Pro17-rhG-CSF, they all retained biological activity *in vitro*. Gly17-rhG-CSF, Ala17-rhG-CSF and Ser17-rhG-CSF had almost the same or higher specific biological activities than rhG-CSF, and the other mutants had lower activities. Pro17-rhG-CSF had no detectable biological activity. These results indicate that the amino acid residue at position 17 affects the biological activity in some degree but does not play an essential role.

Thermostabilities of mutant proteins. To investigate the thermostabilities of mutant proteins, they were incubated at 53°C and their residual biological activities were assayed. Table I shows the results with regard to rhG-CSF, Ala17-rhG-CSF and Ser17-rhG-CSF. Other mutant proteins retained less than 1% of their initial activities after 6 hr incubation. The enhanced thermostability of Ala17-rhG-CSF was further confirmed by gel filtration HPLC analysis. The intermolecular thiol/disulfide interchange caused the formation of oligomers of rhG-CSF. In our preliminary study, we found a linear correlation between the biological activity of rhG-CSF and the amount of its monomer form (data not shown). Hence, we incubated these mutant proteins at 53°C and measured their residual monomer contents (%) by gel filtration HPLC analysis. Figure 2 shows the decrease of the residual monomer content (%) of each mutant protein during the incubation at 53°C.

We calculated the half-life time of the residual monomer content (%) of each mutant protein from the data of Figure 2. The half-life time of monomer content (%) of rhG-CSF or Ser17-rhG-CSF under these conditions was 34 hours and 50 hours, respectively. On the other hand, that of Ala17-rhG-CSF was more than 168 hours (Fig. 3). These results show that the replacement of Cys17 with Ala improved the heat-stability of rhG-CSF greatly and that not only the disulfide bond scrambling of Cys17 but also other properties may be relevant to its heat-stability. As the CD spectra of mutant proteins except Pro17-rhG-CSF at far UV region (200–250 nm) were similar to that of rhG-CSF (Fig. 4), it is thought that those mutant proteins had similar conformations. But some conformational change of the side chain at the residue of position 17 may affect the heat-stability of rhG-CSF. More information about the structure of rhG-CSF, for instance x-ray diffraction data, will help to explain the relationship between the stability of rhG-CSF and the 17th amino acid residue in more detail.

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