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Mutagenesis of human granulocyte colony stimulating factor

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SUMMARY To define the structure-function relationship, we have made a number of mutants of human granulocyte colony-stimulating factor (hG-CSF) by in vitro mutagenesis. The results indicate that most of the mutations located in the internal and C-terminal regions of the molecule abolished the activity, wheras the mutants without N-terminal 4,5,7, or 11 amino acids retained the activity. N-terminal amino acids were also altered by cassette mutagenesis using a synthetic oligonucleotide mixture. Among them, KW2228, in which Thr-1, Leu-3, Gly-4, Pro-5 and Cys-17 were respectively substituted with Ala, Thr, Tyr, Arg and Ser, showed more potent granulopoietic activity than that of intact hG-CSF both in vitro and in vivo.

Colony stimulating factor (CSF) is a family of glycoproteins characterized by their activity to stimulate the proliferation and differentiation of hemopoietic cells in vitro. In the human and mouse system, four CSFs have been identified which specifically stimulate the progenitor cells of granulocyte-macrophage lineage: granulocyte CSF (G-CSF), macrophage CSF (M-CSF), granulocyte-macrophage CSF (GM-CSF) and interleukin 3 (IL-3) (for reviews, see ref 1-3). Among these CSFs, G-CSF exclusively stimulates neutrophilic granulocyte colony formation from bone marrow stem cells and induces the terminal differentiation of leukemic cells such as WEHI-3B D in vitro (4). Two groups of researchers (5,6) have successfully isolated hG-CSF from tumor cell lines and cloned cDNA. Therapeutic potential has also been reported using recombinant hG-CSF (7-

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9). It was reported (10) that alternative splicing produces two different cDNAs which encode polypeptides of 177 a.a. (hG-CSFa) and 174 a.a (hG-CSFb). The amino acid sequences of these two hG-CSF polypeptides are almost identical; hG-CSFa has an insertion of 3 amino acids (Val-Ser-Glu) between Leu-35 and Cys-36. Although it was reported that this insertion greatly reduces the specific activity of hG-CSF, there has not been any report on the structure/function analysis.

Recently, we (11) isolated cDNA which encodes hG-CSF of 174 a.a. from a cDNA library of lipopolysaccharide-stimulated human peripheral blood macrophage and showed that recombinant hG-CSF is useful in the treatment of 5-fluorouracil-induced hematopoietic injury in mice (12-14). In this report, we describe the mutational analysis of hG-CSF and mutants with increased biological activity.

EXPERIMENTAL PROCEDURE

IN VITRO MUTAGENESIS. The hG-CSF expression plasmid, pCfTA1 (11), was used in this study (Fig. 1A). DNA modification enzymes, such as restriction endonucleases, T4 DNA ligase and DNA polymerase I were used to introduce mutations. In some cases, double-strand oligonucleotide linkers were inserted at the resriction endonuclease sites. Site-directed mutagenesis using synthetic oligonucleotides was also used. Oligonucleotides were synthesized by the phosphodiester method on a solid support (15). The base sequences of synthetic oligonucleotides were designed to create new sites for restriction endonucleases in order to be used for the next mutagenesis procedure. The nucleotide sequence of each mutant was determined using chain termination methods (16). All the recombinant DNA techniques were performed as previously described (17).

PREPARATION OF hG-CSF MUTANTS. The production and partial purification of hG-CSF mutants were performed as described previously (11) with slight modifications. To solubilize inclusion bodies, 20 mM Tris-HCl buffer (pH7.2) containing 7 M urea, 10 mM EDTA, and 5 mM DTT, was used in place of 6 M guanidine hydrochloride, 1 mM DTT. The solublized hG-CSF mutants were dialyzed against 20 mM Tris-HCl buffer (pH 8.0). After dialysis, these samples were run through DEAE-Sepharose column equilibrated with 20 mM Tris-HCl buffer (pH 8.0). The hG-CSF mutants were eluted from the column by increasing the concentration of NaCl, and dialyzed against 20 mM Tris-HCl buffer (pH8.0). The purity of the hG-CSF mutant preparations was greater than 95% as determined by SDS-PAGE analysis (Coomassie blue stain) and HPLC analysis.

G-CSF BIOLOGICAL ACTIVITIES. Granulocyte colony-formation by bone marrow cells of C3H/He mice was assayed as described (18) by plating 5×10^4 cells per 0.5 ml of α-MEM containing 20 % fetal calf serum (FCS) and 0.3 % agar (Difco Laboratories, Detroit, MI) in 24-well microplate. Colonies 40 cells were enmerated on day 7. Bone marrow cell-proliferative assay was carried out in 96-well microplate with a modification of Ihle (19) using non-adherent bone marrow cells instead of NFS-60 cells. Samples were serially diluted 1:2 in 100 µl α-MEM containing 10 % FCS and mixed with 100 µl bone marrow cells (5×10^5 cells/ml). Cultures were incubated for 72 hr

at 37°C, and 0.25 μCi of ^{3}H -Thymidine (specific activity, 26 Ci/m mol, Amersham Japan) was added for the last 18 hr. The cells were harvested onto glassfilter with the automated cell harvester (Labo-science, Tokyo) and assayed for ^{3}H -Thymidine incorporation into the cells. For *in vivo* test of hemopoietic effect, C3H/He mice (8 weeks old, male, n=4) recieved 0.2 ml of test samples intravenously twice a day at 9:00 a.m. and 5:00 p.m. for 5 days. Peripheral leukocytes (WBC) were counted by a micro cell-counter (TOA electronics, Hyogo, Japan) 17 hr after the last injection.

RESULTS

Various hG-CSF mutants were obtained by the *in vitro* mutagenesis procedure. One of the structural characteristics of hG-CSF cDNA was that there were 5 PstI sites (CTC-GAG/Leu-Gln) in frame (Fig.1B). Partial digestion with PstI gave cDNA fragments truncated at one of the PstI sites and the ligation of the 5'- and 3'- deleted fragments gave a series of internal deletion and tandem repeat mutants. Other internal deletion mutants were also constructed using two different restriction endonucleases in the same manner as that when two fragments are ligated in the correct frame. In some cases, synthetic single strand or double strand oligonucleotides were used to introduce the mutation. The genetic codons

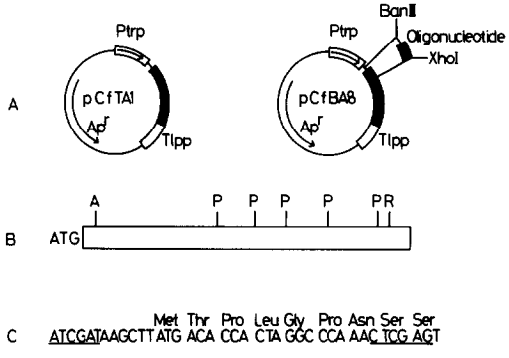

Fig.1. Structure of hG-CSF expression plasmids. (A) Structure of pCfTA1 and pCfBA8. The construction of pCfTA1 was described previously (11). Human G-CSF cDNA (black box) was located between *E. coli* tryptophan operon promoter (Ptrp) and lipoprotein terminator (Tlpp). This plasmid was the prototype plasmid of this study. To produce hG-CSF mutants, hG-CSF cDNA was substituted by mutated cDNA. Plasmid, pCfBA8 was constructed during the course of this study through 5 mutagenic steps and used as a parental plasmid for cassette mutagenesis using synthetic oligonucleotides mixture. (B) Restriction map of hG-CSF cDNA. Initiation codon (ATG) introduced in front of the first amino acid codon of the mature hG-CSF was also indicated. Some restriction endonuclease sites used for mutagenesis were indicated. A, Apa I; P, Pst I; R, Rsa I. (C) Nucleotide sequence aroud initiation codon of pCfBA8. Amino acid sequence of N-terminus was also indicated. BanIII and XhoI sites used for cassette mutagenesis were underlined.

were selected to introduced new sites for restriction endonuclease in order to be used for next mutagenesis.

We constructed approximately one hundred hG-CSF mutants. To assess the effect of each mutation on hG-CSF function, the mutated cDNA were expressed in Escherichia coli (E. coli) under the control of tryptophan promoter. The biological activities of the hG-CSF mutants, partially purified from E. coli lysates, were determined in vitro. The results are summarized in Table 1. Most of the cDNA with a mutation located in the

Table 1. Biological activity of hG-CSF mutants

Position	Mutation	Activity
1	Thr → Ser, Arg or Gly	+
1-3	Thr Pro Leu → Ser Pro Arg	+
1-3	Thr Pro Leu → Ser Leu Ser	+
1-4	Deletion	+
1-4/17	Deletion/Cys → Ser	+
1-6/17	Deletion/Cys → Ser	+
1-7/17	Deletion/Cys → Ser	+
1-11/17	Deletion/Cys → Ser	+
17	Cys → Ser	+
1-18	Deletion	-
33-35	Deletion	-
35	Deletion	-
35	Leu → His, Ala, Phe, Asp, Ser or Thr	-
35-36	Leu Cys → Leu Val Cys	-
36-37	Cys Ala → Cys Asp Ala	-
36-37	Cys Ala → Cys Cys Ala	-
70-89	Deletion	-
70-89	Tandem Repeat	-
74-75	Cys Leu → Ser Cys	-
74-76	Cys Leu Ser → Ser Leu Cys	-
90-106	Deletion	-
90-106	Tandem Repeat	-
91	Ala → Pro or Phe	-
91-97	Deletion	-
95-97	Ile Ser Pro → Thr	-
107-130	Deletion	-
107-130	Tandem Repeat	-
131-157	Deletion	-
131-157	Tandem Repeat	-
137-174	Deletion	-
165-174	Deletion	-

hG-CSF mutant proteins were partially purified as described in EXPERIMENTAL PROTOCOL and subjected to G-CSF assay in vitro. Since the purity of hG-CSF mutants varied from 20 % to 90 %, an accurate estimation of the specific activity for each mutant was not done. When the G-CSF activity of each mutant was not detected at a dose of 100 ng/ml, it was estimated to have no activity.

internal or C-terminal regions abolished the hG-CSF activity. The cDNA mutated near Leu-35 showed no activity or greatly reduced activity. These results agreed with the reduced specific activity of hG-CSFa previously reported (5) and showed the structural importance of this region of hG-CSF. We introduced mutations in or around Cys residues. Although the substitution of Cys-17 with Ser did not affect the activity, the insertion of 1 amino acid just after the Cys-36 and the shift of Cys-74 to position 75 or 76 reduced the activity.

These results showed the significance of Cys residues except for Cys-17 on hG-CSF activity. Conversely, most of N-terminal mutants retained activity. In addition, the deletion of N-terminal 4,6,7 or 11 amino acids did not reduce the activity, but a cDNA mutant without N-terminal 18 amino acids lacked the activity. The results indicated that hG-CSF exhibited biological activity without at least N-terminal 11 amino acids.

Regarding the N-terminal region of hG-CSF, Schrader et al. (18) have reported that the structural homology at the N-termini of cytokines dictates the activity on pluripotential hemopoietic stem cells and their cellular derivatives. They proposed Ala-Pro-Xaa-Arg-Ser as the consensus sequence of N-terminus of such cytokines. As the N-terminal sequence of hG-CSF only agreed with this sequence at Pro-2, the effect of this sequence on hG-CSF activity was thus tested. To do this, the plasmid pCfBA8 (Fig.1A), which was constructed during the course of this study was used. This plasmid encoded a mutant with a new XhoI site: Ala-6 and Cys-17 residues were replaced by Asn and Ser, respectively (Fig.1A,C). Three types of oligonucleotide mixture were synthesized (Table 2) and used to construct N-terminal mutant library. The in vitro hG-CSF activity of these partially purified mutants was shown in Table 3. All of the mutants tested were active. As some of these mutants seemed to be more active than wild type hG-CSF, three representative mutants were purified (>95% purity) and the specific activities were compared with that of wild type hG-CSF. Table

Table 2. Nucleotide sequences of synthetic oligonucleotides

```
                    Met-Ala-Pro-Thr-Arg-Ser-Ala
I     CGATAAGCTT-ATG-GCA-CCA-ACA-AGA-AGC-GCC-
        TATTCGAA-TAC-CGT-GGT-TGT-TCT-TCG-CGG-AGCT

                    Met-(16)-Pro-(8)-Arg-Ser-Ala-
                                 G
II    CGATAAGCTT-ATG-NNT-CCA-ANA-AGA-AGC-GCC-
        TATTCGAA-TAC-NNA-GGT-CNT-TCT-TCG-CGG-AGCT
                             T

                    Met-Ala-Pro-(4)-(4)-(4)-(4)-
III   CGATAAGCTT-ATG-GCA-CCA-NCA-NAT-NGC-GNC-
        TATTCGAA-TAC-CGT-GGT-NGT-NTA-NCG-CNG-AGCT
```

Nucleotide sequences and their coding amino acids sequences of three types of synthetic oligonucleotide mixtures were shown. N in the nucleotide sequences means one of four nucleotides. Number in parenthesis in the amino acid sequences means possible number of amino acids in the positions.

Table 3. Biological activity of hG-CSF mutants with the N-terminal amino acids substitution

N-terminal amino acid sequences				hG-CSF activity	
				I*	II**
Met-Thr-Pro-Leu-Gly-Pro-Ala-------Cys				+	1.00
		Asn-------Ser		+	N.D.
Ala	Thr-Arg-Ser	-------Ser		++	N.D.
	Glu-Lys-Ser	-------Ser		++	N.D.
Val	Ile-Arg-Ser	-------Ser		++	N.D.
Cys	Ile-Arg-Ser	-------Ser		++	N.D.
Tyr	Ile-Arg-Ser	-------Ser		++	1.86
Arg	Thr-Arg-Ser	-------Ser		++	N.D.
	Thr-Arg-Ser	-------Ser		+	N.D.
Asn	Glu-Arg-Ser	-------Ser		++	N.D.
Ile	Thr-Arg-Ser	-------Ser		++	3.05
Ser	Thr-Arg-Ser	-------Ser		++	N.D.
Ala	Thr-Asp-Ser-Asp	-------Ser		+	N.D.
Ala	Thr-Tyr-Arg	-------Ser		++	3.09
Ala	Ser-Asp-Ser	-------Ser		++	N.D.
Ala	Pro-Asn-Arg-Gly	-------Ser		+	N.D.

N-terminal amino acid sequence shown in the top line corresponds to that of hG-CSF. Only the substituted amino acid residues of hG-CSF mutants are shown below the dotted line. In vitro G-CSF activity was determined as described in EXPERIMENTAL PROCEDURE.
I*; results with partially purified samples.
II*; results with highly purified samples (>95 % purity).
Specific activity of the wild-type hG-CSF was around 1.0×10^8 U/mg protein.
N.D. means not done.

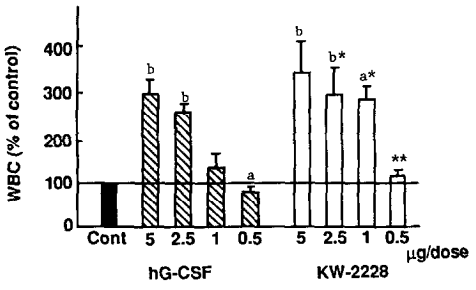

Fig.2. Effects of hG-CSF and KW2228 on granulopoiesis in mice. 0.2 ml of samples were injected intravenously into C3H/He mice (8 weeks old, male, n=4), twice a day for 5 days. Peripheral leukocytes (WBC) were counted daily by a micro cell-counter.
Data represent mean value of WBC 17 hrs after the last injection.
a: significant at $P<0.01$
b: significant at $P<0.001$ compared with control (viechle alone).
*: significant at $P<0.05$
**: significant at $P<0.01$ compared with a respected dose of hG-CSF.

3 shows that 2 of 3 mutants had a specific activity which was 3-fold higher than that of wild type hG-CSF.

One of the mutants (Ala-1, Thr-3, Tyr-4, Arg-5, Ser-17; designated KW2228) was intravenously injected into C3H/He mice twice a day and peripheral white blood cells (WBC) were counted. As shown in Fig.2, KW2228 and wild type hG-CSF showed a dose dependent granulopoietic effect. The maximum levels of WBC stimulated by wild type hG-CSF and KW2228 were not different. However in the dose range of 0.5 to 2.5 g/mouse, KW2228 stimulated granulopoiesis more effectively than wild type hG-CSF. These results indicated that KW2228 had more potent granulopoietic activity than that of the wild type hG-CSF *in vivo* as well as *in vitro*.

DISCUSSION

In this study, we constructed approximately one hundred hG-CSF mutants and the effect of each mutation on hG-CSF activity was determined *in vitro*. Although most of the mutant hG-CSF with mutations located in internal or C-terminal region abolished activity, many with the mutation in the N-terminal region retained activity. The results of these mutational analysis were compared with the evolutionary analysis of the G-CSF

structure. Tsuchiya et al. (21) reported the isolation of cDNA for murine G-CSF. From the comparison of the structure of human and murine G-CSF, it was noted that 73 % of amino acids of G-CSF were conserved but the N-terminal amino acid sequence was highly divergent. Our mutational study showed that the evolutionally-conserved sequence was required for the generation of a functional hG-CSF molecule and G-CSF exhibited its biological activity even without the N-terminal sequence. This finding agrees with the hypothesis that the evolutionally-conserved sequences are essential for function. However, our results also indicated a delicate relationship between the non-essential N-terminal structure and G-CSF activity. The substitution of N-terminal amino acids sequences resulted in a 3-fold increase in specific activity. KW2228 showed a specific activity 2 to 4-fold higher than that of hG-CSF on the human bone marrow stem cells (data not shown). These results suggest that the functionally-essential structure of G-CSF was formed without the N-terminal sequence but that the N-termianl sequence affected G-CSF activity by a mechanism which did not directly change the essential structure. Preliminary experiment showed that KW2228 was more resistant to heat inactivation than wild type hG-CSF (data not shown). Such a physicochemical alteration might contribute to the higher specific activity of KW2228.

Recently, using ^{124}I-labeled KW2228, the presence of the specific receptor (~150K) for human G-CSF was demonstrated on human circulating neutrophils (22). The results also showed that KW2228 had the higher specific binding to the receptor on human neutrophils than the intact G-CSF (22). This is the first example of hG-CSF mutein with the higher specific activity, the higher stability and the higher affinity to its receptor, which suggests it's potential clinical usefulness.

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