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Site-specific mutagenesis of human interleukin-6 and its biological activity

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Amino acid substitutions of human interleukin-6 (IL-6) were performed. Single substitution Met^{162}→Ala and double substitutions $Leu^{159,166}$→Val resulted in a significant decrease of IL-6 activity in the production of immunoglobulin (Ig) from B-cells. Single substitution Leu^{166}→Val or Leu^{159}→Val gave a slight or no significant decrease in the Ig-induction activity, respectively. The receptor-binding activity of each IL-6 mutant was also examined. It was observed that the decrease of the receptor-binding activity was generally in parallel with that of the Ig-induction activity. We therefore suggest that hydrophobic side-chains existing in Met^{162}, Leu^{159}, and Leu^{166} are significantly involved in the receptor-binding of IL-6.

Interleukin-6; Site-specific mutagenesis; B-cell stimulatory activity; Receptor-binding activity; Hydrophobic side-chain; Human

1. INTRODUCTION

Interleukin-6 (IL-6) plays an important role in the growth and differentiation of various cells [1]. The induction of IL-6 production has been associated with a variety of diseases such as rheumatoid arthritis [2,3], severe burns [4], and acquired immunodeficiency syndrome [5-7]. Human IL-6 consists of 185 amino acids (M_r = 21 000) [8], and recombinant human IL-6 has been expressed at a high level in *Escherichia coli* [9]. Human IL-6 receptor [10] associates with a non-ligand-binding membrane glycoprotein, gp130, in the presence of IL-6 and mediates its function [11].

Although biological functions of IL-6 in vitro and in vivo have been examined extensively, only a few studies concerning the structure/function relationship of IL-6 have been reported. Brakenhoff et al. [12] showed that the 28 amino-terminal amino acid residues can be removed without significantly affecting the biological activity of IL-6. Krüttgen et al. [13] showed that the three carboxy-terminal amino acids of IL-6 are essential for its biological function. No study has so far been reported concerning the substitution of amino acids in the IL-6 molecule.

In previous studies, we have partially assigned the ^1H nuclear magnetic resonance (NMR) signals of the aromatic residues in human IL-6 [14], and, on the basis of the chemical modification and ^1H NMR data, we have concluded that Trp^{158}, Met^{162}, His^{165}, and Met^{185} are in spatial proximity, comprising the receptor-binding region [15]. In the present study, on the basis of our previous findings, we have prepared several mutant IL-6 cDNAs by site-directed mutagenesis and examined the effect of the substitution of amino acid(s) on the expression of the IL-6 activity.

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Abbreviations: IL-6, interleukin-6; NMR, nuclear magnetic resonance; ELISA, enzyme-linked immunosorbent assay; Ig, immunoglobulin

2. MATERIALS AND METHODS

2.1. *Construction of plasmids*

pBSF2.38-1 cDNA [9], containing the entire coding region of human IL-6, was digested with *Kpn*I and *Sal*I, and the resulting ca. 1 000 bp cDNA fragment coding IL-6 was inserted into M13mp19 (Takara). For the insertion of IL-6 cDNA into the expression plasmid pSVL (Pharmacia) after site-specific oligonucleotide mutagenesis, *Bam*HI site was introduced by destroying its *Hin*dIII site in M13mp19. Mutant IL-6 cDNA was prepared using an in vitro Mutagenesis System (Amersham) with synthetic nucleotide oligomers, (i) 5'-ACCAGTGGGTCCAGGACA-3' (Leu^{159} → Val); (ii) 5'-ACAACTCACGTGATTCTGCG-3' (Leu^{166} → Val); (iii) 5'-TGCAGGATGCGACAACT-3' (Met^{162} → Ala). For the COS1 cell expression, native and mutant IL-6 cDNAs were digested with *Sac*I and *Bam*HI, and inserted in the expression plasmid pSVL. The nucleotide sequences of mutant clones were verified by the dideoxynucleotide chain termination procedure [16].

2.2. *Transfection of COS1 cells and analysis of expressed proteins*

Each plasmid (10 μg) was transfected into 1×10^6 COS1 cells by the calcium phosphate method [17]. The transfected cells were cultured in Dulbecco's modified Eagle's medium without serum in 10 cm dishes for 3 days. The culture supernatant was collected and concentrated (25×) with an Amicon Centriprep 10 cartridge for the analysis of biological activity. IL-6 and mutant IL-6 concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using recombinant human IL-6 [9] as a standard. Anti-human IL-6 polyclonal an-

tibody (rabbit) was used for ELISA. A mock control was prepared using pSVL vector without the insert.

2.3. *Assay for B-cell stimulatory activity*

B-cell stimulatory activity was assayed as described by Hirano et al. [18]. Four thousand SKW6-CL4 cells, EBV-transformed B-cell lines, were cultured in 200 μl of culture medium in the presence of the test sample. After three days, the concentration of immunoglobulin (Ig) M, which is secreted into the culture medium by SKW-CL4 cells, was determined by ELISA.

2.4. *Assay for the receptor-binding activity*

Ninety-six-well microplate was coated with 100 μl of anti-IL-6 receptor antibody MT18 [19] (2 μg/ml) in 0.1 M sodium-hydrogen carbonate buffer (pH 9.6) at 4°C overnight. After this coating, all experiments were performed at room temperature. The wells were blocked with 100 μl of 1% bovine serum albumin in 10 mM phosphate, 150 mM NaCl/H_2O (pH 7.4) for 2 h, then washed with 10 mM phosphate, 150 mM NaCl/H_2O (pH 7.4) containing 0.05% Tween 20, and 100 μl of soluble IL-6 receptor (50 ng/ml) [20] was incubated for 2 h. After washing, 100 μl of test sample was added to the wells and incubated for 2 h. The wells were washed, and 100 μl of anti-human-IL-6 polyclonal antibody (rabbit) (5 μg/ml) was added. After a 2-h incubation, the wells were washed, and 100 μl of alkaline phosphatase-conjugated anti-rabbit- IgG polyclonal antibody (Cappel) (×1/5000) was added. After a 2-h incubation, the wells were rinsed, and 100 μl of alkaline phosphatase substrate (*p*-nitrophenyl phosphate) (1 mg/ml) was added. The enzyme activity was measured spectrophotometrically at 405 nm.

3. RESULTS AND DISCUSSION

In the previous study [15], effects of oxidation of the Met residues of human IL-6 molecule on its receptor-binding activity have been examined. Of five Met residues, Met^{162} was the only residue in which the receptor-binding activity decreases in parallel with the degree of the oxidation reaction. In this report, we have focused our attention not only on Met^{162} but also on Leu^{159} and Leu^{166}. We have previously shown that Trp^{158} and His^{165} are in close spatial proximity to Met^{162} [15]. Trp^{158} or His^{165} is sequential to Leu^{159} or Leu^{166}, respectively. Leu^{159} and Leu^{166} are highly conserved among mammalian IL-6 species [21]. Four mutants (Met^{162} → Ala, Leu^{159} → Val, Leu^{166} → Val, and $Leu^{159,166}$ → Val) were established by site-directed mutagenesis of a human IL-6 coding sequence cloned in M13mp19. Native IL-6 and mutant IL-6 species were expressed in COS-1 cells and secreted into the culture medium. The amounts of IL-6 proteins detected varied between 2 and 10 ng/ml. We have tested the biological activity of IL-6 proteins in Ig-induction and receptor-binding assays.

Fig. 1 shows the effect of substitution of amino acid(s) on B-cell stimulatory activity. The substitution Met^{162} → Ala resulted in a decrease in the production of IgM from SKW6-CL4 cells. Single substitution Leu^{166} → Val or Leu^{159} → Val gave a slight or no significant decrease in the Ig-induction activity, respectively. However, double substitutions $Leu^{159,166}$ → Val showed a significant decrease in the Ig-induction activity. The relative activity to native IL-6 estimated at half-maximal stimulation of Ig-induction activity (IgM = 50 ng/ml) was 11%, 58%, and 10% for Met^{162} → Ala, Leu^{166} → Val, and $Leu^{159,166}$ → Val mutants, respectively.

Fig. 2 also shows the effect of amino acid substitution(s) on receptor-binding activity. A significant decrease in receptor-binding activity was observed for the Met^{162} → Ala and $Leu^{159,166}$ → Val mutants. Single substitution Leu^{166} → Val slightly decreased the activity and Leu^{159} → Val to a lesser extent. The decrease of the receptor-binding activity induced by amino acid

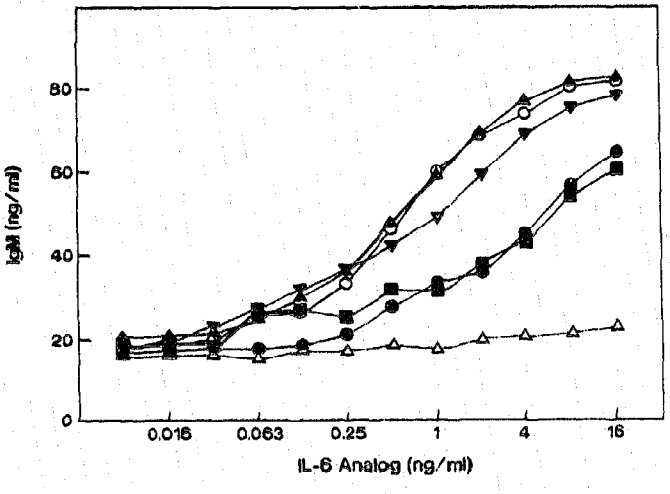

Fig. 1. B-Cell stimulatory assay of native IL-6 (○), Met^{162} → Ala (●), Leu^{159} → Val (▲), Leu^{166} → Val (▼), and $Leu^{159,166}$ → Val (■) mutants, and mock control (△). The concentration of IgM, which is produced in the culture medium of SKW6-CL4 cells, was determined by ELISA. Data represent averages of triplicate determinations.

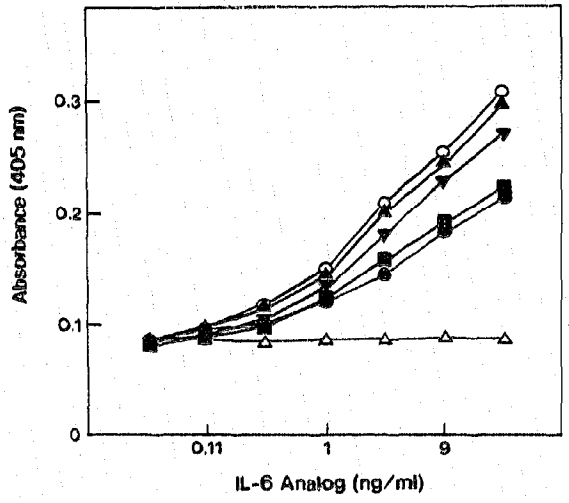

Fig. 2. Receptor-binding assay of native IL-6 , (○), Met^{162} → Ala (●), Leu^{159} → Val (▲), Val (▲), Leu^{166} → Val (▼), and $Leu^{159,166}$ → Val (■) mutants, and mock control (△). The quantity of native or mutant IL-6, which bound to soluble IL-6 receptor, was spectrophotometrically measured. Data represent averages of duplicate determinations.

substitution(s) was generally consistent with that of the Ig-induction activity. We therefore suggest that the decrease of biological activity is due to that of the receptor-binding affinity.

Chemical modification specific to Met residues led us to suggest that Met^{162} plays an important role in the receptor-binding activity of IL-6 [15]. This possibility has been confirmed by the present study showing the decrease in biological activity which was induced by $Met^{162} \rightarrow Ala$ substitution. It is likely that the hydrophobic side-chain of Met^{162} is required for the biological activity of IL-6. Moreover, the importance for receptor-binding of the hydrophobic side-chain of Leu^{159} and Leu^{166} has been shown by double substitutions $Leu^{159,166} \rightarrow Val$. These results are consistent with the conclusion in the previous report that the region between Trp^{158} and His^{165} comprises the receptor-binding region. Since $Met^{162} \rightarrow Ala$ or $Leu^{159,166} \rightarrow Val$ mutant has not completely abolished the biological activity, we cannot exclude the possibility that another residue(s) is (are) also involved, along with Met^{162}, Leu^{159}, and Leu^{166}, in the receptor-binding. The single substitution $Leu^{159} \rightarrow Val$ or $Leu^{166} \rightarrow Val$ resulted in only a small change in the activity. This is probably due to a conserved substitution of Leu residue with Val residue.

The previous circular dichroism and NMR studies [14,15,22] have shown that (i) IL-6 molecule is rich in α-helical structure, and (ii) the side-chains of Trp^{158}, Met^{158}, Met^{162} and His^{165} are in spatial proximity. If the Leu^{152}-Leu^{166} segment takes on an α-helix conformation, the side chains of three Leu residues, Leu^{152}, Leu^{159}, and Leu^{166}, can be aligned in the tertiary structure . In the present studies, double substitutions $Leu^{159,166} \rightarrow Val$ resulted in a significant decrease of the IL-6 activity. This is reminiscent of a significant decrease in DNA-binding activity, which was previously reported for the double substitutions of Leu residues at 7-amino acid interval with Val residues that exist in the leucine zipper region of DNA-binding proteins [23-25]. An intriguing possibility is that this region in the IL-6 molecule takes a structure like the leucine zipper motif [26]. Further NMR analyses are being carried out in our laboratory for a better understanding of the structure in this region of the IL-6 molecule.

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