Structure-function studies on human granulocyte-macrophage colony-stimulating factor

Timothy Robert Hercus, B.Sc. (Hons.)

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Division of Human Immunology
Institute of Medical and Veterinary Science
Department of Microbiology and Immunology
University of Adelaide
Adelaide, South Australia

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Summary

The aim of the work described in this thesis was to study the structure-function properties of the human cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF) in order to generate molecules with novel biological properties. The approach used was to generate mutated forms of GM-CSF cDNA, express mutant proteins and examine their biological activity and receptor binding properties.

At the time the work for this thesis commenced the structure of GM-CSF had not yet been determined. However, the predicted structure of GM-CSF was a bundle of four alpha helices containing hydrophilic regions. Initial studies using COS and CHO cell expression systems focussed on residues within the first predicted helix of GM-CSF that contribute to a prominent hydrophilic peak. Deletion analysis and substitution mutagenesis of residues 20 and 21 indicated that Glu^{21} was a functionally significant residue as charge reversal mutations at this position reduced GM-CSF activity 300-fold. A variety of amino acid substitutions at position 21 indicated a hierarchy of tolerance with Glu,Asp > Asn > Ala > Ser > Gln > Lys,Arg. The results indicated that Glu^{21} was essential for multiple GM-CSF activities including cell proliferation and mature cell activation.

The GM-CSF receptor comprises a GM-CSF specific, low affinity receptor α chain (GMRα) and a β chain (β_c) that does not by itself detectably bind GM-CSF but confers high affinity binding when co-expressed with the α chain and is required for signal transduction. The β_c chain is shared with the IL-3 and IL-5 receptors. Receptor binding studies with residue 21 analogues indicated that Glu^{21} is essential for binding to the high affinity (GMR$\alpha\beta_c$) but not the low affinity (GMRα) receptor. The results identified the presence of two functional domains of GM-CSF required for either GMRα

or GMR$\alpha\beta_c$ interaction and demonstrated that GM-CSF stimulation of both proliferation and mature cell activation are mediated through high affinity receptors.

The functional role of other residues in the first alpha helix was examined in light of the critical role determined for Glu^{21}. Oligonucleotide cassette mutagenesis (OCM) was developed to generate large numbers of mutants for expression in COS cells. Residues 14 and 17 to 28 of the first alpha helix of human GM-CSF were subjected to extensive substitution mutagenesis. Mutation of most amino acids buried in the hydrophobic core did not significantly impair biological activity or receptor binding apart from a modest decrease in biological activity observed with mutation of residues Ala^{22} and Leu^{26}. Mutation of Ile^{19} produced a marked decrease in biological activity and receptor binding, probably as a result of structural perturbations. Mutation of amino acids located on the surface of the first helix did not significantly impair biological activity or receptor binding, with the notable exception of Glu^{21} (discussed above). The conclusion was that residue 21 is the only significant β_c chain contact on the surface of the first helix.

The carboxy terminus of GM-CSF contains a prominent hydrophilic peak centred over the fourth alpha helix with a number of charged amino acids conserved across several species. The importance of these residues was examined by charge reversal mutagenesis and indicated a role in GM-CSF biological activity for Asp^{112}. Binding studies showed a reduction in high- and low-affinity binding for the D112R analogue. In this respect residue 112 appears to be functionally distinct from residue 21 and is apparently involved in binding to the GMRα chain.

An *E. coli* expression system was used to produce larger quantities of GM-CSF residue 21 analogues for detailed receptor binding studies and to enable structural characterisation. The analogues which included hydrophobic (Ala, Phe), hydrophilic (Gln) and basic (His, Lys, Arg) substitutions at position 21, proved refractive to expression but techniques were devised that enabled their expression and purification.

The *E. coli*-derived GM-CSF residue 21 analogues displayed a range of biological activities, yet they all exhibited low affinity binding characteristics on both GMRα and GMRαβ_c, similar to CHO cell-derived analogues. The E21R and E21K charge reversal analogues, were surprisingly devoid of activity. The lack of activity from the *E. coli*-derived E21R analogue was in marked contrast to the activity observed for the CHO cell-derived E21R analogue. These results demonstrated that although in some cases low affinity binding can lead to biological signalling, in other cases such as with the E21R and E21K analogues, low affinity binding can be dissociated from receptor activation and biological signalling.

The *E. coli*-derived E21R and E21K analogues, which bind GMRα chain without eliciting a functional response, were tested for antagonistic activity. Both analogues were effective antagonists of the GM-CSF-mediated proliferation of leukaemic cells and the GM-CSF-mediated release of superoxide anions from neutrophils. The antagonism was effective against glycosylated and non-glycosylated forms of GM-CSF and was specific for GM-CSF in that no antagonism of IL-3-mediated leukaemic cell proliferation or TNF-α-mediated neutrophil superoxide production was observed.

On the basis of the observations presented in this thesis, a model for the interaction of GM-CSF with the receptor complex is proposed that should also be applicable to related cytokines, IL-3 and IL-5 in particular. The critical feature of this model is that GM-CSF contains two functionally distinct receptor binding sites, one for the GMRα chain and another for the β_c chain. The GMRα chain binding site includes residues from the fourth helix and in particular the Asp^{112} residue. The β_c chain binding site includes only Glu^{21} of the first helix. A model to account for the antagonism of GM-CSF activity is presented which proposes that the antagonist sequesters the available GMRα chain into complexes unable to associate with the β_c chain and therefore prevents wild type GM-CSF interaction with the GM-CSF receptor complex. Possible clinical implications for GM-CSF antagonists are discussed.