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Site-Specific PEGylation of Engineered Cysteine Analogs of Recombinant Human Granulocyte-Macrophage Colony-Stimulating Factor

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Abstract

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) stimulates proliferation of hematopoietic cells of the macrophage and granulocyte lineages and is used clinically to treat neutropenia and other myeloid disorders. Because of its short circulating half-life GM-CSF is administered to patients by daily injection. We describe here the engineering of highly potent, long acting human GM-CSF proteins through site-specific modification of GM-CSF cysteine analogs with a cysteine-reactive polyethylene glycol (PEG) reagent. Thirteen cysteine analogs of GM-CSF were constructed, primarily in non-helical regions of the protein believed to lie away from the major receptor binding sites. The GM-CSF cysteine analogs were properly processed, but insoluble following secretion into the *Escherichia coli* periplasm. The proteins were refolded and purified by column chromatography. Ten of the cysteine analogs could be modified with a 5 kDa-maleimide PEG and seven of the mono-PEGylated proteins were purified by ion-exchange column chromatography. Biological activities of the 13 cysteine analogs and seven PEGylated cysteine analogs were comparable to that of wild type GM-CSF in an *in vitro* cell proliferation assay using human TF-1 cells. One cysteine analog was modified with larger 10 kDa-, 20 kDa- and 40 kDa-PEGs, with only minimal loss of *in vitro* bioactivity. Pharmacokinetic experiments in rats demonstrated that the PEGylated proteins had up to 47-fold longer circulating half-lives than wild type GM-CSF. These data demonstrate the utility of site-specific PEGylation for creating highly potent, long-acting GM-CSF analogs, and provide further evidence that the non-helical regions of human GM-CSF examined are largely non-essential for biological activity of the protein.

INTRODUCTION

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) stimulates the proliferation, differentiation and functional activation of hematopoietic cells of the macrophage and granulocyte lineages. Recombinant human GM-CSF is used clinically to treat a variety of hematopoietic disorders, including reducing the severity of chemotherapy-induced neutropenia, accelerating hematopoietic recovery following bone marrow transplantation and mobilizing blood progenitor cells for transplantation (1,2). Recombinant GM-CSF also has shown promise as a treatment for Crohn's disease and as an adjuvant therapy for melanoma (3,4). A limitation of current GM-CSF products is the fact that GM-CSF has a short circulating half-life (1,2) and must be administered to patients by daily injection for optimal effectiveness.

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Development of longer acting forms of GM-CSF that require less frequent injections would be of significant benefit to patients and healthcare providers.

One method that has been used to prolong the circulating half-lives of protein therapeutics is to modify the protein with polyethylene glycol (PEG) (5–8). Attachment of PEG to a protein increases the protein's effective size, thus reducing its clearance rate by the kidney and prolonging its circulating half-life. The most commonly used PEG reagents attach to primary and secondary amines on proteins, generally at lysine residues and/or at the N-terminal amino acid. This approach is not optimal for human GM-CSF because the protein contains six lysine residues in addition to the N-terminal amino acid. Five of these lysine residues are located in regions of the protein implicated in receptor binding (9,10). Site-directed mutagenesis studies showed that Lys72, Lys74 and Lys85 in Helix C, and amino acids adjacent to these residues, Gly75 and Gln86, are required for optimal binding of GM-CSF to its receptor (9,10). The large number and critical locations of these lysine residues makes GM-CSF a poor target for modification with amine-reactive PEG reagents. Indeed, covalent modification of GM-CSF with an amine-reactive PEG reagent resulted in a heterogeneous mixture of mono- and multi-PEGylated GM-CSF variants that could not be separated from each other or from unmodified GM-CSF by conventional column chromatographic procedures (11). Additionally, partial loss of bioactivity was observed for the PEGylated GM-CSF mixture, but the effect was difficult to quantitate due to heterogeneity of the sample and inability to separate PEGylated protein from nonPEGylated protein (11).

An alternative and potentially more selective method for PEGylating proteins covalently attaches PEG to cysteine residues using cysteine-reactive PEGs. At near neutral pH, these PEG reagents selectively attach to the thiol groups of “free” cysteine residues, i.e., cysteine residues not involved in disulfide bonds. Most proteins, including GM-CSF, do not contain surface-exposed, free cysteine residues. By using *in vitro* mutagenesis it is possible to introduce a non-native, free cysteine residue into a protein. The non-native cysteine residue can serve as the site for targeted modification of the protein with a cysteine-reactive PEG reagent, thus preserving biological activity of the modified protein (12). In this report we describe the application of this technology to the rational design of highly potent, long acting human GM-CSF analogs.

EXPERIMENTAL PROCEDURES

Cloning of GM-CSF and Construction of GM-CSF Cysteine Analogs

A cDNA encoding human GM-CSF was cloned by the reverse transcriptase – polymerase chain reaction (PCR) method (13), using total RNA isolated from the human bladder carcinoma cell line 5637 (obtained from the American Type Culture Collection, Rockville, MD). The leader sequence of the *E. coli* heat-stable enterotoxin (STII) gene (14), preceded by an *Nde* I restriction site, was fused to the amino-terminal coding sequence of mature GM-CSF using PCR. At the same time, several changes in the GM-CSF gene were made to optimize the protein for expression in *E. coli*. A TAA stop codon, followed by an *Eco* RI restriction site, was added following the carboxy-terminal residue, E127, codons for prolines at positions 2, 6, 8, 12, 117, and 124 were all changed to CCG, and the codon for leucine at position 114 was changed to CTG. The STII-GM-CSF gene was cloned as a *Nde* I – *Eco* RI fragment into pUC18 (Sigma-Aldrich, Inc., St. Louis, MO), to create pBBT268, and its DNA sequence confirmed. The *Nde* I - *Eco* RI fragment of pBBT268 was subcloned subsequently into plasmid pBBT257, creating pBBT271. Plasmid pBBT257 was derived from the expression vector pCYB1 (New England BioLabs, Beverly, MA) by deleting the ampicillin resistance gene of pCYB1 and replacing it with the gene for tetracycline resistance derived from plasmid pBR322 (15). In both pBBT257 and pCYB1, expression of the cloned gene is under the control of the *tac* promoter. Thirteen mutant GM-CSF genes were constructed by site-directed PCR-based

mutagenesis (16,17) using pBBT268 as the DNA template. PCR products were digested with appropriate restriction endonucleases and cloned into pBBT268 vector DNA. Correct DNA sequences of all mutants were confirmed.

Expression and Purification of Wild Type GM-CSF and GM-CSF Cysteine Analogs

Plasmids encoding wild type GM-CSF and GM-CSF cysteine analogs were transformed into *E. coli* strain W3110. An overnight culture of each strain was inoculated at an optical density at 600 nm of ~ 0.05 in 400 mL of Luria Broth media containing 10 µg/mL tetracycline. When the culture reached an optical density of ~ 0.6, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and the induced culture was incubated overnight for about 16 h. The cells were pelleted by centrifugation and stored at -80°C. The cell pellet was thawed and treated with 5 mL of B-PER™ bacterial protein extraction reagent (Pierce Chemical Company, Rockford, IL) according to the manufacturer's protocols. Insoluble material was recovered by centrifugation and resuspended in B-PER. This mixture was treated with lysozyme (200 µg/mL) for 10 min to further disrupt the cell walls, and MgCl₂ (10 mM final concentration) and protease-free DNase (2 µg/mL) were added. Insoluble GM-CSF was collected by centrifugation, resuspended in water and recentrifuged. The insoluble pellet was dissolved in 10 mL of 8 M urea, 25 mM cysteine, 20 mM Tris Base, stirred for 30 min at room temperature and diluted into 100 mL of 20 mM Tris, 40 µM copper sulfate, 15% glycerol, pH 8.0. The refold mixture was held at 4°C for 2 days, centrifuged and loaded onto a 5 mL Q-Sepharose HiTrap column (Amersham Biosciences Corporation, Piscataway, NJ) equilibrated in 20 mM Tris, pH 8.0 (Buffer A). The bound proteins were eluted with a linear salt gradient from 0–35% Buffer B (1M NaCl, 20 mM Tris, pH 8). Column fractions were analyzed by non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fractions containing primarily GM-CSF were pooled. The Q-Sepharose pool was diluted with an equal volume of 30% ammonium sulfate and loaded onto a 1 mL Phenyl HP HiTrap column (Amersham Biosciences Corporation) previously equilibrated with 15% ammonium sulfate in 20 mM sodium phosphate, pH 7.5. GM-CSF was recovered from the column by elution with a reverse salt gradient (15% ammonium sulfate to 0% ammonium sulfate in 20 mM sodium phosphate, pH 7.5). Column fractions were analyzed by non-reducing SDS-PAGE and fractions containing GM-CSF and no detectable contaminants were pooled.

PEGylation of GM-CSF Cysteine Analogs

Aliquots of 200 to 300 µg of the purified GM-CSF cysteine analogs were incubated with a 15-fold molar excess of Tris[2-carboxyethyl]phosphine]hydrochloride (TCEP, Pierce Chemical Company) and a 20-fold molar excess of a linear 5 kDa-maleimide-PEG (Nektar, Inc., Huntsville, AL). After 1.5 h at room temperature, the PEGylation mixture was diluted 20X with ice cold 20 mM Tris, pH 8.0 and loaded onto a 1 mL Q-Sepharose HiTrap column (Amersham Biosciences Corporation) equilibrated in 20 mM Tris, pH 8.0. Bound proteins were eluted as described above. Column fractions were analyzed by non-reducing SDS-PAGE and fractions containing predominantly the monoPEGylated GM-CSF cysteine analog were pooled and stored at -80°C. This procedure was scaled up to prepare the A3C analog modified with linear 10 kDa- and 20 kDa-maleimide PEGs, and a branched 40 kDa-maleimide PEG (Nektar, Inc.).

Biochemical Methods

N-terminal protein sequencing was performed by Macromolecular Resources (Colorado State University, Fort Collins, CO) using an Edman degradation procedure. Protein samples were analyzed by SDS-PAGE on precast Tris-glycine polyacrylamide gels (Invitrogen Corporation, Carlsbad, CA) and stained with Coomassie Blue. 2-mercaptoethanol was added to a final concentration of 1–5 % (V/V) in sample buffer to reduce the proteins. Protein concentrations

were determined using a Bradford protein assay kit (Bio-Rad Laboratories, Richmond, CA), using bovine serum albumin as the protein standard. Endotoxin levels of the proteins were measured using a chromogenic Limulus Amebocyte Lysate kit (QCL-1000, BioWhittaker, Walkersville, MD). Western blots were performed using standard methods. The primary antibody was polyclonal goat anti-human GM-CSF antisera (R&D Systems, Inc., Minneapolis, MN) and the secondary antibody was alkaline phosphatase-conjugated rabbit anti-goat immunoglobulin antisera (Pierce Chemical Company). Western blots were developed using a NBT/BCIP staining kit (Promega Corporation, Madison, WI).

***In Vitro* Bioactivity Assays**

The human TF-1 cell line was obtained from the American Type Culture Collection. The cells were maintained in RPMI 1640 media supplemented with 10% FBS, 50 units/mL penicillin, 50 µg/mL streptomycin and 2 ng/mL recombinant human GM-CSF (*E. coli*-derived; R&D Systems). For bioassays, the TF-1 cells were washed and resuspended at a concentration of 1×10^5 /ml in RPMI 1640 media containing 10% FBS, 50 units/mL penicillin and 50 µg/mL streptomycin (assay media). Fifty µL (5×10^3 cells) of the cell suspension was aliquotted per test well of a flat bottom 96 well tissue culture plate. Serial dilutions of the protein samples to be tested were prepared in assay media. Serial dilutions of a commercial recombinant human GM-CSF standard (*E. coli*-expressed; R&D Systems) were analyzed in parallel. Fifty µL of the diluted protein samples were added to the test wells and the plates incubated at 37°C in a humidified 5% CO₂ tissue culture incubator. Protein samples were assayed in triplicate wells. After 3 days, 20 µL of CellTiter 96 AQueous One Solution (Promega Corporation) was added to each well and the plates incubated at 37°C in the tissue culture incubator for 1–4 h. Absorbance of the wells was read at 490 nm using a microplate reader.

Pharmacokinetic Experiments

Animal experiments were performed with the approval of BolderPATH's Institutional Animal Care and Use Committee. Groups of three male Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN), weighing 340–370 g each, received a single intravenous injection (lateral tail vein) of Leukine® (sargramostim, a recombinant yeast-derived human GM-CSF containing a R23L amino acid substitution, Schering AG), wild type *E. coli*-expressed GM-CSF prepared by us, or the GM-CSF A3C cysteine analog modified with 10 kDa-, 20 kDa-, or 40 kDa-maleimide PEG, each at a dose of 100 µg protein/kg. At selected time points, blood samples (0.4 mL) were drawn from the rats into EDTA anticoagulant tubes. The blood samples were centrifuged and the plasma samples stored at –80°C. Blood samples were drawn at 0.25, 1, 2, 4, 10, 24, 48, 72, 96, and 120 h post injection. A pre-dose sample was drawn one day prior to injection of the test compounds. Plasma levels of the test proteins were quantitated using human GM-CSF ELISA kits (R & D Systems, Inc.). Serial dilutions of plasma samples from one animal in each test group were analyzed in the *in vitro* bioassay to identify dilutions that fell within the linear range of the ELISA (7.8 to 500 pg/mL). Duplicate samples of appropriate dilutions of plasma samples from all rats were then analyzed in the ELISA. A subcutaneous pharmacokinetic experiment was performed in the same manner except that the protein samples were injected subcutaneously into the lateral sides of the rats. Blood samples were drawn at 0.16, 0.5, 1, 2, 4, 10, 24, 48, 72, 96 and 120 h post-injection. Pharmacokinetic parameters were analyzed using the WinNonlin software package (Pharsight, Inc., MountainView, CA).

RESULTS

Expression of GM-CSF in *E. coli* Using the STII Signal Sequence

Human GM-CSF was engineered to be secreted to the *E. coli* periplasm by fusing the mature coding region of GM-CSF to the *E. coli* enterotoxin STII signal sequence. The protein was cloned under control of the *tac* promoter and expression of the protein induced by addition of

IPTG to the cultures. SDS-PAGE analysis of the overnight induced cultures showed expression of a new 14 kDa protein, which is consistent with mass of mature GM-CSF. These data suggested that the STII leader peptide had been removed, which was confirmed by the N-terminal sequencing studies presented below. Although GM-CSF appeared to be properly processed and secreted, the recombinant protein was largely insoluble. The insoluble protein was refolded using the procedure described in **Experimental Procedures** and purified from the refold mixture by Q-Sepharose and Phenyl-Sepharose column chromatography. N-terminal sequencing of purified GM-CSF yielded the sequence APARSPS, which matches the first seven amino acids of mature human GM-CSF (18,19), and indicates that the N-terminus is correctly processed. Purified GM-CSF co-migrated with an *E. coli*-expressed human GM-CSF standard under reducing and non-reducing conditions (Figure 1). Western blots were used for this analysis because the commercial GM-CSF standard is formulated with a protein carrier. Both recombinant GM-CSF proteins exhibited a mobility shift to a higher apparent molecular weight under reducing conditions, presumably due to reduction of the two native intramolecular disulfide bonds, C54-C96 and C88-C121 (20). Bioactivities of the proteins were compared in an *in vitro* cell proliferation assay using human TF-1 cells. Our purified GM-CSF and the GM-CSF standard stimulated proliferation of human TF-1 cells to the same extent and had similar mean EC₅₀s of 105 and 97 pg/mL, respectively (Table 1).

Expression and *In Vitro* Bioactivities of GM-CSF Cysteine Analogs

Human GM-CSF has a compact globular structure, comprising four alpha helices joined by loops (10,20,21). The four alpha helices are termed A-D beginning from the N-terminus of the protein. The loop regions are referred to by the helices they join, e.g., the A-B loop joins helices A and B. We constructed 13 GM-CSF cysteine analogs, primarily in the non-helical regions of the protein, which lie away from the identified receptor binding domains. Five cysteine analogs were constructed in the amino-terminal region preceding helix A [*1C (the addition of a cysteine residue preceding the natural amino terminus), A1C, A3C, S5C and S7C]; one cysteine analog was constructed in the B-C loop [S69C]; three cysteine analogs were constructed in the C-D loop [E93C, T94C, and T102C]; and three cysteine analogs were constructed in the carboxy-terminal region following helix D [V125C, Q126C and *128C (the addition of a cysteine residue to the natural carboxy-terminus)]. We also constructed one cysteine analog at an N-linked glycosylation site (N27C), which is the last amino acid in helix A. The GM-CSF cysteine analogs were expressed in *E. coli* using the STII signal sequence. Expression studies indicated that the cysteine analogs appeared to be correctly processed but largely insoluble. The cysteine analogs were refolded and purified using the protocols described for wild type GM-CSF. Recoveries of the purified GM-CSF cysteine analogs ranged from 0.4 to 2.2. mg/400 mL of culture compared to 2.6 mg/400 mL of culture for wild type GM-CSF. The cysteine analogs were recovered predominantly as monomers that co-migrated with wild type GM-CSF under reducing and non-reducing SDS-PAGE conditions. Non-reducing SDS-PAGE analysis of the 13 purified cysteine analogs is shown in Figure 2A. Mean EC₅₀s of the 13 cysteine analogs ranged from 80 – 134 pg/mL in the TF-1 cell proliferation assay and were similar to the EC₅₀ of wild type GM-CSF (Table 1). Dose response curves for representative cysteine analogs are shown in Figure 2B.

Preparation and *In Vitro* Characterization of PEGylated GM-CSF Cysteine Analogs

The 13 purified GM-CSF cysteine analogs were reacted with a 5kDa maleimide-PEG under partial reducing conditions, as described in **Experimental Procedures**. These conditions yielded significant amounts of monoPEGylated protein for 10 of the cysteine analogs. No di- or tri-PEGylated proteins were detected for any of the cysteine analogs. PEGylated GM-CSF cysteine analogs were purified from non-PEGylated protein by Q-Sepharose column chromatography (Figure 3A). Figure 3B, lane 3, shows non-reducing SDS-PAGE analysis of the PEGylation reaction for a representative cysteine analog, S7C. The only visible PEGylated

species is the PEG-S7C monomer, which migrates with an apparent molecular mass of 26 kDa. Fractionation of the S7C PEGylation reaction mixture by Q-Sepharose column chromatography yielded two major peaks. SDS-PAGE analysis of the column fractions indicated that the early eluting peak was mono-PEGylated S7C, and the later eluting peak was unmodified S7C protein (Fig. 3B). Fractions enriched for the mono-PEGylated S7C protein were pooled. The other GM-CSF cysteine analogs were PEGylated and purified by the same protocol. PEGylation efficiencies for the cysteine analogs were estimated from the peak areas of the Q-Sepharose column chromatograms and ranged from 11% (T102C) to 89% (S7C), with most analogs being in the 40–70% range (Table 2). No PEGylated products were observed for the three cysteine analogs near the C-terminus of the protein, V125C, Q126C or *128C using these conditions. Wild type GM-CSF did not PEGylate under the conditions used to PEGylate the cysteine analogs (Figure 3C), which strongly suggests that the site of PEGylation in the cysteine analogs is the newly introduced cysteine residue and not one of the four native cysteine residues.

Non-reducing SDS-PAGE analysis of the 10 purified PEGylated cysteine analogs is shown in Figure 4A. The PEGylated proteins displayed similar apparent molecular masses (26 kDa) by non-reducing SDS-PAGE, except for the PEG-E93C and PEG-T94C analogs, which displayed slightly smaller apparent molecular masses. The reason for this difference is unknown, although it may be significant that these analogs are adjacent residues in the C–D loop. Sufficient quantities of seven cysteine analogs (*-1C, A1C, A3C, S5C, S7C, S69C and E93C) modified with a 5 kDa-PEG were purified for accurate protein concentration and bioactivity measurements. Mean EC₅₀s for the PEG-GM-CSF cysteine analogs ranged from 80 – 123 pg/mL in the TF-1 cell proliferation assay and were similar to the EC₅₀ of wild type GM-CSF (Table 2). Dose- response curves for representative PEGylated GM-CSF cysteine analogs are shown in Figure 4.

The A3C protein was PEGylated with larger 10 kDa- and 20 kDa-maleimide PEGs, and a 40 kDa-branched maleimide PEG, and the PEGylated proteins purified by Q-Sepharose column chromatography as described above. SDS-PAGE analysis of the purified PEG-A3C proteins is shown in Figure 5A. Bioactivity experiments yielded mean EC₅₀s of 78, 113 and 300 pg/mL for the 10 kDa-, 20 kDa-, and 40 kDa-PEG-A3C proteins in the TF-1 cell proliferation assay (Figure 5B and Table 2).

Pharmacokinetic Experiments With PEGylated A3C Proteins

Circulating half-lives of the A3C protein modified with 10 kDa- and 20 kDa-PEGs were determined by ELISA following intravenous administration to rats (Figure 6A). A commercial yeast-derived GM-CSF analog (Leukine[®]) and our *E. coli*-derived wild type GM-CSF were analyzed in parallel. Blood levels of the proteins were measured from 0.25 h to 120 h post-injection. Both Leukine[®] and wild type GM-CSF cleared rapidly from the rats and were undetectable 24h post-injection. Terminal half-lives for Leukine[®] and wild type GM-CSF were 0.9 h and 1h, respectively. In contrast, the PEGylated A3C proteins possessed significantly longer circulating half-lives. Both the initial distribution phase and the terminal half-life phase were longer for the PEGylated A3C proteins than for the wild type GM-CSF proteins. Terminal half-lives for 10 kDa- and 20 kDa-PEG-A3C proteins were 19 h and 22 h, respectively. In a separate experiment the terminal half-life of the 40 kDa-PEG-A3C was determined to be 37 h following intravenous administration to rats (data not shown).

Half-lives of Leukine[®] and the 10 kDa-, 20 kDa- and 40 kDa-PEG-A3C proteins also were determined following subcutaneous administration to rats. Blood levels of the proteins were measured from 0.16 h to 120 h post-injection and are shown in Figure 6B. Leukine[®] reached maximum blood levels between 30 and 60 min post-injection and decreased by greater than 10,000-fold to undetectable levels by 24 h post-injection. The terminal half-life was 1 h. In

contrast, blood levels of the 10 kDa-, 20 kDa-, and 40 kDa-PEG-A3C proteins reached maximum levels at 10, 24, and 48 h post-injection, respectively, indicating that the PEGylated proteins are absorbed more slowly than Leukine[®] from the injection site. Terminal half-lives for the proteins were 14 h (10 kDa-PEG-A3C), 23 h (20 kDa-PEG-A3C) and 47 h (40 kDa-PEG-A3C).

DISCUSSION

We found that wild type human GM-CSF and human GM-CSF cysteine analogs can be secreted efficiently into the *E. coli* periplasm using the *E. coli* STII signal sequence. Although the STII signal sequence is correctly processed, the secreted proteins are insoluble and must be refolded into a biologically active form. Refolded GM-CSF co-migrates with intracellular expressed *E. coli* GM-CSF and has a comparable specific activity in an *in vitro* cell proliferation assay using human TF-1 cells. Similar results were obtained using the bacterial ompA signal sequence to secrete human GM-CSF into the *E. coli* periplasm (22,23).

To identify sites in GM-CSF where a cysteine residue can be introduced and PEGylated without significantly affecting biological activity of the protein, we constructed a series of cysteine analogs in regions of the protein believed to lie away from the major receptor binding sites, which have been localized to helices A, C and D (9,10,24–27). The 13 GM-CSF cysteine analogs analyzed possessed *in vitro* biological activities comparable to wild-type GM-CSF. The large number of highly active GM-CSF cysteine analogs identified was somewhat unexpected, but probably biased by our choice of mutation sites. These data provide support for the notion that the regions of GM-CSF chosen for mutagenesis (the region preceding helix A, the B-C loop, the C-D loop, the region following helix D, and the N27 glycosylation site at the C-terminal end of helix A) are largely nonessential for proper *in vitro* biological activity of GM-CSF. The nonessential nature of these regions was explored further by attaching a large polymer, PEG, to the proteins at the cysteine residues introduced into these regions. All of the cysteine analogs, except those analogs located near the C-terminus of the protein, reacted with a maleimide-PEG using our standard reaction conditions, yielding monoPEGylated proteins. Seven of the monoPEGylated proteins were purified and their *in vitro* biological activities found to be similar to that of wild type GM-CSF. These data strongly suggest that the amino acids analyzed (*-1, A1, A3, S5, S7, S69 and E93) and the regions in which they are located (the region preceding helix A, the B-C loop and the C-D loop) are largely nonessential for *in vitro* biological activity of GM-CSF. Most of the *in vitro* bioactivity measurements for the GM-CSF cysteine analogs and PEGylated GM-CSF cysteine analogs were obtained from single preparations of the proteins. It remains to be determined whether the minor differences observed in *in vitro* bioactivities of the proteins are due to intrinsic differences in bioactivities of the proteins or to other variables associated with purification, PEGylation and quantitation of the proteins.

The three cysteine analogs introduced near the C-terminus of the protein, V125C, Q126C or *128C, did not PEGylate using our standard PEGylation conditions. Based upon the published structure of GM-CSF (20,21), these residues appear to be surface-exposed and should be accessible to react with the PEG reagents. The C-terminal region of GM-CSF contains a short loop held together by a disulfide bond between residues C88 and C121 (20). It is possible that this disulfide loop, or another feature of this region, alters the local structure of the C-terminal region in a way that makes these residues unreactive with the PEG reagent. Other reaction conditions or use of other types of cysteine-reactive PEG reagents may permit modification of these residues. Studies with human growth hormone revealed that only selected surface-exposed lysine residues are modified by amine-reactive PEG reagents (28). These data, and those described here, indicate that surface accessibility is only one factor determining whether a particular amino acid residue will react with a PEG reagent.

With the A3C analog we explored the effect of different size PEGs on *in vitro* biological activity and circulating half-life of the protein. We found that modification of the A3C protein with linear 5-, 10- and 20-kDa-PEG reagents did not substantially affect *in vitro* biological activity of the protein, as measured by EC₅₀ values in the TF-1 cell proliferation assay. Only when the protein was modified with a large, 40 kDa-branched maleimide PEG did we detect an obvious decrease in *in vitro* bioactivity, but even this decrease was moderate (3-fold). Although this result is based upon a single preparation of the 40 kDa-PEG A3C protein, it is consistent with results by us (29, and unpublished results) and others (8) showing greater loss of *in vitro* bioactivity when proteins are modified with 40 kDa-branched PEGs than with smaller, linear PEGs. Pharmacokinetic experiments demonstrated that the PEGylated GM-CSF (A3C) proteins have up to 47-fold longer circulating half-lives than unmodified GM-CSF proteins in rats. Circulating half-lives of the proteins correlated with the size of the PEG molecule, similar to what has been reported for other PEGylated proteins (6). Human GM-CSF is inactive in rodents due to a failure to bind rodent GM-CSF receptors (19). Since receptor-mediated endocytosis is one mechanism for promoting *in vivo* clearance of hematopoietic factors (30), the pharmacokinetic parameters obtained for the PEG-A3C protein in rats probably reflect primarily clearance of the protein by kidney filtration. The failure of human GM-CSF to function in rodents precluded us from measuring biological activities of the PEG-A3C proteins in these experiments. In studies to be reported elsewhere, we find that a site-specific PEGylated mouse GM-CSF homologue of human GM-CSF (A3C) is much more potent than mouse GM-CSF at stimulating granulopoiesis in rats (Lee, Rosendahl, Hughes, Cox and Doherty, manuscript in preparation). Other researchers reported that a PEGylated murine GM-CSF protein was more effective than unPEGylated murine GM-CSF at expanding and stimulating differentiation of dendritic cells in mice (31,32).

The PEG-GM-CSF cysteine analogs described here have significantly different structural and biological properties than previously described non-specific, amine-PEGylated GM-CSF proteins (11,33). Amine-PEGylated GM-CSF comprises a heterogeneous mixture of different molecular weight PEG-GM-CSF species modified at multiple amine groups and containing different numbers of PEG molecules. The various amine-PEGylated GM-CSF species could not be purified from each other or from non-PEGylated GM-CSF by conventional chromatography procedures, which prevented specific activity measurements of the various isoforms from being determined. An amine-PEGylated murine GM-CSF protein in which PEG is preferentially attached to the N-terminus of the protein has been described (31,32), but *in vitro* bioactivity and physical properties of the protein have not been reported. By contrast, each of the PEG-GM-CSF cysteine analogs described here is homogeneously modified at a single unique residue, can be readily purified from non-PEGylated GM-CSF by ion-exchange chromatography and possesses a specific activity in *in vitro* bioassays similar to wild type GM-CSF. The high potency and long circulating half-lives of these site-specific PEGylated GM-CSF analogs warrant further investigation of their potential utility as human therapeutics.

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Figure 1. SDS-PAGE analysis of purified wild type GM-CSF. Western blot of a wild type GM-CSF standard (lanes 1 and 3; R & D Systems, Inc.) and our purified wild type GM-CSF (lanes 2 and 4) following non-reducing (lanes 1 and 2) and reducing (lanes 3 and 4) SDS-PAGE. Positions of molecular weight standards are shown to the left.

Figure 2.

SDS-PAGE analysis and *in vitro* bioactivities of purified wild type GM-CSF and GM-CSF cysteine analogs. Panel A shows non-reducing SDS-PAGE analysis of column pools of the purified GM-CSF cysteine analogs. Lane 1, molecular weight standards; Lane 2, wild type GM-CSF; Lane 3, *-1C; Lane 4, A1C; Lane 5, A3C; Lane 6, S5C; Lane 7, S7C; Lane 8, N27C; Lane 9, S69C; Lane 10, E93C; Lane 11, T94C; Lane 12, T102C; Lane 13, V125C; Lane 14, Q126C; and Lane 15, *128C. Proteins were stained with Coomassie Blue. Panel B shows dose response curves for our wild type GM-CSF and representative GM-CSF cysteine analogs for stimulating proliferation of TF-1 cells. Data are means of triplicate wells $\pm$ SD from representative experiments. SD were generally less than 5% of the means.

Figure 3.

Purification of the PEGylated GM-CSF S7C protein and inability of wild type GM-CSF to react with maleimide-PEG. Panel A shows Q-Sepharose column chromatography of the S7C PEGylation reaction products. Panel B shows non-reducing SDS-PAGE analysis of the Q-Sepharose column fractions. Lane 1, molecular weight standards; Lane 2, S7C; Lane 3, S7C PEGylation reaction products; Lanes 4–12 represent Q-Sepharose column fractions 30–38. Fractions 30–34 (Lanes 4–8) contain predominantly PEGylated S7C. Fractions 36–38 (Lanes 10–12) contain unreacted S7C. Panel C is non-reducing SDS-PAGE analysis of wild type GM-CSF PEGylation reaction products. Lane 1, molecular weight standards; Lane 2, wild type

GM-CSF; Lane 3, GM-CSF +5 kDa-maleimide-PEG; Lane 4, GM-CSF + 5 kDa-maleimide-PEG + TCEP.

Figure 4.

SDS-PAGE analysis and *in vitro* bioactivities of purified 5 kDa-maleimide PEG-GM-CSF cysteine analogs. Panel A shows non-reducing SDS-PAGE analysis of column pools of purified PEG-GM-CSF cysteine analogs. Lane 1, molecular weight standards; Lane 2, wild type GM-CSF; Lane 3, 5 kDa-PEG-*1C; Lane 4, 5 kDa -PEG-A1C; Lane 5, 5 kDa -PEG-A3C; Lane 6, 5 kDa -PEG-S5C; Lane 7, 5 kDa -PEG-S7C; Lane 8, 5 kDa -PEG-N27C; Lane 9, 5 kDa -PEG-S69C; Lane 10, 5 kDa -PEG-E93C; Lane 11, 5 kDa -PEG-T94C; Lane 12, 5 kDa -PEG-T102C. Proteins were stained with Coomassie Blue. Panel B shows dose-response curves for 5 kDa-PEG GM-CSF cysteine analogs and our wild type GM-CSF for stimulating proliferation of TF-1 cells. Data are means of triplicate wells $\pm$ SD from representative experiments. SD were generally less than 5% of the means.

Figure 5. SDS-PAGE analysis and *in vitro* bioactivities of the GM-CSF A3C protein modified with different size PEGs. Panel A shows non-reducing SDS-PAGE analysis of the purified proteins. Lane 1, molecular weight standards; Lane 2, wild type GM-CSF; Lanes 3–6 are column pools of the A3C protein modified with 5 kDa-, 10 kDa-, 20 kDa- and 40 kDa-maleimide PEGs, respectively. Panel B shows dose response curves for the 10 kDa-, 20 kDa-, and 40 kDa-PEG A3C proteins for stimulating proliferation of TF-1 cells. SD were generally less than 5% of the means.

Figure 6.

Circulating plasma levels of Leukine®, *E. coli*-derived wild type GM-CSF and the GM-CSF A3C protein modified with different size PEGs following (A) intravenous or (B) subcutaneous administration in rats. Data are means $\pm$ SD for three rats per group and were measured using human GM-CSF ELISAs. SD were generally less than 25% of the means. The 120 h time point samples for the 10 kDa-PEG-A3C protein in the subcutaneous study shown in (B) were lost and could not be analyzed.

Table 1
In Vitro Bioactivities of GM-CSF and GM-CSF Cysteine Analogs

GM-CSF protein	added cysteine location ^a	EC ₅₀ (pg/ml) ^b
GM-CSF ^c	-	97 ± 5
GM-CSF ^d	-	105 ± 8
*-1C	N-terminus	111 ± 5
A1C	N-terminus	80 ± 0
A3C	preceding helix A	108 ± 3
S5C	preceding helix A	125 ± 6
S7C	preceding helix A	106 ± 6
N27C	helix A	134 ± 30
S69C	B-C loop	103 ± 10
E93C	C-D loop	103 ± 14
T94C	C-D loop	120 ± 4
T102C	C-D loop	114 ± 3
V125C	following helix D	110 ± 0
Q126C	following helix D	126 ± 9
*128C	C-terminus	124 ± 3

^aBased on the GM-CSF structure in reference 20.

^bMean ± SD for at least 4 assays per protein in the TF-1 cell proliferation assay.

^cWild type GM-CSF standard (*E. coli*-derived, R&D Systems, Inc.).

^dWild type GM-CSF prepared by us.

Table 2
PEGylation Efficiencies and *In Vitro* Bioactivities of PEGylated GM-CSF Cysteine Analogs

GM-CSF analog	PEG size	PEGylation efficiency (%) ^a	EC ₅₀ (pg/ml) ^b
*-1C	5 kDa	45, 55	96 ± 5
A1C	5 kDa	55	115 ± 4
A3C	5 kDa	62	106 ± 3
A3C	10 kDa	70	78 ± 3
A3C	20 kDa	75	113 ± 5
A3C	40 kDa	70, 70	300 ± 50
S5C	5 kDa	76	80 ± 11
S7C	5 kDa	50, 70, 89	123 ± 15
S69C	5 kDa	71	88 ± 6
E93C	5 kDa	62	86 ± 5

^aPercent PEGylated protein estimated by anion-exchange column chromatography of PEGylation reactions. Numbers represent results from individual PEGylation reactions.

^bMean ± SD of at least 4 assays for each protein in the TF-1 cell proliferation assay.