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Improved Conjugation of Cytokines Using High Molecular Weight Poly(ethylene glycol): PEG-GM-CSF as a Prototype

Mark G. P. Saifer,[a] L. David Williams,[a] Merry R. Sherman,[a] John A. French,[a] Larry W. Kwak[b] and Joost J. Oppenheim[b]

[a]Mountain View Pharmaceuticals, Inc., San Carlos, CA 94070
[b]National Cancer Institute, Frederick, MD 21702

Introduction

For several enzymes, the increased half-life in plasma that results from conjugation of many strands of 5-kDa poly(ethylene glycol) (PEG) more than compensates for the significant loss of enzymatic activity observed *in vitro*[1]. On the other hand, small receptor-binding proteins, such as cytokines and peptide hormones, differ from most enzymes in having fewer available sites for attachment of PEG and losing substantial receptor-binding activity as a result of coupling many strands of PEG. As a consequence, success in enhancing cytokine potency by covalent coupling to PEG (PEGylation) has been limited[2-4]. Studies of PEG conjugates of interleukin-2[5], granulocyte-colony stimulating factor (G-CSF)[6], tumor necrosis factor[7] and human growth hormone[8] have demonstrated that the plasma persistence of these small proteins increases with the coupling of additional strands of 5- or 10-kDa monomethoxyPEG (mPEG). Such studies have also revealed that each additional strand of mPEG coupled to the protein decreases its receptor-binding activity *in vitro*[6-9]. Thus, in contrast to the results obtained with many enzymes, the most extensively PEGylated preparations of cytokines and peptide hormones are not the most potent *in vivo*[7,8]. Consistent with these observations, Satake-Ishikawa *et al.*[6] found that when G-CSF was coupled to two or three strands of 10-kDa mPEG, it displayed higher potency and longer duration of action *in vivo* than when it was coupled to five strands of 5-kDa mPEG.

The current studies were undertaken to assess the effects of PEGylating the pluripotent cytokine, granulocyte-macrophage colony-stimulating factor (GM-CSF)[2,4], with a small number of strands of mPEG of high molecular weight (≥18 kDa). For example, conjugates with a single strand of 20- or 40-kDa mPEG were expected to have similar molecular radii, and hence similar rates of renal clearance, to conjugates containing an average of four or eight strands of 5-kDa mPEG, respectively. On the other hand, the mono-PEGylated conjugates were expected to be more homogeneous and to retain more receptor-binding activity than preparations that were extensively substituted with 5-kDa mPEG.

Experimental Methods

Recombinant murine GM-CSF (rmuGM-CSF) was from PeproTech (Rocky Hill, NJ) and recombinant human GM-CSF (rhuGM-CSF) was a generous gift from Immunex Corporation (Seattle, WA). MonomethoxyPEGs of various molecular weights (18, 36, 42, and 60 kDa) and PEG standards for column calibration were from Polymer Laboratories, Ltd. (Church Stretton, Shropshire, UK). MonomethoxyPEG aldehydes were from Polymer Laboratories or from Shearwater Polymers (Huntsville, AL). Sodium cyanoborohydride and most other reagents were from Aldrich (Milwaukee, WI). The succinimidyl carbonate derivative of 36-kDa mPEG (SC-PEG) was prepared and coupled to rmuGM-CSF by modifications of published methods[10]. The method used for coupling PEG aldehydes to rhuGM-CSF was adapted from Friedman *et al.*[11] Samples of PEGylation reaction mixtures were characterized by size-exclusion chromatography in phosphate-buffered saline on a TSK 5000 PW_{XL} column or on TSK 4000 PW_{XL} and TSK 2500 PW_{XL} columns in series (TosoHaas, Montgomeryville, PA). Elution profiles were analyzed using the program PeakFit (Jandel Scientific, San Rafael, CA). Methods for *in vivo* testing in mice were adapted from those of Metcalf *et al.*[12]

Results

Incubation with a six-fold molar excess of 42-kDa mPEG aldehyde and $NaBH_3CN$ at 0-4°C converted rhuGM-CSF to PEG-GM-CSF conjugates with a predominance of one mPEG strand after one day and a predominance of two mPEG strands after 14 days (Fig. 1). In the absence of a reducing agent, only traces of PEG_1-GM-CSF were detected after 14 days. During incubation of the reaction mixture illustrated in Fig. 1 for two weeks, the PEG_1-GM-CSF that was initially formed was slowly converted to conjugates containing two, three or four strands of mPEG (Fig. 2).

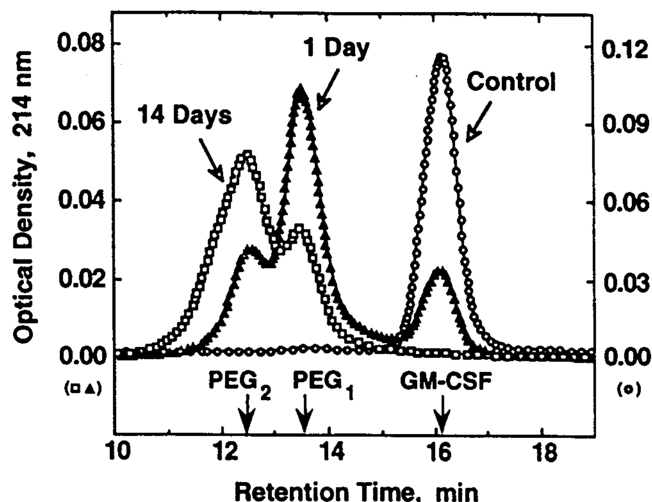

Fig. 1. Size-exclusion HPLC of rhuGM-CSF incubated at 0 to 4°C with a six-fold molar excess of 42-kDa mPEG aldehyde for one day or 14 days in the presence of $NaBH_3CN$, or 14 days in the absence of reducing agent (Control). Retention times are indicated for rhuGM-CSF and for conjugates with one or two strands of mPEG (PEG_1- or PEG_2-GM-CSF).

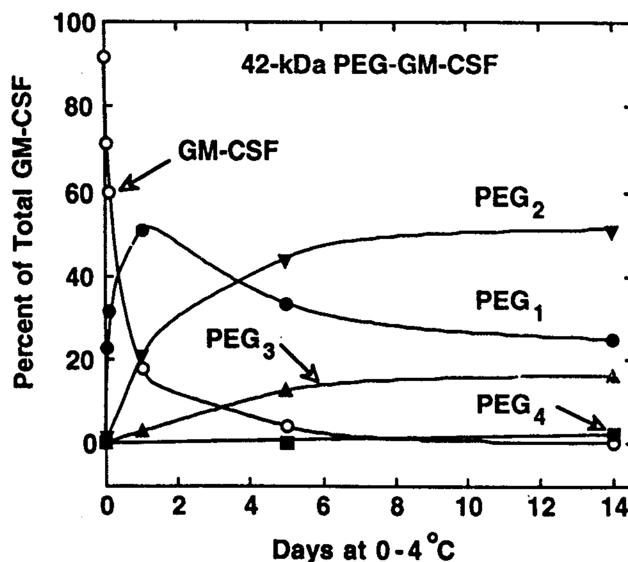

Fig. 2. Sequential formation of conjugates of rhuGM-CSF with one to four strands of 42-kDa mPEG. Size-exclusion HPLC elution patterns were analyzed using the program PeakFit; conjugates containing the indicated number of strands of mPEG (PEG_1- through PEG_4-GM-CSF) were identified using PEG and protein calibration curves.

Injection of mice with a chromatographic pool containing PEG-GM-CSF with one or two strands of 36-kDa mPEG per molecule of rmuGM-CSF induced dramatic, dose-dependent increases in the numbers of eosinophils, neutrophils and monocytes in peripheral blood (Fig. 3) and in the total white blood cell counts (not shown). At the same doses, the unmodified cytokine had negligible effects on the numbers of circulating white blood cells.

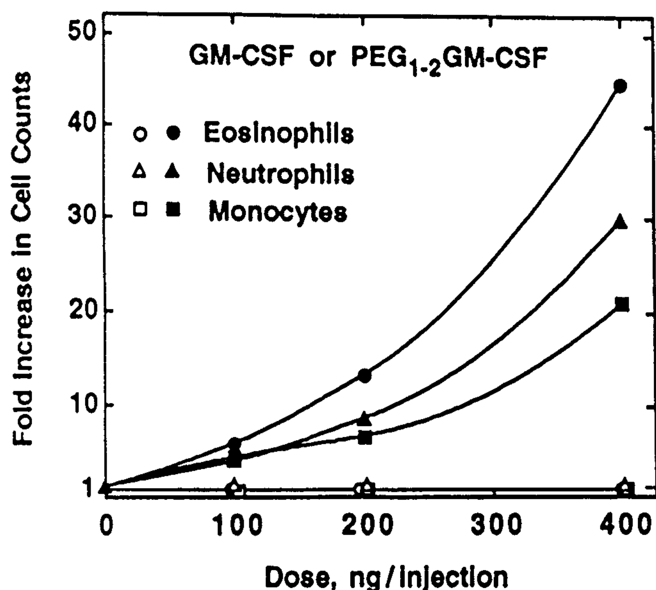

Fig. 3. Hematologic responses in mice treated with rmuGM-CSF (open symbols) or PEG-GM-CSF conjugates containing one to two strands of 36-kDa mPEG (filled symbols). Mice received two injections per day of the indicated doses on Days 1-6 and blood was sampled on Day 7. Increases in counts of each cell type were calculated relative to counts in mice injected with 100 ng GM-CSF.

Discussion

The results presented above are the first to demonstrate enhancement of the *in vivo* potency of GM-CSF by PEGylation. Prior reports of the PEGylation of GM-CSF with 5-kDa mPEG provided evidence of increased half-life in plasma and, at best, conservation of bioactivity *in vitro*[2-4]. The present conjugates have been produced and purified in high yield after the reaction of rmuGM-CSF or rhuGM-CSF with two- to six-fold molar excesses of SC-PEG or PEG aldehyde, respectively. An advantage of coupling high molecular weight PEGs to proteins is the potential for the efficient production of adducts with a specified, low degree of conjugation (see Fig. 1). From the standpoints of both regulatory acceptability and cost-effectiveness, mono- or di-PEGylated conjugates offer substantial advantages over the usual mixtures of multiply-PEGylated species formed when proteins are derivatized with PEGs of lower molecular weight.

Conjugates of cytokines or peptide hormones with a few strands of 5-kDa mPEG have somewhat delayed clearance from plasma[8,13], with half-lives that increase directly with the number of strands of mPEG[5-9]. The most persistent conjugates have hydrodynamic radii that are equal to or larger than that of serum albumin, as determined by size-exclusion chromatography. A single strand of 18-kDa mPEG has a larger hydrodynamic radius than that of serum albumin. Therefore, coupling one strand of mPEG of at least 18 kDa to a cytokine or peptide hormone would be expected to markedly decrease its rate of clearance from the plasma. In contrast, the elimination constant measured for 6-kDa PEG corresponds to a half-life of only five minutes in mice[14]. Consequently, coupling only one or two strands of 5-kDa mPEG to a cytokine or peptide hormone that has a radius similar to that of 5-kDa PEG would produce conjugates that would be smaller than serum albumin and would be cleared from the plasma rather rapidly[5,8]. Thus, only by coupling PEG strands of higher molecular weight is it possible to optimize both the specific activity and the duration of action of small proteins such as cytokines.

These considerations may help to account for the dramatic increase in the *in vivo* potency of GM-CSF observed after the attachment of one or two strands of high molecular weight PEG.

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