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International Journal of Pharmaceutics 261 (2003) 93–103
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

The sustained granulopoietic effect of progenipoietin encapsulated in multivesicular liposomes

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Received 7 February 2003; received in revised form 2 May 2003; accepted 2 May 2003

Abstract

Progenipoietin (ProGP), a dual receptor agonist of fetal liver tyrosine kinase-3 (flt3) and granulocyte colony-stimulating factor (G-CSF) receptors, has been shown to significantly enhance production of both polymorphonuclear leukocytes and dendritic cells (DCs) in the peripheral blood and spleen of mice, when administered as daily s.c. injections for about 10 days. Here, we have successfully designed a sustained-delivery formulation for this novel chimeric protein using multivesicular liposomes (DepoFoam), and studied the effects of changing both the triglyceride and phospholipid composition of the lipid matrix to modulate its delivery profile. Encapsulation of ProGP in these particles led to retention of its structural integrity, and maintenance of its biological activity in vivo. Administration of a single s.c. dose of 1 mg/kg of an optimized DepoProGP formulation in rats, led to significant elevation of absolute neutrophil counts (ANC) that were maintained at levels >10,000 μl^{-1} for 9–11 days, in a reproducible manner. In contrast, administration of the unencapsulated ProGP at the same dose, resulted in elevation of neutrophils by day 1, followed by a quick decline to base line levels by day 3. These data suggest the possibility of administering a single dose of DepoFoam-encapsulated ProGP to improve hematopoietic recovery time after chemotherapy, and for other indications that require multiple daily doses of ProGP.

Keywords: Progenipoietin; Hematopoietic growth factors; Multivesicular liposome; DepoFoam; Sustained granulopoiesis; G-CSF

1. Introduction

Neutrophils constitute the first line of host defense against infections by pathogenic organisms. Granulocyte colony-stimulating factor (G-CSF) enhances both neutrophil production and its phagocytic function, and has been recently explored as an adjunct to antibiotic therapy for bacterial as well as fungal infections in non-neutropenic subjects (Hollingshead and Goa, 1991; Dempke et al., 2000). Homeostasis is maintained through the combined actions of multiple growth factors (Moore, 1991; Metcalf, 1992). Fetal liver tyrosine kinase-3 (flt3) ligand (FL) is a growth factor that acts on primitive progenitor cells (Zeigler et al., 1994), and when combined with other growth

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doi:10.1016/S0378-5173(03)00291-6

factors such as stem cell factor (SCF), G-CSF, or GM-CSF, it markedly enhances the proliferation of multiple hematopoietic lineages (Sudo et al., 1997). Treatment with a combination of FL and G-CSF results in an extensive increase in peripheral blood hematopoietic progenitor cells, neutrophils, and dendritic cells (DCs) in rodents and primates (Molineux et al., 1997; Papayannopoulou et al., 1997)

Progenipoietin (ProGP) molecules (Mr 39,000–48,000) are novel dual receptor agonists that bind and activate flt3 and the G-CSFR, and exhibit superior activity when compared to the combination of FL and G-CSF, or either individual cytokine (Streeter et al., 2001). Mice treated daily for 10 days with ProGP, stimulated increases in a variety of hematopoietic lineages in peripheral blood and spleen. Treatment with ProGP at 40 μg/mouse/day (∼1 mg/kg/day, total dose = 10 mg) yielded significant increases in peripheral blood white blood cells, polymorphonuclear leukocytes, and DCs after 10 days (Streeter et al., 2001). Mobilization of hematopoietic stems cells, progenitor cells, and white blood cells by ProGP has also been shown in non-human primates (MacVittie et al., 1998). In addition to stimulation of neutrophils, ProGP molecules have a potent DC effect, thereby making them attractive candidates for use in ex vivo cancer-vaccine immunotherapy (Fleming et al., 2001; Ishioka et al., 2001; Streeter et al., 2001).

The $t_{1/2}$ of ProGP in circulation, administered s.c., is very short (6.8 h in rats and 5.4 h in rhesus monkeys, data on File). Therefore, the protein needs to be administered daily for 10–12 days for its therapeutic utility in cancer vaccine immunotherapy, as shown recently in a mouse tumor model (Bjorck et al., 2002). ProGP has also been demonstrated, with daily injections for 10 days, to be superior to G-CSF in the prevention of Graft-versus-Host disease induced by allogenic stem cell transplantation (MacDonald et al., 2002). Furthermore, the current dose schedule of G-CSF treatment for chemotherapy-induced neutropenia, involves daily administration for several days until recovery of neutrophil levels. Hence, it would be useful to develop a sustained-delivery formulation of ProGP to meet a convenient dose schedule for these various indications, which would replace several daily doses, and improve patient compliance.

PEGylation (covalent attachment of polyethylene glycol) is one option for providing sustained systemic exposure of growth factors. PEGylated G-CSFs have provided sustained pharmacodynamic (PD) effect (elevated neutrophil levels) in rodents (single dose of 1 mg/kg), although only for 5–6 days (Bowen et al., 1999; Molineux et al., 1999). Further, PEGylation of the protein can sometimes reduce its bioactivity (Bowen et al., 1999). Based on the data from preclinical animal models with ProGP (Bjorck et al., 2002; MacDonald et al., 2002), there is a clear need to improve the duration of its PD effect to about 10 days.

DepoFoam®, a unique multivesicular liposome (MVL), distinct from conventional liposomes in composition, structure and size, is the only class of commercial liposomes that has been demonstrated for depot-delivery of both small molecule and peptide/protein drugs in a sustained manner from days to several weeks (Kim et al., 1983; Katre et al., 1998; Ye et al., 2000; Howell, 2001; Ramprasad et al., 2002). These MVLs are characterized by the presence of a continuous outer bilayer membrane, with numerous internal aqueous compartments that are contiguous and separated by bilayer septums (Kim et al., 1983). Due to their large size (average diameter 10–20 μm), these MVLs are not rapidly cleared by tissue macrophages and can act as a drug-depot, with high encapsulation efficiency, providing slow release of drugs delivered through different routes of administration. Further, these particles have higher mechanical strength and are more stable than traditional liposomes. However, every therapeutic protein has unique physiochemical and structural properties that pose distinct challenges to encapsulation, retention of biological activity and stability, and providing the necessary duration of release utilizing this drug delivery technology. With ProGP, the bioactive form is a non-covalent dimer (Violand et al., 2002) which has to be retained in this state within the encapsulated system, in order to maintain activity. Here, we designed a delivery system for ProGP using MVLs, by both identifying the appropriate aqueous conditions to preserve its biological activity, and by optimizing the lipid (triglyceride and the phospholipid) components, in order to achieve a sustained biological effect that could be maintained for 9–11 days with a single s.c. dose in rats.

2. Materials and methods

2.1. Materials

Recombinant human progenipoietin (ProGP) was expressed in *E. coli* and purified as described earlier (Fleming et al., 2001). The protein was >99% pure as determined by analytical reverse phase-HPLC (RP-HPLC). The bulk protein solution was used either as such, or after diafiltration and concentration in an Amicon filtration unit using 10 mM sodium phosphate buffer, pH 7.5. The concentrated stock protein at 8.45 mg/ml in 10 mM sodium phosphate buffer was stored at 4 °C for 2–3 weeks and used for the DepoFoam formulation studies during that period. Phospholipids, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; $C_{18:1}$ PC), 1,2-dierucoyl-*sn*-glycero-3-phosphocholine (DEPC; $C_{22:1}$ PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG), and the triglyceride, triolein ($C_{18:1}$ TG) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA), while the triglyceride, tricaprylin ($C_{8:0}$ TG), was obtained from Sigma (St. Louis, MO, USA). Cholesterol was supplied by Spectrum Chemical Manufacturing Corp. (Gardena, CA). L-Lysine was procured from Degussa Corp. (Marceau, France), and dextrose (50%) was from McGaw Inc. (Irvine, CA, USA). L-Arginine and glycyl-glycine were obtained from Sigma.

UV absorbance was determined with a U2000 UV-Vis spectrophotometer from Hitachi Instruments Inc. (San Jose, CA). Extinction coefficient of 0.99 mg ml^{-1} cm^{-1} at 280 nm was used to calculate the concentration of the stock ProGP solution. RP-HPLC was performed on a C18 column (Jupiter 250 mm × 4.6 mm, 5 μm particle size with a pore size of 300 Å; Phenomenex, Torrance, CA) using a Hewlett-Packard Model 1100 HPLC system equipped with a Diode Array Detector and a Fluorescent Detector (Hewlett-Packard GmbH, Waldborn, Germany). Light microscopy of the MVL particles was performed using an Olympus (BH-2) microscope (Olympus Optical Co., Tokyo, Japan) and the images recorded photographically. The size distribution of the DepoProGP particles was determined with a Horiba model LA-910 light scattering particle size detector (Horiba Instruments Inc., Irvine, CA).

2.2. Preparation of multivesicular liposome formulations containing ProGP (DepoProGP formulations)

The multivesicular particles containing ProGP were prepared by a two-step water-in-oil-in-water double emulsification process (Katre et al., 1998; Ramprasad et al., 2002). Briefly, the first step is the formation of a water-in-oil emulsion. Six milliliters of a lipid solution made of 19.8 mM PC, 30 mM cholesterol, 4.2 mM DPPG, and 3.75 mM triglyceride in chloroform was emulsified with 5 ml of an aqueous solution (the first aqueous solution) containing 2 mg/ml ProGP in 25 mM arginine-containing buffers (pH varied from 4 to 8) and 2.5–7% (w/v) sucrose to produce a water-in-oil emulsion (the first emulsion). The emulsification conditions were 10,000 rpm for 12 min with an Omni Mixer. A subsequent emulsification (4500 rpm for 2 min) with a second aqueous solution containing 3.2% glucose (w/v) and 40 mM lysine resulted in a water-in-oil-in-water double emulsion (the second emulsion). Chloroform was removed by flushing nitrogen over the surface of the second emulsion at 37 °C. The resulting MVLs were washed with PBS (phosphate-buffered saline) to remove any unencapsulated ProGP, and harvested by centrifugation at 600 × *g*. After washing, the DepoProGP particles were resuspended in PBS. Blank DepoFoam, containing no ProGP in the first aqueous solution, was also prepared using the same process.

2.3. Determination of percent encapsulation, lipocrit, percent free ProGP, particle size distribution, and drug loading

Drug loading, percent encapsulation, and lipocrit were determined as described earlier (Ye et al., 2000). Isopropanol:2N HCl (90:10) solvent was used to extract the protein from the formulations (10-fold dilution in solvent), and this extract was used to determine the amount of encapsulated protein by RP-HPLC analyses. Percent recovery of ProGP is the percent ratio of the amount of drug in the final liposome suspension to the total amount of ProGP used in the first aqueous solution. Lipocrit is analogous to hematocrit, the percent ratio of the pellet volume of MVL particles to the suspension volume. Percent free ProGP is the percent ratio of the amount of ProGP in the supernatant to the

amount of ProGP in the final suspension multiplied by (1 − lipocrit). ProGP loading is the amount of ProGP encapsulated in the particle fraction of the suspension.

2.4. Pharmacodynamic (PD) studies

All formulations were prepared under aseptic conditions and had endotoxin levels <1 endotoxin unit/mg protein, based on a limulus amebocyte assay (Associates of Cape Cod, Inc., Woods Hole, MA). Endotoxins tend to partition into the lipid phase, and therefore, the formulations underwent a procedure to solubilize the lipids with a surfactant, Pyrosperse (Biowhitaker, MD), in order to release the aqueous phase components for the endotoxin testing. The endotoxin assay for DepoFoam suspensions is a validated assay at SkyePharma, and has been accepted by the FDA. Bioburden analysis of the formulations showed <10 cfu/ml of DepoProGP suspension.

PD studies were performed in male Sprague–Dawley rats from Harlan (∼11–12 weeks old). Rats ($n = 4$) were injected s.c. with a single dose of 1 mg/kg of either free ProGP or the DepoProGP formulations in PBS. Blood samples (250–300 μl) were collected into EDTA-coated tubes from unanesthetized animals via the saphenous vein. Blood samples at pre-injection, and at specified time points post injection of test articles, were analyzed for total and differential white blood cell counts at a contract facility (Rabbit and Rodent Diagnostic Associates, San Diego, CA).

3. Results

3.1. DepoProGP formulation characterization

The solubility and stability of ProGP under various conditions, and the stability of the liposomal matrix to prevent leakage of drug, was used as the basis for the formulation of ProGP in the MVL particles. We initially screened DepoProGP formulations using various low pH (4–5) and higher pH (7.8–8.1) first aqueous buffers containing the protein with arg/citrate or arg/gly-gly. The sucrose (osmotic spacer) content was varied from 2.5 to 7%, keeping the protein concentration constant at 1.5 mg/ml. These preliminary screening studies revealed that the higher pH (7.9–8.1) formulations were better in percent drug encapsulation and recovery. Formulations containing 4% sucrose gave slightly higher protein loading than those with 5 or 7% sucrose; and formulations with 2.5%

Table 1
The relative triglyceride and phospholipid % ratios in the formulations that were tested in vivo in rats

Formulation	Tricaprylin ($C_{8:0}$)/triolein ($C_{18:1}$) (mol% ratio)	DEPC ($C_{22:1}$)/DOPC ($C_{18:1}$) (mol% ratio)
1	100/0	100/0
2	90/10	100/0
3	0/100	100/0
4	0/100	25/75
5	0/100	90/10
6	50/50	50/50
7	90/10	90/10

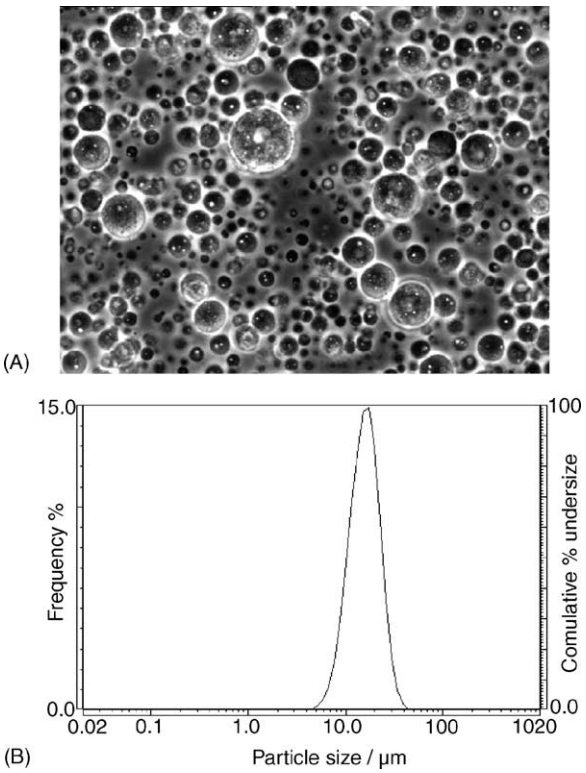

Fig. 1. (A) Light micrograph of DepoProGP particles from a representative formulation (#6 described in Table 1) at 400× magnification. (B) Particle size distribution of DepoProGP particles (formulation #6 described in Table 1) obtained using a Horiba LA910 laser light scanning particle size analyzer. The profile shows a narrow, monomodal distribution of particles with a median size of 14.7 μm.

sucrose had the lowest osmolality and the highest protein loading, but had lower recoveries than the 4% sucrose formulations.

Based on these screening studies, the following 1st aqueous solution was chosen for further formulation development: 1.5–2 mg/ml protein in 25 mM arg/gly-gly buffer, pH 7.9, containing 4% sucrose. Seven formulations with varying lipid compositions were prepared for evaluation of pharmacodynamic response in rats. The rationale for varying the triglyceride and PC compositions was to study their effects on the duration of sustained PD effect in vivo. The phospholipid and triglyceride compositions of these formulations are shown in Table 1. A light microscopic image of a representative MVL formulation containing encapsulated ProGP (formulation #6) is shown in Fig. 1A. The picture shows the smooth, spherical, and multivesicular nature of these unique liposomal particles. All the DepoProGP formulations studied had similar morphology. Fig. 1B shows a volume-weighted size distribution profile of the DepoProGP formulation depicted in Fig. 1A by laser scattering particle size analysis. The profile shows that the formulation had a monomodal particle size distribution, with a median size of 14.7 μm. Over 95% of the DepoProGP particles were in the size range of 8–26 μm, with no particles <4 or >50 μm in size. The particle size distribution of this formulation was typical of the DepoProGP formulations studied and the median size range of these formulations was 14–16 μm.

Drug loading in the formulations ranged from 1.5 to 2.0 mg/ml, with protein recoveries of 80–90%. There was no detectable free ProGP in the DepoProGP suspensions, suggesting that the particles were not leaky after manufacture. Fig. 2A and B show the RP-HPLC profiles of the bulk ProGP and of a DepoProGP

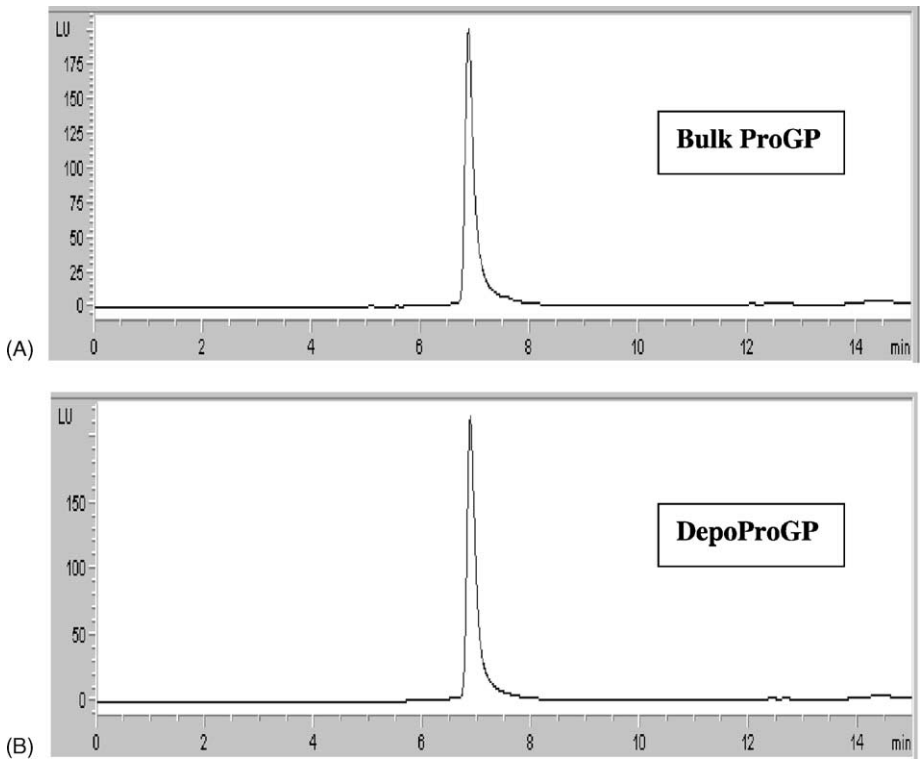

Fig. 2. RP-HPLC analyses comparing bulk ProGP (A) with the ProGP extracted from a representative DepoProGP formulation (B). Extraction of protein from the DepoFoam matrix was performed using acidified IPA. A gradient (30–85% for 8 min) of acetonitrile in water containing 0.1% trifluoroacetic acid was used for elution. The chromatogram shows the fluorescence absorption monitored (at 280 nm excitation, and 340 nm emission) relative to time in minutes.

formulation, respectively. The profile for the DepoProGP formulation (Fig. 2B) shows a single protein peak (340 nm fluorescence emission) at 6.8 min retention time, similar to that of the bulk protein used in these formulations (Fig. 2A). Notably, we did not observe any methionine-oxidation or degradation products that can be resolved using the RP-HPLC methods (Katre et al., 1998), either with unencapsulated ProGP, or in the DepoFoam formulations.

3.2. Pharmacodynamic studies

For the studies described here, the ability of the G-CSF component of ProGP to stimulate the production of neutrophils was monitored. The PD profiles showing the change in absolute neutrophil counts (ANC) after single bolus injections of the seven DepoProGP formulations described in Table 1 are shown in Figs. 3–5. Fig. 3 shows the ANC elevations in rats upon single injections of 1 mg/kg of DepoProGP formulations #1–3 (Table 1), with varying triglyceride ratios, relative to the insignificant change in ANC levels observed with administration of the DepoFoam vehicle alone. The PD profiles of the DepoProGP formulations show that there is a significant increase in ANC levels on day 1 (relative to $t = 0$), dropping thereafter, and returning to baseline levels (represented by blank DepoFoam treated group) by day 3. It can be seen that changing the triglyceride composition from 100% triolein ($C_{18:1}$) (formulation #3) to 90/10% tricaprylin/triolein (formulation #2) or 100% tricaprylin ($C_{8:0}$) (formulation #1) had little impact on the duration of the PD effect. However, there was a difference in the extent of elevation of neutrophils between these formulations, with the 100% tricaprylin formulation showing the highest elevation.

Fig. 4 shows the change in ANC levels in rats after single bolus injections of DepoProGP formulations #4 and 5 (Table 1), with two distinct PC ratios. Formulation #5 containing 90/10 DEPC/DOPC maintained extended levels of elevated ANC above baseline values for approximately 7 days. Formulation #4 containing a higher concentration of DOPC (25/75 DEPC/DOPC) had a very high elevation of neutrophil counts on day 1, with a rapid decline by day 3. Formulations containing intermediate ratios of DEPC and DOPC were

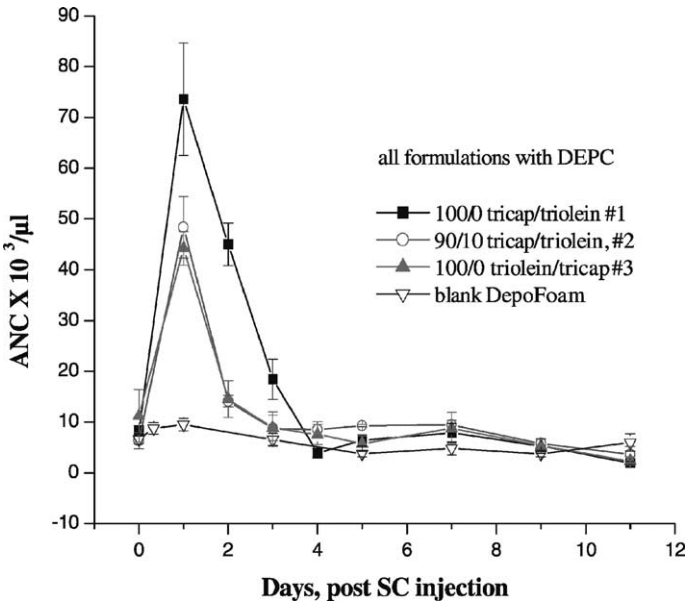

Fig. 3. Effect of variation of triglyceride composition on the PD profiles (S.E.M., $n = 4$) of various DepoProGP formulations. Formulations #1–3 listed in Table 1, containing different ratios of tricaprylin ($C_{8:0}$) and triolein ($C_{18:1}$), were injected s.c. at a single dose of 1 mg/kg into male Sprague–Dawley rats. Blank DepoFoam was injected at the same volume as the DepoProGP formulations. Whole blood was collected into EDTA tubes at various indicated time periods, and analyzed for absolute neutrophil counts.

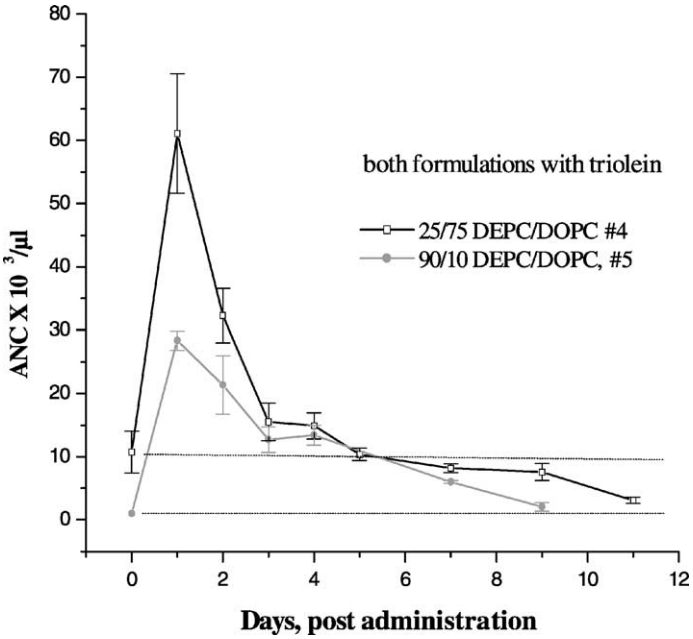

Fig. 4. Effect of variation of phospholipid composition on the PD profiles (S.E.M., $n = 4$) of various DepoProGP formulations. Formulations #4 and 5 listed in Table 1, containing different ratios of DEPC ($C_{22:1}$) and DOPC ($C_{18:1}$) were injected s.c. at a single dose of 1 mg/kg into male Sprague–Dawley rats. Whole blood was collected at indicated time periods and analyzed for ANC. The dashed lines represent the ANC values at $t = 0$ for the individual groups of rats receiving two separate formulations.

tested, but did not show any significant improvement in extending the duration of PD effect.

Based on the results from these two studies, we next varied both the triglyceride and the PC ratios in the DepoProGP formulations to determine the ideal combination of PC and triglyceride components needed to obtain the desired duration of the PD effect of ∼10 days. Fig. 5 shows the ANC levels in rats after single injections of DepoProGP formulations #6 and 7 (Table 1), with two distinct variations in both the triglyceride and PC ratios. The profile obtained with administration of unencapsulated ProGP at the same dose of 1 mg/kg demonstrates that the PD effect lasts for only ∼2 days. In contrast, the DepoProGP formulations with variation in both triglyceride and PC showed sustained elevation of ANC levels for 9 days. Further, formulation #6 (containing equimolar ratios of tricaprylin/triolein, and DEPC/DOPC, respectively) had the best PD profile, showing an increase in ANC levels by day 1 and a maintenance of ANC over 10,000 μl^{-1} for at least 9 days, which is significantly higher than the pre-treatment levels of ∼4000 μl^{-1}. Fig. 6 demonstrates reproducibility of the sustained PD effect (9–11 days of elevated ANC) obtained with three different batches of the optimized DepoProGP formulation (Table 1, formulation #6). In Fig. 6, with all three batches of the optimized DepoProGP formulation in rats, a slight decrease in the elevated neutrophil levels was observed on day 3. Despite the trough on day 3, the neutrophils were elevated over 10,000 μl^{-1} for 9–11 days in a reproducible manner.

4. Discussion

The encapsulation of protein therapeutics in delivery vehicles poses many challenges, namely, the maintenance of structural integrity of the encapsulated protein to preserve its bioactivity and stability, and encapsulation of sufficient amount in order to provide sustained and reproducible pharmacological effects with a single injection. The RP-HPLC data on DepoProGP suggest that the integrity of the protein was retained after encapsulation in DepoFoam, which is

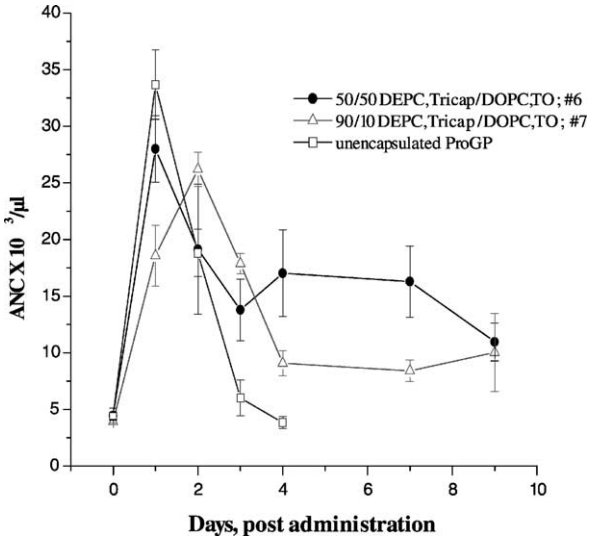

Fig. 5. Effect of variation of both triglyceride and phospholipid composition on the PD profiles (S.E.M., $n = 4$) of DepoProGP formulations. Formulations #6 and 7 listed in Table 1, containing different ratios of tricaprylin/triolein and DEPC/DOPC, were injected s.c. at a single dose of 1 mg/kg into male Sprague–Dawley rats. Unencapsulated ProGP in PBS was also injected at the same dose as the DepoProGP formulations. Whole blood was collected at indicated time periods and analyzed for ANC.

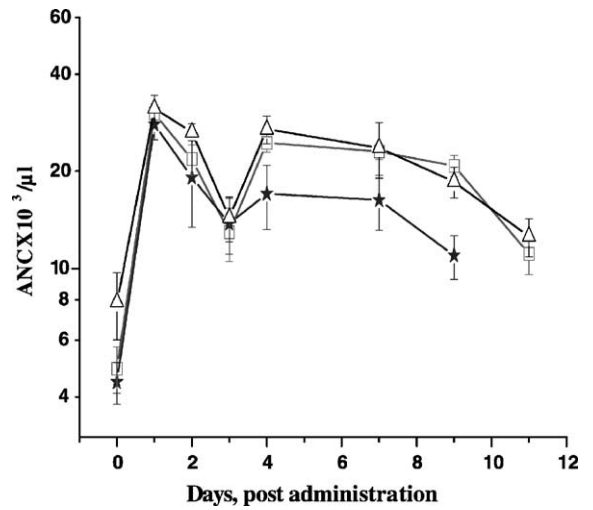

Fig. 6. Reproducibility of PD profiles (shown as the change in ANC), (S.E.M., $n = 4$) of three batches of DepoProGP formulation #6 (Table 1) containing equimolar ratios of tricaprylin/triolein, and DEPC/DOPC, respectively. The formulations were injected s.c. at a single dose of 1 mg/kg into male Sprague–Dawley rats. Whole blood was collected and analyzed for ANC at the indicated time points.

consistent with retention of biological activity in the animal studies. The data from the PD studies showed that a single s.c. dose of 1 mg/kg of DepoProGP formulation #6 (containing equimolar ratios of DEPC and DOPC, and equimolar ratios of triolein and tricaprylin), resulted in elevated neutrophil counts in rats by day 1, with levels >10,000 μl^{-1} being maintained for at least 9–11 days (Figs. 5 and 6). In contrast, a single bolus injection of the unencapsulated protein results in elevated circulating neutrophil levels for only 2 days. Thus, we were able to encapsulate sufficient ProGP in the optimized formulation to achieve a target PD effect for 10 days. Further, the optimized formulation could be manufactured reproducibly to obtain consistent elevation and duration of biological effect. Although stimulation of DC production was not studied here, we anticipate based on the sustained PD effect of elevated ANC, that a single s.c. dose of DepoProGP would be sufficient to yield a significant elevation of DCs by 9 days.

Selection of the aqueous solution containing ProGP for encapsulation is important, because the proper medium maintains the protein bioactivity and stability during storage of the formulations, and perhaps for the duration of release in vivo. Several aqueous conditions were screened in order to identify the appropriate and unique conditions for the encapsulation of ProGP in MVL. Structural studies on DepoFoam particles using transmission electron microscopy (Kim et al., 1983); confocal microscopy utilizing fluorescence-labeled lipids, and ^{13}C nuclear magnetic resonance study (Ellena et al., 1999), have indicated that the internal chambers of the particles meet at phospholipid bilayer junctions that are filled with bulk triglyceride molecules, which impart the unique multivesicular structure. The rate of release of encapsulated drug in DepoFoam can be modulated by changing the molar ratios of a “slow-releasing” triglyceride, and a “fast-releasing” triglyceride (Willis, 1999). Generally, the “slow-releasing” neutral lipid is triolein ($C_{18:1}$), and the “fast-releasing” triglyceride is tricaprylin ($C_{8:0}$) (Willis, 1999; Ye et al., 2000). The rate of drug release can also be modulated by use of phospholipids with varying chain lengths (Willis, 1999). The effect of fatty acid chain length of phospholipids on selective permeability properties of conventional liposomes has been known for a long time (Blok et al., 1975). Furthermore, the duration of drug release from

DepoFoam particles can be also modulated by varying the concentration of the triglyceride, as demonstrated recently (Langston et al., 2003). Based on in vitro release experiments using fluorescent-labeled lipids and aqueous-phase markers, several mechanisms of drug release from these MVLs in vivo have been postulated. These include: (a) diffusion; (b) surface erosion of outer vesicles and presentation of internal vesicles to the tissue milieu; and (c) reorganization of the lipid membranes and redistribution of the lipids, leading to extrusion of the lipids from the particle surface (Willis, unpublished data; Langston et al., 2003). Data presented in this paper and the results presented in earlier reports support the hypothesis that chain lengths of both phospholipid and triglyceride components of the MVLs impact on drug delivery, and can be used as a tool to optimize the in vivo delivery profiles. While one can use these basic guidelines to encapsulate protein therapeutics in DepoFoam for sustained delivery, the actual delivery profiles in vivo can vary with different proteins, and with different aqueous and lipid components. Therefore, screening several formulations with varying lipid compositions in animals is often needed in order to determine the ideal composition that provides the most favorable PD response and duration.

Consistent with the above hypothesis that the triglycerides and PCs with shorter chain fatty acids would release encapsulated drug faster, the results in Figs. 3 and 4 illustrate that DepoProGP formulations #1 and 4 (containing 100% tricaprylin and 75% DOPC, respectively) showed a rapid and high elevation of neutrophils, followed by a rapid decline in ANC. The studies described in this paper demonstrate that modulation of both the triglyceride as well the phospholipid components in the DepoProGP lipid matrix was necessary to obtain the desirable elevation of neutrophils (>10,000 μl^{-1}), and an optimal duration of the biological effect (~10 days). The three- to sevenfold increase in ANC levels that were maintained for 9–11 days with a single dose of 1 mg/kg DepoProGP is consistent with the fold increase in neutrophil levels that would be expected with 1/10th the dose of unencapsulated ProGP administered as a daily injection for 10 days. Therefore, these results suggest that a single dose of DepoProGP may be sufficient to improve the hematopoietic recovery time after each chemotherapy cycle. However, dose-ranging efficacy studies using neutropenic animal models need to be performed to confirm this hypothesis.

The peak (day 1) and trough (day 3) in ANC observed with the optimized DepoProGP formulation shown in Fig. 6 may be explained as follows. The G-CSF component of ProGP primarily stimulates committed myeloid progenitor cells in the bone marrow to divide and mature into neutrophils, which are rapidly mobilized into circulation. Hence, there is a rapid appearance of mature neutrophils in the blood (day 1) after DepoProGP (or ProGP) injection. Once the committed progenitors are depleted, however, it takes some time for replacements to be formed from early pluripotent stem cells that are normally quiescent. During this time, the circulating neutrophils may be dying, resulting in the trough observed on day 3. As more committed progenitors are being produced from early stem cells, there is still sufficient circulating ProGP present in the animals injected with DepoProGP to stimulate production of more mature neutrophils after day 3 which are maintained at elevated levels through 9–11 days.

Sustained PD effects have been observed in rodents with PEGylated versions of G-CSFs (Bowen et al., 1999; Molineux et al., 1999; de Haan et al., 2000), PEGylated leridistim (Hills et al., 2001), and PEGylated ProGP (Finn et al., 2001). All of these forms involve protein modification with polyethylene glycol (PEGylation), to obtain long-circulating variants of the native entities that are cleared slowly from the body. Sustained elevation of neutrophils was observed for 5–6 days in mice after single injections of PEGylated G-CSF at a dose of 1 mg/kg. PEGylation can offer several advantages, which include longer circulation times of the proteins, enhanced solubility and stability, and reduced immunogenicity of recombinant proteins (Katre, 1990, 1993). However, disadvantages of this technology include reduced potency, frequently requiring increased doses, and poor recoveries resulting from the need to isolate monopegylated species (Veronese, 2001).

Optimal efficacy of native ProGP is dependent on its daily administration over 10 days (1 mg/kg/day) (Streeter et al., 2001; Bjorck et al., 2002; MacDonald et al., 2002). A monopegylated formulation of ProGP was recently reported to decrease this dose frequency to once every 3 days in mice, but at a very high dose of 300 μg/mouse (~12 mg/kg) per injection (Finn et al.,

2001). In contrast, the results from our study support the conclusion that a single injection of DepoProGP at 1 mg/kg is sufficient to obtain reproducible, elevated neutrophil counts that are sustained for 9–11 days in rats. Although an optimal dose of DepoProGP has not yet been determined, the significantly lower dose needed in rats may be due to several reasons: the bioavailability and potency of ProGP may be higher in rats than in mice; the use of the native protein without chemically modifying it, coupled with the retention of its biological activity in the MVLs; and/or, enhanced efficacy of the molecule originating from its continuous, and slow release from the s.c. administration site. In support of the latter possibility, it has been demonstrated that a very low dose of G-CSF is sufficient to effectively ameliorate neutropenia in non-Hodgkin's lymphoma patients receiving chemotherapy, when administered as a continuous s.c. infusion (Furuya et al., 1995).

Taken together with both its biocompatibility and biodegradability, this unique MVL technology offers a safe and convenient sustained-release platform to deliver growth factors, such as ProGP, for further evaluation in preclinical efficacy models and the ensuing clinical applications.

Acknowledgements

We are grateful to Mario Flores and Maria Esguerra, SkyePharma, for their timely and dedicated assistance with the animal experiments. We would like to thank Melissa Langston, SkyePharma, for her assistance with preparation of some formulations.

References

Bjorck, P., Lie, W.R., Woulfe, S.L., Klein, B.K., Olson, W., Storkus, W.J., 2002. Progenipoietin-generated dendritic cells exhibit anti-tumor efficacy in a therapeutic murine tumor model. Int. J. Cancer 100, 586–591.

Blok, M.C., van der Neut-Kok, E.C., van Deenen, L.L., de Gier, J., 1975. The effect of chain length and lipid phase transitions on the selective permeability properties of liposomes. Biochim. Biophys. Acta 406, 187–196.

Bowen, S., Tare, N., Inoue, T., Yamasaki, M., Okabe, M., Horii, I., Eliason, J.F., 1999. Relationship between molecular mass and duration of activity of polyethylene glycol conjugated granulocyte colony-stimulating factor mutein. Exp. Hematol. 27, 425–432.

de Haan, G., Ausema, A., Wilkens, M., Molineux, G., Dontje, B., 2000. Efficient mobilization of haematopoietic progenitors after a single injection of pegylated recombinant human granulocyte colony-stimulating factor in mouse strains with distinct marrow-cell pool sizes. Br. J. Haematol. 110, 638–646.

Dempke, W., Von Poblozki, A., Grothey, A., Schmoll, H.J., 2000. Human hematopoietic growth factors: old lessons and new perspectives. Anticancer Res. 20, 5155–5164.

Ellena, J.F., Le, M., Cafiso, D.S., Solis, R.M., Langston, M., Sankaram, M.B., 1999. Distribution of phospholipids and triglycerides in multivesicular lipid particles. Drug Deliv. 6, 97–106.

Finn, R.F., McGarity, K., Thurman, T., Nicastro, P., Walker, V., Klover, J., Hills, R., LaPorte, M., Vickery, L., Donovan, M., Klein, B., Siegel, N., 2001. Pegylated progenipoietin: synthesis and characterization. In: Proceedings of the 28th International Symposium on Controlled Release of Bioactive Materials. Abstract #6168.

Fleming, W.H., Mulcahy, J.M., McKearn, J.P., Streeter, P.R., 2001. Progenipoietin-1: a multifunctional agonist of the granulocyte colony-stimulating factor receptor and fetal liver tyrosine kinase-3 is a potent mobilizer of hematopoietic stem cells. Exp. Hematol. 29, 943–951.

Furuya, H., Wakayama, T., Ohguni, S., Yamauchi, K., Tanaka, J., Hatazoe, T., Kato, Y., 1995. Effect of continuous subcutaneous administration of a small dose of granulocyte colony stimulating factor (G-CSF) by the use of a portable infusion pump in patients with non-Hodgkin's lymphoma receiving chemotherapy. Int. J. Hematol. 61, 123–129.

Hills, R., Finn, R., Nicastro, P., Thurman, T., Thayer, K., Durham, W., Klover, J., Smith, W., Siegel, N., 2001. PEGylated leridistim: multiple approaches to lead selection. In: Proceedings of the 28th International Symposium on Controlled Release of Bioactive Materials. Abstract #6157.

Hollingshead, L.M., Goa, K.L., 1991. Recombinant granulocyte colony-stimulating factor (rG-CSF). A review of its pharmacological properties and prospective role in neutropenic conditions. Drugs 42, 300–330.

Howell, S.B., 2001. Clinical applications of a novel sustained-release injectable drug delivery system: DepoFoam technology. Cancer J. 7, 219–227.

Ishioka, G.Y., Fikes, J., Qin, M., Gianfrani, C., Chesnut, R.W., Kahn, L.E., Streeter, P.R., Woulfe, S.L., Sette, A., 2001. Dendritic cells generated in vivo by a chimeric hematopoietic growth factor, progenipoietin-4, demonstrate potent immunological function. Vaccine 19, 3710–3719.

Katre, N.V., 1990. Immunogenicity of recombinant IL-2 modified by covalent attachment of polyethylene glycol. J. Immunol. 144, 209–213.

Katre, N.V., 1993. The conjugation of proteins with polyethylene glycol and other polymers. Adv. Drug Deliv. Rev. 10, 91–114.

Katre, N.V., Asherman, J., Schaefer, H., Hora, M., 1998. Multivesicular liposome (DepoFoam) technology for the sustained delivery of insulin-like growth factor-I (IGF-I). J. Pharm. Sci. 87, 1341–1346.

Kim, S., Turker, M.S., Chi, E.Y., Sela, S., Martin, G.M., 1983. Preparation of multivesicular liposomes. Biochim. Biophys. Acta 728, 339–348.

Langston, M.V., Ramprasad, M.P., Kararli, T.T., Galluppi, G.R., Katre, N.V., 2003. Modulation of the sustained delivery of myelopoietin (Leridistim) encapsulated in multivesicular liposomes (DepoFoam). J. Control. Release 89, 87–99.

MacDonald, K.P., Rowe, V., Filippich, C., Thomas, R., Clouston, A.D., Welply, J.K., Hart, D.N., Ferrara, J.L., Hill, G.R., 2002. Donor pretreatment with progenipoietin-1 is superior to G-CSF in preventing Graft-versus-Host disease after allogeneic stem cell transplantation. Blood 101, 2033–2042.

MacVittie, T.J., Farese, A.M., Smith, D.L., Casey, D.B., Smith, W.G., Woulfe, S.L., Streeter, P.R., McKearn, J.P., 1998. Members of the progenipoietins, a family of chimeric FLT-3 ligand and G-CSF receptor agonists mobilize hematopoietic progenitor cells with different kinetics and efficiency in nonhuman primates. Blood 92, 682a.

Metcalf, D., 1992. Hemopoietic regulators. Trends Biochem. Sci. 17, 286–289.

Molineux, G., McCrea, C., Yan, X.Q., Kerzic, P., McNiece, I., 1997. Flt-3 ligand synergizes with granulocyte colony-stimulating factor to increase neutrophil numbers and to mobilize peripheral blood stem cells with long-term repopulating potential. Blood 89, 3998–4004.

Molineux, G., Kinstler, O., Briddell, B., Hartley, C., McElroy, P., Kerzic, P., Sutherland, W., Stoney, G., Kern, B., Fletcher, F.A., Cohen, A., Korach, E., Ulich, T., McNiece, I., Lockbaum, P., Miller-Messana, M.A., Gardner, S., Hunt, T., Schwab, G., 1999. A new form of Filgrastim with sustained duration in vivo and enhanced ability to mobilize PBPC in both mice and humans. Exp. Hematol. 27, 1724–1734.

Moore, M.A., 1991. Review: Stratton Lecture 1990. Clinical implications of positive and negative hematopoietic stem cell regulators. Blood 78, 1–19.

Papayannopoulou, T., Nakamoto, B., Andrews, R.G., Lyman, S.D., Lee, M.Y., 1997. In vivo effects of Flt3/Flk2 ligand on mobilization of hematopoietic progenitors in primates and potent synergistic enhancement with granulocyte colony-stimulating factor. Blood 90, 620–629.

Ramprasad, M.P., Anantharamaiah, G.M., Garber, D.W., Katre, N.V., 2002. Sustained-delivery of an apolipoprotein E-peptidomimetic using multivesicular liposomes lowers serum cholesterol levels. J. Control Release 79, 207–218.

Streeter, P.R., Minster, N.I., Kahn, L.E., Hood, W.F., Vickery, L.E., Thurman, T.L., Monahan, J.B., Welply, J.K., McKearn, J.P., Woulfe, S.L., 2001. Progenipoietins: biological characterization of a family of dual agonists of fetal liver tyrosine kinase-3 and the granulocyte colony-stimulating factor receptor. Exp. Hematol. 29, 41–50.

Sudo, Y., Shimazaki, C., Ashihara, E., Kikuta, T., Hirai, H., Sumikuma, T., Yamagata, N., Goto, H., Inaba, T., Fujita, N., Nakagawa, M., 1997. Synergistic effect of FLT-3 ligand on the granulocyte colony-stimulating factor-induced mobilization of hematopoietic stem cells and progenitor cells into blood in mice. Blood 89, 3186–3191.

Veronese, F.M., 2001. Peptide and protein PEGylation: a review of problems and solutions. Biomaterials 22, 405–417.

Violand, B.N., Minnerly, J.C., Feng, Y.G., Polazzi, J.O., Mathis, K.J., Thomas, K.E., Zeng, D.L., 2002. Purification and characterization of progenipoietins produced in *Escherichia coli*. Protein Expr. Purif. 26, 275–283.

Willis, R.C., 1999. Multivesicular liposomes utilizing neutral lipids to modify in vivo release. United States Patent #5962016.

Ye, Q., Asherman, J., Stevenson, M., Brownson, E., Katre, N.V., 2000. DepoFoam technology: a vehicle for controlled delivery of protein and peptide drugs. J. Control. Release 64, 155–166.

Zeigler, F.C., Bennett, B.D., Jordan, C.T., Spencer, S.D., Baumhueter, S., Carroll, K.J., Hooley, J., Bauer, K., Matthews, W., 1994. Cellular and molecular characterization of the role of the flk-2/flt-3 receptor tyrosine kinase in hematopoietic stem cells. Blood 84, 2422–2430.