Pharmacokinetic characterization of ^{14}C-vascular endothelial growth factor controlled release microspheres using a rat model

Tae-Kyoung Kim and Diane J. Burgess

Abstract

The objectives of this study were to characterize the pharmacokinetics of vascular endothelial growth factor (VEGF) in poly(lactic-*co*-glycolic) acid (PLGA) microspheres using a rat model, and to develop a pharmacokinetic model for this controlled release formulation. ^{14}C-VEGF was encapsulated using a solid-in-oil-in-water emulsification method. The microspheres were administered subcutaneously to rats and the pharmacokinetic parameters were compared with those of protein solutions. Intravenous administration of protein solutions resulted in short half-lives and subcutaneous administration resulted in rapid clearance from the subcutaneous tissue, with high plasma concentrations as expressed by rapid absorption and elimination. The subcutaneous administration of the VEGF microspheres produced low plasma concentrations and high subcutaneous concentrations over a period of 7 weeks. The area under the curve (AUC), the time required to achieve the maximum concentration (t_{max}), the maximum concentration (C_{max}) in blood samples and the elimination rate constant (k_{el}) values at the subcutaneous tissue site were selected to compare the pharmacokinetic characterization of VEGF microspheres with that of protein solutions. The in-vivo release profiles of the proteins were slower than the in-vitro release profiles and they followed the same trend as the in-vitro and in-vivo PLGA degradation rates. The PLGA microsphere degradation was the determinant step for VEGF release from the microspheres and its absorption at the subcutaneous site. Microspheres appear to be an attractive system for the localized rate-controlled delivery of VEGF. ^{14}C-Methylation via reductive alkylation of VEGF did not affect its mitogenic activity, however approximately 25% activity was lost following release from PLGA microspheres. This loss of activity may be due to degradation in an acidic environment as a result of PLGA degradation.

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Introduction

The subcutaneous implantation of biosensors and other devices may cause severe tissue responses, including acute and chronic inflammation, undesired immune reactions and fibrosis (Tang & Eaton 1995). These inflammatory reactions can affect and even prevent implant functionality. A strategy to modify the tissue response is to correct the vascular supply required for cellular inflow of nutrients, outflow of waste products, and gas exchange to the tissue and organs (Renkin 1989; Alifrangis et al 2000). Vascular endothelial growth factor (VEGF) is a selective and direct mitogen of endothelial cells, and a critical factor for the induction of neovascularization (angiogenesis) (Ferrara & Henzel 1989; Christinger et al 1996; Muller et al 1997). However, subcutaneous injection of VEGF alone, as with most proteins, is likely to have very low efficiency since it will probably be rapidly cleared from the site and it is possible that the native protein may cause undesired immune reactions. These problems may be overcome by encapsulation within polymeric microspheres.

Microspheres have been used as a controlled release dosage form for many therapeutic proteins to alter pharmacokinetic parameters and undesired immune response in the body (Reuning et al 1983). Poly(lactic-*co*-glycolic) acid (PLGA) microspheres are most commonly used, since they have been shown to be biocompatible and controlled release of weeks to several months can be achieved by alteration of the copolymer ratio, molecular weight and microsphere size (Crotts & Park 1998). Controlled release PLGA

microspheres containing leuprolide acetate depot (Lupron-depot) are available on the market (Machluf et al 2000) and several PLGA microsphere systems are in clinical trials, such as human growth hormone (Cleland et al 1997).

There have been no previous reports of VEGF pharmacokinetics, either alone or within controlled release dosage forms. In fact, little work has been reported on the pharmacokinetic modelling for controlled release parenteral dosage forms. Burgess & Davis (1988) reported a single point (level C) correlation of in-vitro and in-vivo release of steroids from albumin microspheres, and Reuning et al (1983) characterized the pharmacokinetic parameters of a copolymer delivery system of naltrexone in monkeys. Sun et al (1999) described the pharmaco-kinetic/pharmacodynamic modelling of growth hormone from microspheres. Although pharmacokinetic modelling has been reported for oral controlled release products (Welling 1997), those products are usually limited to release over hours compared with weeks to several months for parenteral controlled release products. In addition, the environment for dissolution and absorption differs greatly between the oral and parenteral routes. Therefore, pharmacokinetic models developed for controlled release oral products are unlikely to be useful for controlled release parenteral products.

In the present study, the pharmacokinetics of VEGF in controlled release microspheres, implanted at the subcutaneous site, were characterized and compared with those of the carrier protein, rat serum albumin (RSA), and with those of the two protein solutions alone. An attempt was made to develop a pharmacokinetic model for this controlled release microsphere delivery system. The carrier protein was necessary because of the potency of VEGF. In addition, this carrier possessed a buffer capacity, which should reduce/avoid any negative effect of acid catalysed PLGA degradation on the biological activity of VEGF (Kim & Burgess 2001). RSA was selected since these pharmacokinetic studies were conducted in rats. The method of preparation of the VEGF/PLGA microspheres and the in-vitro release of VEGF from these microspheres has been reported previously (Kim & Burgess 2001). VEGF activity following ^{14}C labelling and processing into microspheres was determined using an in-vitro mitogenic assay.

Materials and Methods

Materials

Male Sprague-Dawley rats (200–224 g) were purchased from Harlan (Indianapolis, IN). VEGF and RSA were purchased from R & D Systems (Minneapolis, MN) and Sigma (St Louis, MO), respectively. The radioactive materials, ^{3}H-thymidine, ^{3}H-NaBH$_4$ (^{3}H-sodium borohydride) and ^{14}C-HCHO (^{14}C-formaldehyde) were purchased from Amersham Pharmacia Biotech (Piscataway, NJ) and NEN Life Science Products (Boston, MA), respectively. PLGA (50:50, MW 60 000, Resomer RG504) was purchased from Boeringer Ingelheim (Germany). All chemical reagents (propanal, cyanosodiumborohydride and nickel chloride) were purchased from Sigma.

Methods

VEGF and RSA were radiolabelled with ^{14}C and ^{3}H, respectively, using reductive alkylation on the amino groups (Mean & Feeney 1995). The administration of native protein was conducted by intravenous and subcutaneous injection into rats (five rats per group, per time period). The time intervals were 5 min, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 21, 22, 23 and 24 h for the administration of the native proteins. The VEGF microspheres were injected subcutaneously into rats (five rats per group, per time period). The time intervals were 1, 3, 5, 7, 14, 21 and 28 days. The specific radioactivity was determined for blood and subcutaneous tissue samples and any residual radioactivity in the microspheres was also determined at each time interval. The specific radioactivities of ^{3}H and ^{14}C were separated using different energy levels (Fox 1976). Modelling of the pharmacokinetic data was attempted using a two-compartment model for the solution intra-venous injection. A one-compartment open model with first order absorption and elimination was used for both the solution and microsphere subcutaneous studies (Welling 1997).

Reductive methylation of VEGF for ^{14}C labelling

VEGF (200 l g) was dissolved in 200 l L HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) buffer (0.2 M, pH 7.4) containing 15 mM cyanosodiumborohydride (NaBH$_3$CN) and 10 mM nickel chloride (NiCl$_2$). ^{14}C-HCHO solution in water (^{14}C-HCHO/water, 1:99, 1.06 l mol, 50 l Ci, 9 times excess of amino group on VEGF) was added to the protein solution. The solution was incubated with gentle stirring at room temperature for 24 h, followed by dialysis against 0.9% (w/v) NaCl solution and lyophilization.

Reductive propylation of RSA for ^{3}H labelling

RSA (200 mg) was dissolved in 5 mL HEPES buffer containing 1.049 mL propanal (14.55 mmol, 100 times excess of amino group on RSA). Sodium borohydride (5 mCi; ^{3}H-NaBH$_4$) solution in 0.1 M sodium hydroxide was added to this solution. The solution was incubated with gentle stirring at room temperature for 24 h followed by dialysis against 0.9% (w/v) NaCl solution and lyophilization. The lyophilized samples were reconstituted and used on a volume basis so that the amount of RSA was not affected by the weight of NaCl.

Determination of protein concentration and specific radioactivity

A protein mixture of 4.5 l g VEGF and 650 l g RSA in 200 l L 0.9% (w/v) NaCl solution was prepared. This solution was mixed with 10 mL EcoLite(+) (ICN Phar-

maceuticals, Aurora, OH) and the specific radioactivity was counted (in counts min^{-1}) using a Beckman LS 1801 liquid scintillation counter. The specific radioactivities of ^{3}H and ^{14}C were separated using an isotope exclusion method. The conversion of counts min^{-1} to disintegrations min^{-1} was performed manually (disintegrations min^{-1} = counts $min^{-1} \times$ efficiency^{-1}; where the efficiency was 0.4 from channels 0 to 400 for ^{3}H and 0.6 from channels 400 to 1000 for ^{14}C).

VEGF/RSA microsphere preparation

^{3}H-RSA (100 mg) and ^{14}C-VEGF (200 l g) in powder form were dispersed in 4 mL PLGA (50:50) solution (25%, w/v) in CH_2Cl_2 using a homogenizer at 7500 rev min^{-1} for 2 min. Polyvinylalcohol (PVA) solution (20 mL, 1%) was added to this mixture and homogenized at 7500 rev min^{-1} for a further 2 min. This emulsion was poured into 300 mL of a 0.1% (w/v) PVA solution and stirred for 1 h under vacuum to achieve rapid evaporation of the organic solvent. The hardened microspheres were centrifuged, filtered and washed three times with distilled water, and subsequently dried for 24 h under vacuum.

In-vitro release study

Three vials containing 50 mg of microspheres were prepared in 1 mL PBS (pH 7.4) for each of the following time intervals: 2, 4, 7, 8, 9, 10, 12, 14, 16, 18, 23, 28 and 30 days. The release profile of protein from the microspheres was monitored at 37°C during a 30-day period using the method of Sah (1997). The microspheres and the protein were dissolved in dimethylsulfoxide to determine the amount of residual protein in the microspheres. The degraded microspheres were dissolved in 4 mL distilled tetrahydrofuran (4%, w/v) and filtered to measure molecular weight using gel permeability chromatography at 37°C. The in-vitro release studies were conducted in triplicate, and mean values and standard deviations were calculated.

In-vitro mitogenic assay using VEGF, ^{14}C-VEGF and VEGF microspheres

Each well of 96-well plates, except the peripheral wells of plates to prevent potential contamination, was coated with 50 l L of solution containing 40 l g mL^{-1} type I collagen for 2 h at room temperature, followed by overnight at 4°C. Human umbilical vein endothelial cells (HUVEC; Clontics, Walkersville, MD) were plated at 5×10^3 cells/well in 50 l L of assay medium and incubated for 72 h at 37°C under 5% CO_2. Serial dilution of VEGF, ^{14}C-VEGF and released ^{14}C-VEGF from microspheres were prepared in an assay medium (Medium 199 with 10 mM HEPES, 10% heat-inactivated fetal calf serum and antibiotics) and 50 l L of each diluted sample was added per well. Samples were prepared in triplicate. ^{3}H-thymidine (0.5 l Ci/10 l L/well)

was added to each well and agitation-labelled with ^{3}H-thymidine for the last 16–24 h of the total 72-h incubation period. At the end of the incubation process, the cells were harvested onto a glass fibre filter and the amount of ^{3}H-thymidine incorporated into DNA elongated in HUVEC cells was determined from the specific radioactivity as described above.

Pharmacokinetic study in rats

Intravenous and subcutaneous protein solutions

All animal studies were conducted at the University of Connecticut in accordance with IACUC guidelines using an IACUC approved protocol (no. E290 1201). Intravenous injection of 400.4 l g/500 l L of native mixed proteins (VEGF/RSA, 1:1000) was conducted through the lateral tail vein, or subcutaneously into the back of the rats. Total blood samples were taken through the thoracic cavity and subcutaneous and dermis tissue samples were removed from each sacrificed rat. Five rats were sacrificed at each of the following time intervals: 5 min, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 21, 22, 23 and 24 h. The blood samples were centrifuged to separate the plasma and blood cells. The subcutaneous tissue samples were homogenized, centrifuged and the supernatant removed. Extracted samples from blood and subcutaneous tissues were separated via a 10% acrlyamide gel, using native VEGF and RSA as standards. The bands corresponding to native proteins were cut and mixed with EcoLite($+$) to count the specific radioactivity using a liquid scintillation counter. All radioactivity measurements were converted into disintegrations min^{-1} (g of sample)$^{-1}$.

Subcutaneous injection of microspheres and sampling of tissues

PLGA microspheres containing RSA and VEGF (RSA/VEGF, 1000:1) were prepared under aseptic conditions. The microspheres were hydrated in filtered 0.9% (w/v) NaCl solution for 24 h before injection. Microspheres (50 mg) containing 400 ng VEGF and 400 l g RSA were suspended in 500 l L filtered 0.9% NaCl (w/v) solution and injected subcutaneously into the backs of the rats using 1-mL syringes fixed with 18-gauge needles.

Five rats were sacrificed at each of the following time intervals: 1, 3, 5, 7, 14, 21, 28, 35, 42 and 49 days. Blood samples were taken through the thoracic cavity. Subcutaneous tissue, including the microspheres, was removed from the injection site. Microspheres (40 mg) were separated manually from the tissue samples to analyse the VEGF concentration remaining in the microspheres and the extent of microsphere degradation. The protein content of the degraded microspheres in-vivo was determined according to the method of Sah (1997) using dimethylsulfoxide as a dissolving solvent. The molecular weight of the degraded PLGA removed from the site by dissolving with tetrahydrofuran was determined using gel permeability chromatography, as described above. The concentration of the proteins at the subcutaneous site was calculated by subtracting the amount of protein remaining

in the microspheres from the total amount of protein injected.

Pharmacokinetic modelling (intravenous injection of protein solutions)

Following intravenous injection, the pharmacokinetic profiles of the proteins may follow either the one-compartment open model (Equation 1) or the two-compartment open model (Equation 2). These two models were compared to determine the best fit.

$$C = C_0 e^{-k_{el}*t} \tag{1}$$

$$C = Ae^{-at} + Be^{-bt} \tag{2}$$

where $A = (D(k_{21}-a))/(V_1(b-a))$ and
$\qquad B = (D(k_{21}-b))/(V_1(a-b))$

where $a = 0.5\{(k_{12}+k_{21}+k_{el})+[(k_{12}+k_{21}+k_{el})^2$
$\qquad\qquad -4k_{21}k_{el}]^{1/2}\}$
$\qquad b = 0.5\{(k_{12}+k_{21}+k_{el})$
$\qquad\qquad -[(k_{12}+k_{21}+k_{el})^2-4k_{21}k_{el}]^{1/2}\}$

where C is the concentration of the proteins in the plasma; C_0 is the initial concentration of protein at zero time; k_{el} is the elimination constant containing urinary excretion and metabolism; D is the protein amount; V_1 is the volume of the first compartment; and k_{12} and k_{21} are the microscopic rate constants between the two compartments. All disintegrations min^{-1} g^{-1} values were corrected into the corresponding amount of protein using the method described above. Data fitting was conducted using Prism 3.0 GraphPad software.

Pharmacokinetic modelling (subcutaneous injection of protein solutions and microspheres)

When a drug is taken orally, or by intramuscular or subcutaneous injection, the resulting drug in plasma profiles can frequently be described by a pharmacokinetic model that incorporates first-order absorption and elimination (Welling 1997). The protein concentration profiles in each organ were modelled using Equation 3 over a period of 28 days.

$$C = (FD/V)(k_a/(k_a-k_{el}))(e^{-k_{el}*t}-e^{-k_a*t}) \tag{3}$$

Where C is the concentration of protein as a function of time; F is the absorption efficiency; D is the absorbed quantity; V is the volume of the compartment; k_a is the absorption constant; and k_{el} is the elimination constant.

The application of the pharmacokinetic model to observed data was conducted until the goodness-of-fit attained was over 95% of confidence, by changing equations. All data fitting were conducted using Prism 3.0 GraphPad software, which includes the least square method linear regression analysis. Any statistical difference between the two groups of data, concentration in blood and that at the subcutaneous site, was evaluated using analysis of variance.

Since there is no established pharmacokinetic model for implanted microsphere dosage forms, the in-vivo release profile from the microspheres and the protein con-

centration in each organ were utilized to develop a pharmacokinetic model for this dosage form.

Results and Discussion

Intravenous and subcutaneous injection of RSA and VEGF solutions

The specific radioactivity profiles as a function of time after intravenous injection of the protein mixture showed an exponential decrease. The two-compartment model resulted in a better fit to the data than the one-compartment model (Figure 1). The basic pharmacokinetic parameters for the intravenous and subcutaneous injections were identified (Table 1). The microscopic rate constants, k_{12}, k_{21} and k_{el}, for VEGF and RSA were calculated using the parameters a and b in Equation 2, and assuming equilibrium between the central and second compartments. For both VEGF and RSA, the uptake process (k_{12}), according to the level in the peripheral tissues or the second compartment (including subcutaneous tissue, liver, spleen, lung, kidney and other organs), may be significantly more rapid than the elimination process (k_{el}). At steady state, the rates are the same between the two compartments ($C_1 \times k_{12} = C_2 \times k_{21}$, or $V_1 \times k_{12} = V_2 \times k_{21}$) and two different situations may
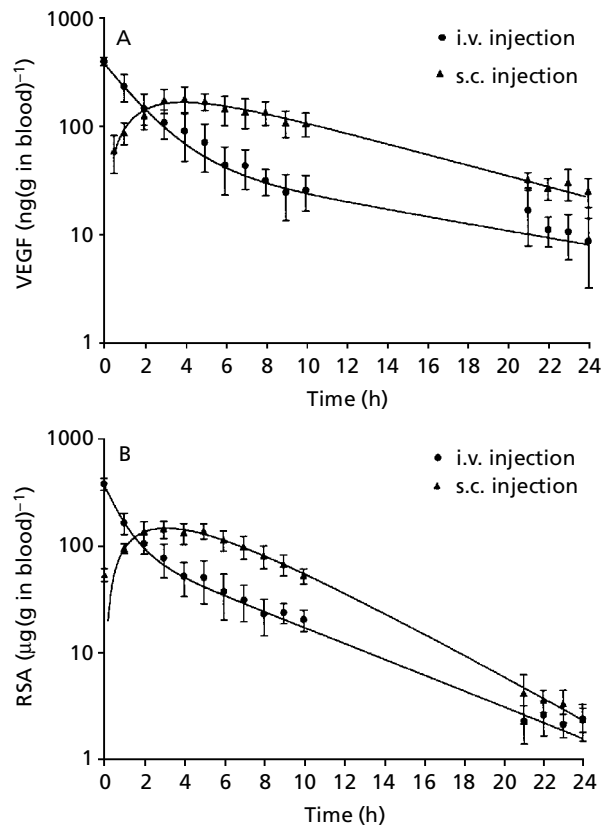
Figure 1 Concentration–time profile of ^{14}C-VEGF (A) and ^{3}H-RSA (B) in blood (error bars represent s.d.). VEGF, vascular endothelial growth factor; RSA, rat serum albumin.

Table 1   The basic pharmacokinetic parameters for intravenous and subcutaneous injections of native proteins.

|	Intravenous injection					
	A (ng (g of sample)$^{-1}$)	a (h^{-1})	t$_{1/2(a)}$ (h)	B (ng (g of sample)$^{-1}$)	b (h^{-1})	t$_{1/2(b)}$ (h)
VEGF±s.d.	703.08±57.8	0.576±0.083	1.203±0.109	103.95±35.5	0.078±0.023	8.885±2.870
RSA±s.d.	596.4±53.3	1.154±0.183	0.601±0.098	206.8±35.0	0.174±0.021	3.983±0.327
	Subcutaneous injection					
	FD V^{-1} (ng (g sample)$^{-1}$)	k$_a$ (h^{-1})	k$_{el}$ (h^{-1})	t$_{max}$ (h)		
VEGF±s.d.	285.2±57.5	0.414±0.116	0.126±0.037	4.135		
RSA±s.d.	287.2±44.3[a]	0.461±0.084	0.231±0.043	3.002		

VEGF, vascular endothelial growth factor; RSA, rat serum albumin. [a]Unit is l g.

occur: $k_{12} \ll k_{el}$, resulting in rapid intrinsic elimination; or $k_{12} \gg k_{el}$, resulting in very low intrinsic elimination. It is considered that the second of the two possibilities described above is more relevant since k_{12} (0.369$_{VEGF}$, 0.523$_{RSA}$) $\gg k_{el}$ (0.041$_{VEGF}$, 0.292$_{RSA}$). Accordingly, the pharmacokinetic parameters described in Table 1 were obtained.

During the first 24 h, the absorption and elimination profiles of both proteins in blood were modelled using the one-compartment open model with first-order absorption and elimination (Equation 3). However, three different situations may have occurred when using this model: (i) k_a may be greater than k_{el}; (ii) k_a may be smaller than k_{el}; and (iii) the two constants may have the same, or approximately the same, numerical value. On analysis of the data, it was determined that k_a was larger than k_{el}. This means that the absorption rates of the two proteins were always more rapid than their elimination from the blood. Figures 1A and 1B are the concentration profiles of the two radio-labelled proteins (VEGF and RSA) as a function of time during the first 24 h, which show that most of the administered protein was absorbed and eliminated during the first 24 h. When the plasma concentration profiles of VEGF and RSA were observed for a period of 28 days, there was no significant concentration for either protein detected between Days 1 to 28. VEGF had a similar absorption efficiency to that of RSA, whereas the elimination constant of VEGF was smaller than that of RSA (Table 1). The half-life of VEGF in both phases (a and b) was longer than that of RSA.

Subcutaneous injection of microspheres

The subcutaneous injection of the protein solutions (Figure 1) showed that most of the protein was cleared from the site within 4 h for VEGF and 3 h for RSA. The majority of these proteins (>70%) were located in the blood, liver, spleen, lung and kidneys. Only trace quantities were observed in the subcutaneous tissue, the target site, compared with the other organs. In the case of the microsphere injections, the concentration of VEGF and RSA at the
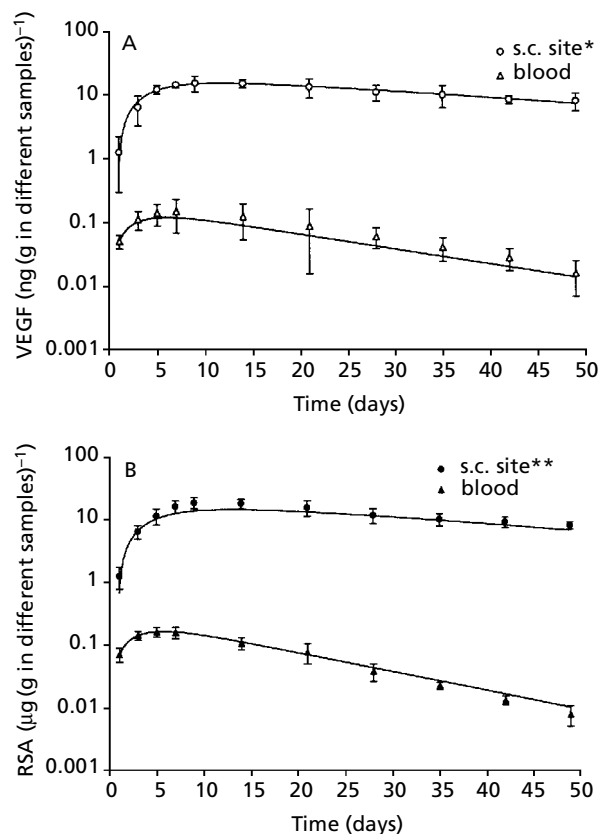
Figure 2 ^{14}C-VEGF (A) and ^{3}H-RSA (B) concentration–time profile in the subcutaneous site and blood after subcutaneous microsphere injection (error bars represent s.d.). VEGF, vascular endothelial growth factor; RSA, rat serum albumin. *$P < 0.0001$, **$P < 0.013$ compared with blood level data.

subcutaneous site was much higher (63 times) than that in the blood. The microsphere formulation pharmacokinetic data appear to be consistent with one absorption and a single elimination phase (Figure 2). The protein con-

Table 2 Modelling of vascular endothelial growth factor (VEGF) and rat serum albumin (RSA) concentration profile in the subcutaneous site with one-compartment open model with first-order absorption and elimination.

Parameters	FD V_1^{-1} (ng (g of sample)$^{-1}$)	k_a (day^{-1})	k_e (day^{-1})	FD V_1^{-1} (ng (g of sample)$^{-1}$)	k_a (day^{-1})	k_e (day^{-1})	R (ng)
VEGF							
Blood ± s.d.	0.166 ± 0.027	0.379 ± 0.139	0.055 ± 0.010				
Subcutaneous site ± s.d.	21.13 ± 2.99	0.173 ± 0.045	0.026 ± 0.007	23.65 ± 2.31	0.255 ± 0.059	0.016 ± 0.004	4.486 ± 2.507
RSA							
Blood ± s.d.	0.245 ± 0.056[a]	0.357 ± 0.167	0.071 ± 0.021				
Subcutaneous site ± s.d.	28.18 ± 4.29[a]	0.134 ± 0.073	0.036 ± 0.019	29.14 ± 4.918[a]	0.203 ± 0.099	0.021 ± 0.012	4.815 ± 4.553[a]

R, residual concentration. [a]Unit is l g.

Table 3 Comparison of the pharmacokinetic parameters in blood following subcutaneous injection of protein solution and their microspheres dosage forms.

	AUC$_{0-\infty}$ (ng h mL^{-1})	t_{max} (h)	C_{max} (ng)
VEGF solution	2229	4.135	169.46
RSA solution	1287[a]	3.002	143.38[b]
VEGF microspheres	72.6	143	0.120
RSA microspheres	83.5[a]	136	0.165[b]

VEGF, vascular endothelial growth factor; RSA, rat serum albumin. [a]Unit is l g h mL^{-1}; [b]unit is l g.

centration profile at the subcutaneous site was modelled using a one-compartment open model with first-order absorption and elimination, as described in Equation 3, with and without residual concentration, R (Equation 4). Absorption at the subcutaneous site was defined as release of protein from the microspheres and elimination from the subcutaneous site was defined as absorption into the bloodstream. The additional parameter, R, represents the amount of protein available for absorption, which is defined as protein that has been released from the microspheres, but that has not yet been eliminated from the subcutaneous site (absorbed into the bloodstream).

$$C = (FD/V)(k_a/(k_a - k_{el}))(e^{-k_{el}*t} - e^{-k_a*t}) + R \qquad (4)$$

The standard deviation was smaller when modelled with than without the residual concentration at the subcutaneous site (Table 2). Therefore, the existence of the residual concentration was accepted. This implies that the microspheres released the proteins slowly for a period of 49 days at the subcutaneous site, with a minimum of 4.486 ± 2.507 ng VEGF and 4.815 ± 4.553 l g RSA released continually.

The absorption rate constants (protein release) from the microsphere injections at the subcutaneous site (k_a = 0.255 ± 0.059 day^{-1}, or 0.011 ± 0.003 h^{-1} for VEGF; k_a =

0.203 ± 0.099 day^{-1} or 0.009 ± 0.004 h^{-1} for RSA) are much slower than those of the subcutaneous injections of the protein solutions (k_a = 0.414 ± 0.116 h^{-1} for VEGF; k_a = 0.461 ± 0.084 h^{-1} for RSA). The microsphere dosage forms resulted in sustained release of the proteins. According to the complexity of the compartment composition, including both the sustained release dosage form and the subcutaneous site, the absorption rate constant was considered as the sum of two rate constants: slow first-order protein release from the microspheres and fast first-order absorption of released protein at the subcutaneous site. The appearance (elimination from subcutaneous site) of VEGF in the blood was much faster for the protein solutions compared with the microsphere formulation. Therefore, it appears that the release rate of protein from the microspheres is the determinant step for protein absorption from the subcutaneous site.

The protein concentration in the blood was insignificant compared with that at the subcutaneous site and, consequently, modelling the blood concentration profiles without the residual concentration was not significantly different from that with the residual concentration. The low concentration of the proteins in blood may be explained by the slow release rate from the microspheres and hence the high efficiency of local delivery to the subcutaneous site. The attempt to compare the pharmacokinetic parameters between VEGF solution and that of microspheres was not possible because of different time intervals, such as 24 h for VEGF solution and 49 days for VEGF microspheres.

The area under the curve (AUC), the time required to achieve the maximum concentration (t_{max}), the maximum concentration (C_{max}) in blood samples and the elimination rate constant (k_{el}) at the subcutaneous site were selected to compare the pharmacokinetic characteristics of the protein solutions and microspheres following injection at the subcutaneous site. AUC represents the loss of protein from the subcutaneous site to the blood, and this is undesirable for the purpose of localized delivery. Using the microsphere system, the plasma AUC values were considerably smaller than for the protein solutions (31 times smaller for VEGF and 15 times smaller for RSA). The t_{max} for the microsphere

dosage forms was much longer than that for the protein solution. The C_{max} was much lower for the microspheres compared with the solution dosage forms (approximately 1412 times lower for VEGF and 869 times for RSA) (Table 3). The lower C_{max} value implies that the microspheres dosage form may be safer than the solution injection with respect to the potential to generate an immune response. Unexpected immune response is a significant problem in the use of therapeutic proteins, and the higher the C_{max}, the more likely it is that this may occur. The k_{el} from the subcutaneous site was approximately 189 times smaller (VEGF) and 264 times smaller (RSA) for the microsphere formulation compared with the protein solutions. These four parameters (AUC_{plasma}, t_{max}, C_{max} and k_{el}) can be considered as indications that the microsphere formulation has achieved the goal of localized slow release.

The parameter, AUC_{plasma}, in the case of intravenous injection of VEGF and RSA solution was 1270 ng h mL^{-1} and 891 1 g h mL^{-1} for VEGF and RSA, respectively. These values are smaller than those for subcutaneous injection; this may be a result of difficulty in intravenous injection through the rat tail vein.

In-vitro and in-vivo release profiles of VEGF and degradation of PLGA microspheres

The in-vitro and in-vivo release profiles of VEGF from PLGA microspheres were compared (Figure 3). The in-vitro release data are zero order between Days 10 and 30, with 65% of the total protein released at 30 days. The in-vivo release profiles, which were calculated from the amount of protein remaining in the microspheres, were much slower, with 15% released at 30 days and 63% released at 49 days. Although good in-vitro/in-vivo cor-

relation has been obtained for oral dosage forms using standardized USP dissolution methods (Halperin 1995), there is little published data on in-vitro/in-vivo correlation for parenteral products. The subcutaneous route is more complex and less well studied than the oral route. For example, sink conditions may not apply at the subcutaneous site and the body's inflammatory and immune responses may complicate drug release from delivery devices such as PLGA microspheres. The lack of sink conditions at the subcutaneous site may explain the slower in-vivo release rate obtained in the present study when compared with the in-vitro data. The in-vivo release rate was slow initially with less than $7.4 \pm 2.3\%$ or 29.6 ± 9.0 ng released within the first 2 weeks. Subsequently, the in-vivo release profile showed a slight exponential increase. The overall in-vivo release profile was too complex to define using a single mathematical model.

The in-vitro and in-vivo release data follow the same rank order as the in-vitro and in-vivo PLGA degradation (Figure 3), suggesting that release of VEGF from the microspheres is controlled by the degradation of polymer. The heterogeneous in-vivo release profile was in agreement with the PLGA microsphere degradation profile. The average molecular weights and the polydispersity of the degraded polymer were more variable for the in-vivo samples compared with the in-vitro samples.

In-vitro mitogenic assay of VEGF, ^{14}C-VEGF and ^{14}C-VEGF microspheres

An in-vitro cell culture assay, using HUVEC, was used to determined the activity of VEGF following ^{14}C labelling and subsequent processing into the PLGA microspheres. The activities of the native and methylated ^{14}C-VEGF were
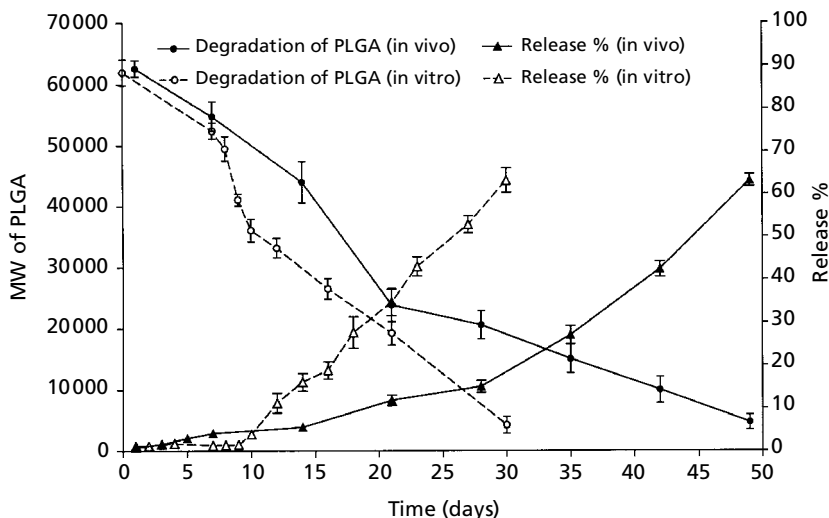
Figure 3 Degradation of PLGA microspheres and percent release of vascular endothelial growth factor (VEGF) from the microspheres in-vitro and in-vivo (in-vitro release condition: PBS pH 7.4 at 37°C and 100 rev min^{-1}; degradation of PLGA determined using gel permeability chromatography) (error bars represent s.d.).
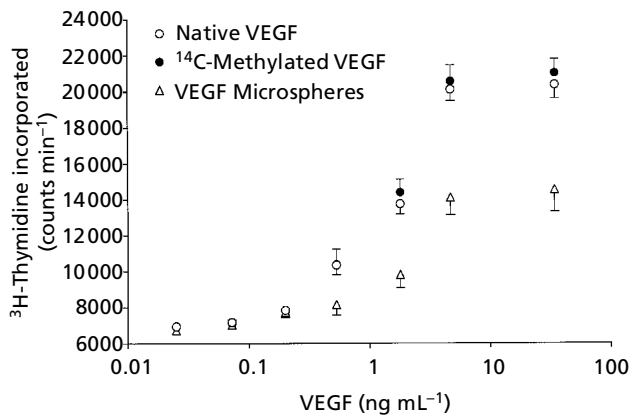
Figure 4 Comparison of biological activities of vascular endothelial growth factor (VEGF), ^{14}C-VEGF and ^{14}C-VEGF microspheres determined via a cell culture (human umbilical vein endothelial cells) mitogenic assay (error bars represent s.d.).

not significantly different (Figure 4). It therefore appears that reductive methylation of VEGF does not affect the critical domain for mitogenic activity (residues 111–165) (Keyt et al 1996). Those results are consistent with those for other proteins, such as hen egg lysozyme (Rypniewski et al 1993) and calbindin D9k (Zhang et al 1994).

On the other hand, the ^{14}C-VEGF released from microspheres had less activity (approx. 25% less) compared with the same amount of native VEGF. These results indicate that the VEGF released from microspheres included both active VEGF (75%) and degraded VEGF (25%). Degradation is probably due to the acidic environment within the microspheres that results from PLGA degradation.

Conclusions

The administration of protein solutions via intravenous or subcutaneous injection may not be effective as a result of their rapid clearance rates. PLGA microspheres are a promising strategy for the controlled delivery of therapeutic proteins. Release of VEGF from the PLGA microspheres was measured using ^{14}C-methylation of VEGF. ^{14}C-Methylation did not affect the mitogenic activity of VEGF. However, the activity of VEGF was reduced by 25% following release from the PLGA microspheres. This was considered to be a result of acid degradation as a consequence of the acid environment created in the microspheres following PLGA degradation. The release profile of VEGF was different in the in-vitro and in-vivo environments. Therefore, the in-vitro release kinetics of VEGF from the controlled release PLGA microsphere system could not be directly applied to predict the in-vivo absorption rate owing to complex biological responses to the PLGA and the released proteins. However, in-vitro and in-vivo release did follow the same rank order as the in-vitro and in-vivo degradation of the PLGA microspheres.

In order to model the pharmacokinetic profile of VEGF from microspheres injected at the subcutaneous site, the conventional one-compartment open model was modified. An additional parameter, R, was included to describe the sustained release profile, where R is the concentration of protein in the tissue that has been already released from the microspheres. The protein absorption at the subcutaneous site included both the release rate from microspheres, which was the dominant step, and the absorption rate at the subcutaneous site. Finally, we propose that four pharmacokinetic parameters (AUC, t_{max}, C_{max} in blood and k_{el} at the subcutaneous site) were useful to characterize this sustained release dosage form and to compare the data with those from protein solution.

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