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Lymphatic Transport and Catabolism of Therapeutic Proteins Following Subcutaneous Administration to Rats and Dogs

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Nonstandard abbreviations

AUC, area under the serum concentration-time curve; CERA, continuous erythropoietin receptor activator; C_{max} , maximum serum concentration; Conc, concentration; EPO, erythropoietin; F,

DMD/2011/043604

bioavailability; IV, intravenous or intravenously; LDC, lymph duct cannulation; MW, molecular weight; PEG, polyethylene glycol; ng-eq/ml, radioactive equivalent concentration ng/ml; PK, pharmacokinetics; SC, subcutaneous or subcutaneously; SD rats, Sprague Dawley rats; TCA, trichloroacetic acid

DMD/2011/043604

Abstract

The mechanism underlying subcutaneous (SC) absorption of macromolecules and factors that can influence this process were studied in rats using PEGylated erythropoietins (EPOs) as model compounds. Using a thoracic lymph duct cannulation (LDC) model, we showed that PEGylated EPO was absorbed from the SC injection site mainly via the lymphatic system in rats, which is similar to previous reports in sheep. Following SC administration, the serum exposure was reduced by ~70% in LDC animals when compared to the control animals, and most of the systemically available dose was recovered in the lymph. In both LDC and intact rats, the total radioactivity recoveries in excreta following SC administration were high (70-80%), indicating that catabolism, not poor absorption, was the main cause for the observed low bioavailability (30-40%). Moreover, catabolism of PEGylated EPO was found with both rat SC tissue homogenate and lymph node cell suspensions, and a significant amount of dose-related breakdown fragments was found in the lymph of LDC rats. In addition, the bioavailability of PEGylated EPOs were shown to be 2- to 4- fold lower in "fat rats", indicating that physiological features pertinent to lymphatic transport can have a profound impact on SC absorption. Limited studies in dogs also suggested similar SC absorption mechanisms. Collectively, our results suggest that the lymphatic absorption mechanism for macromolecules is likely conserved among commonly used preclinical species, e.g. rats and dogs, and that mechanistic understanding of SC absorption mechanism and associated determinants should be helpful in biologics drug discovery and development.

DMD/2011/043604

Introduction

Almost all protein-based biologics drugs are administered parenterally. Subcutaneous (SC) administration is the much preferred route of administration compared to intravenous (IV) administration. There are more than 30 marketed biologics drugs that are administered SC, including most of the therapeutic proteins/peptides (McDonald et al., 2010). Despite its wide application, our understanding of the exact mechanism underlying SC absorption and the factors that can influence this process remain limited (Porter and Charman, 2000; Lin, 2009).

Biologics drugs can exhibit a wide range of bioavailability following SC administration in humans (Tang et al., 2004). Many physiological factors have been identified as covariants for the pharmacokinetics (PK) of macromolecules administered SC in humans, e.g. age, body weight, injection site (Macdougall et al., 1991; Chan et al., 2003; Fishbane et al., 2007; Olsson-Gisleskog et al., 2007; Kakkar et al., 2011). But the type of factors and extent of their impact are apparently compound-dependent. For example, when the PK of peginterferon alfa-2a and peginterferon alfa-2b was compared in a randomized 36-patients trial, peginterferon alfa-2a showed much larger variability in patient exposure than peginterferon alfa-2b (38% vs. 20%), and body weight appeared to be a covariant for peginterferon alfa-2a, but not for peginterferon alfa-2b (Silva et al., 2006). Currently there is no established method to predict SC absorption in humans a priori, i.e. bioavailability, potential covariants, or the magnitude of PK variability. Therefore, there is a great need of more mechanistic understanding of the SC absorption process, as well as more preclinical and in vitro tools to help gain insights into its underlying mechanism.

Our current understanding of the SC absorption process mostly came from a sheep lymphatic cannulation model, where a series of studies demonstrated a molecular weight (MW)-dependent lymphatic contribution to the SC absorption of macromolecules, i.e. macromolecules with

DMD/2011/043604

MW >16 kDa are absorbed mainly via the lymphatic system (Supersaxo et al., 1990; Porter and Charman, 2000; Porter et al., 2001; McLennan et al., 2006). In addition, the observation of dose-dependent SC bioavailability for an anti-CD4 mAb also provided indirect evidence that mAbs are absorbed via the lymphatic pathway in mice (Davis and Bugelski 1998). This theory aligns well with our knowledge of the anatomic structural differences between blood and lymph capillaries. The lymphatic system is a unidirectional system that transports excess fluid from interstitium to systemic circulation. Unlike blood capillaries in the SC space whose tight endothelial junctions make it difficult for macromolecules to pass through, lymphatic capillaries have incomplete basal lamina which enables almost unrestricted drainage of macromolecules from the interstitial space (Swartz, 2001). Due to the technical challenges of cannulating lymph vessels in smaller-sized laboratory animals, there have been very limited reports on the contribution of lymphatic system in SC absorption of macromolecules in commonly used preclinical species. Unfortunately, the few available reports in rats and rabbits presented contradictory evidence (Bocci et al., 1986; Bocci et al., 1988; Kojima et al., 1988; Kagan et al., 2007). In the more recent report by Kagan et al., the contribution of lymphatic system to SC absorption of macromolecules was systematically examined in a rat thoracic lymph duct cannulation (LDC) model, using bovine insulin (5.6 kDa), recombinant human erythropoietin (EPO) (30.4 kDa) and bovine albumin (66 kDa) as model compounds. The results suggested minimal contribution of the lymphatic system for all three molecules, regardless of their MWs, i.e. minimal reduction of serum exposure in the thoracic LDC animals, and < 3% drug recovery in thoracic duct lymph (Kagan et al., 2007). The only report where a significant percentage (up to 29%) of SC dosed radioactivity was recovered in the rat thoracic duct lymph was a study using PEGylated polylysine dendrimers, though the amount of dose recovered in the lymph was

DMD/2011/043604

still considerably lower than the systemic bioavailability (94-104%) (Kaminskas et al., 2009).

These results questioned whether contribution of the lymphatic system in the SC absorption of macromolecules is conserved among preclinical species, and challenged the value, if any, of studying SC absorption in these commonly used preclinical species.

Here we report our efforts in elucidating the mechanism of SC absorption of macromolecules in thoracic LDC models in rats and dogs. Two PEGylated recombinant human EPOs were selected as model compounds as they are likely to be absorbed via the lymph, and their PK had been well characterized in rats and dogs. Their catabolism following SC administration and the impact of physiological features pertinent to lymphatic absorption/transport, e.g. fat content, level of physical activity, on SC absorption were also evaluated in these preclinical models.

DMD/2011/043604

Materials and Methods

Materials

Recombinant human EPO is a 30.4 kDa therapeutic protein. PEG30-EPO, or Continuous erythropoietin receptor activator (CERA, Mircera™), is a commercially available PEGylated recombinant human EPO that contains a ~30 kDa methoxy polyethylene glycol (PEG) chain linked via amide bonds to the N-terminal amino group or the ε-amino group of lysines (predominantly lysine-52 or lysine-45) (MacDougall, 2005). CERA was purchased from Myoderm (Norristown, PA). PEG40-EPO is a PEGylated recombinant human EPO produced in-house, which has a ~40 kDa linear PEG conjugated preferably to the amino-terminus of the recombinant human EPO (Nett et al., 2012). In addition to the difference in PEG sizes, PEG30-EPO was produced from CHO cells with enriched tetra-antennary sialylated glycoforms at the three N-glycosylation sites of EPO, while PEG40-EPO was produced from engineered strains of *Pichia pastoris* with terminally sialylated bi-antennary N-glycan structures (Nett et al., 2012).

¹²⁵Iodine labeling

¹²⁵I labeling of PEG30-EPO and PEG40-EPO was conducted using Iodobeads according to the manufacturer's protocol with minor modification (Pierce, Rockford, IL). Briefly, 100 µg of protein dialyzed into phosphate buffered saline was incubated with 1 mCi of Iodine-125 (Perkin Elmer, Waltham, MA) and two Iodobeads for 15 min at room temperature. The labeled protein was purified with a Zeba column (Pierce), and stored at 4°C. The radiochemical concentration was determined by gamma counting using a Wallac 1470 automated gamma counter (Perkin Elmer). The purity of labeled proteins was determined by size-exclusion high-performance liquid chromatography (HPLC) equipped with a gamma detector. The percentage of free Iodine-125

DMD/2011/043604

was less than 2% in all preparations. The integrity of labeled proteins was also analyzed with a product-specific immunoassay (Quantikine IVD human erythropoietin kits, R&D system, Minneapolis, MN) and all labeled proteins showed comparable concentration-response curves to corresponding unlabeled materials.

For in vivo dosing, the dosing solution was prepared by mixing unlabeled compound, ^{125}I -labeled compound and formulation buffer to achieve the desired specific activity and final protein concentration.

In vivo studies in SD rats and Beagle dogs

All protocols were approved by the Merck Institutional Animal Care and Use Committee.

Thoracic lymph duct cannulation (LDC) in rats

Male Sprague Dawley (SD) rats (350-400 g, Taconic, Germantown, NY) were used for all rat lymph cannulation studies. Rats were given 0.5-1.0 ml of olive oil by oral gavage, 0.5-1 h prior to the surgery to provide enhanced visualization of lymph vessels. The thoracic duct cannulation technique was based on previous reports (Lee and Hashim, 1966; Ionac, 2003) with modifications for continuous lymph collection.

Briefly, rats were anesthetized with isoflurane. The thoracic duct was located and separated from the psoas muscle and dorsal aorta. The duct was cannulated with a heparin (500 units/ml) saline filled catheter. All accessory branches into the main lymph duct were either bypassed by the cannula tip or ligated. Heparin (500 units/ml) was also infused at a constant flow rate of 50 $\mu\text{l/h}$ into the catheter which contains collected lymph to prevent the coagulation of lymph fluid ex vivo and therefore maintain the free flow of lymph fluid. Once the lymph flow had been established, the cannula was tied down by silk ligatures tunneled subcutaneously and

DMD/2011/043604

exteriorized at the back between the shoulder blades. A spring tether system was installed and attached to a swivel to allow free movement for the animals. Following the surgical procedure, the rats were monitored until they regained full consciousness. The rats were provided with *ad libitum* rodent chow and recovery gel (Diet Gel, Clear H₂O). There was a 48 h period of recovery and stabilization with continuous lymph fluid collection, prior to the study initiation.

For in vivo dosing, ¹²⁵I-labeled PEG30-EPO (25 µg/kg, 13.5 µCi/kg) or PEG40-EPO (36 µg/kg, 14 µCi/kg) was administered SC to the lateral left lower hind leg (just below the knee), of the thoracic LDC rats as well as sham operated ones. Thoracic duct lymph was continuously collected for all cannulated animals during the entire 7 day study period and the volumes were recorded. The lymph flow ranged from 0.5-5 ml/h for LDC rats and the total volume of lymph collected per animal ranged between 25-650 ml during the study period. Only data from animals with total collected lymph volume of > 70 ml were used. Series of blood samples were collected for all animals from the carotid artery via implanted cannula at predefined time intervals and serum was prepared. Lymph and serum samples were stored at -70°C until analysis.

For the study with PEG30-EPO, the animals were kept in metabolic cages and urine and feces samples were also collected.

Thoracic lymph duct cannulation (LDC) in Beagle dogs

Thoracic LDC in Beagle dogs were carried out similarly to that in SD rats. Briefly, adult male Beagle dogs were surgically prepared 16 h prior to dosing. The lymph duct was cannulated close to the thoracic duct ampulla with a silastic catheter which was tunneled under the skin to a small incision over the animal's shoulder region and exteriorized. All accessory branches into the main lymph duct were either bypassed by the cannula tip or ligated. Following the surgical procedure,

DMD/2011/043604

the dogs were monitored until they regained full consciousness. Electrolyte mix was administered SC to the dogs to help compensate for the liquid loss and maintain the lymph flow. The lymph flow after surgery was monitored and it ranged from 1-50 ml/h for LDC dogs and the total volume of lymph collected per animal ranged between 10-700 ml during the study period. Only the animals that maintained a stable lymph flow at the time of study initiation were used for the following studies.

For in vivo dosing, ^{125}I -labeled PEG40-EPO (10 $\mu\text{g/kg}$, 7.5 $\mu\text{Ci/kg}$) was administered either IV or SC (at the lateral left lower hind leg, just below the knee) to thoracic LDC animals. Thoracic duct lymph was continuously collected and the volumes were recorded. Only data from the two animals with sustained lymph flow for > 48 h were used. Blood samples were also collected from the jugular vein at predefined time intervals and serum was prepared. Lymph and serum samples were stored at -70°C until analysis.

PK study in "fat rats" to assess impact of fat on SC absorption

"Fat rats" were obtained by feeding 2-3 months old regular SD rats (~ 250 g) with high-fat diet for ~ 3 months. These "fat rats" had an average body weight of 500-600 g, approximately double the body weights of their normal counterparts. The back (scruff of the neck) was used as the SC injection site in this study, where the "fat rats" had significantly more SC fat, as evidenced by the skinfold thickness. To determine the F, 36 $\mu\text{g/kg}$ of either PEG30-EPO or PEG40-EPO was administered IV and SC in the "fat rats" ($n = 3/\text{IV}$ group; $n = 6/\text{SC}$ group). As a control, the F of PEG40-EPO was also determined in the regular rats following 36 $\mu\text{g/kg}$ IV and SC administration ($n = 6/\text{group}$). Blood samples were obtained from the jugular vein at specific time points. Serum samples were prepared and stored at -70°C until analysis.

DMD/2011/043604

PK study in Beagle dogs to assess impact of physical activity on SC absorption

Beagle dogs with apparent different physical activity levels were used in the study. The dog's physical activity levels were divided into two categories based on cage side observation: high = excited barking, frequent jumping up and down, and vigorous tail wagging; low = quiet, usually laid down with occasional tail wagging.

To evaluate the impact of physical activity on SC absorption, ^{125}I -labeled PEG30-EPO or PEG40-EPO (10 $\mu\text{g}/\text{kg}$, $\sim 10 \mu\text{Ci}/\text{kg}$) was administered SC to the posterior dorsal region, $\sim 10 \text{ cm}$ in front of the tail. A modified hand-held Sodium Iodine (NaI) detector was used to monitor disappearance of radioactivity from the injection site with minimal interference of signals from internal organs such as the thyroid. The NaI detector was collimated with lead to limit the detection area to the injection site. Upon administration, the injection site was monitored at specific time points and radiation measurements were recorded. Blood samples were obtained from the jugular vein at specific time points. Serum samples were prepared and stored at -70°C until analysis.

Analytical method and PK analysis

When ^{125}I -labeled compounds were used for animal studies, the total radioactivity levels in lymph, serum, urine or feces samples were first determined by gamma counting (pre-TCA). The protein-associated radioactivity was subsequently quantified by Trichloroacetic acid (TCA) precipitation (Bensadoun and Weinstein, 1976). The TCA precipitation was conducted as described previously (Vugmeyster et al., 2010). Serum or lymph protein drug levels were calculated from TCA precipitable (post-TCA) radioactivity using the specific activity of the dosing solutions with correction for ^{125}I decay ($\text{ng-eq}/\text{ml}$). The %TCA of a sample, calculated as $\text{post-TCA}/\text{pre-TCA} (\%)$, is an indication of integrity of the labeled protein, i.e. what percentage

DMD/2011/043604

of the radioactivity is associated with protein, instead of being free iodine or very small degraded fragments.

When unlabeled compounds were used for animal studies, the serum drug levels were determined by an immunoassay based on Quantikine IVD human erythropoietin kit (R&D systems), and validated for quantifying PEG30-EPO and PEG40-EPO. The assay range was established with spiked quality control (QC) samples. The accuracy and precision were within $100 \pm 20\%$ and lower limit of quantitation (LLOQ) was 0.1 ng/ml. The drug levels of selected ^{125}I -labeled PEG30-EPO and PEG40-EPO serum/lymph samples were also determined by the immunoassay as a comparison to those calculated from post-TCA radioactivity. The results from both assays generally agree with each other and the differences were less than 40%.

The serum drug concentrations were used for noncompartmental PK analysis using WinNonlin (Enterprise Version 5.2.1, Pharsight Corp, Mountain View, CA). Both PEG30-EPO and PEG40-EPO exhibited approximately linear PK over the dose ranges ($>10 \mu\text{g/kg}$) used in our studies, and the systemic bioavailability (F, %) was calculated as: $\text{AUC}_{\text{SC}} * \text{Dose}_{\text{IV}} / \text{AUC}_{\text{IV}} * \text{Dose}_{\text{SC}}$.

In vitro stability in rat SC tissue homogenate and lymph node cell suspensions

The SC tissue from the back area of naïve SD rats was excised and isolated with a scalpel. After mincing with a scissor, the SC tissue was mixed with 1.5x volume of tissue weight of ice-cold homogenization buffer (50mM Tris-HCl at pH7.4, 150mM NaCl, 0.25M Sucrose, 1% Tween 20, 1% Triton X-100) and homogenized with a polytron homogenizer (Kinematica Inc., Newark, NJ) on ice. The lysate were centrifuged at $14,000 \times g$ at 4°C for 20 min. The supernatant was removed for further analysis.

DMD/2011/043604

For lymph node cell suspensions, multiple lymph nodes from naïve SD rats were excised. The cells were released by gently teasing using a tweezer and then collected by straining through 40 μ m cell strainers (BD Falcon, Franklin Lakes, NJ). The number of viable cells was determined by Trypan Blue dye exclusion with a hemacytometer (Gibco Cell Culture, Invitrogen, Calsbad, CA). The specified numbers of viable cells were incubated with 125 I-labeled PEG30-EPO or PEG40-EPO in a 6-well tissue culture plate in RPMI-1640 medium with 10% fetal calf serum, 5% CO₂ at 37°C for 24 h.

For in vitro stability experiments, 125 I-labeled compounds were spiked into freshly prepared SC tissue homogenate, serum, lymph fluid or lymph node cell suspensions, and incubated at 37°C for 24 h. Following incubation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate potential degradants and the bands were detected by phosphorimaging (GE HealthCare, Piscataway, NJ).

DMD/2011/043604

Results

Role of lymphatic system in SC absorption of PEG30-EPO and PEG40-EPO in rats

To determine the contribution of lymphatic system in the SC absorption of macromolecules in commonly used preclinical species, we first used a rat thoracic LDC model established in-house. This model allows continuous thoracic duct lymph fluid collection in conscious and unrestrained SD rats for up to 7 days, considerably longer than what had been reported previously (Kagan et al., 2007; Kaminskas et al., 2009).

Prior to carrying out experiments in LDC rats, we examined the PK profiles of ^{125}I -labeled PEG30-EPO and PEG40-EPO, where the drug levels were calculated from post-TCA radioactivity, and found them to be similar to the corresponding PK profiles of unlabeled compounds, where the drug levels were determined by a product specific immunoassay: for PEG30-EPO, C_{\max} was 114 ± 30 vs. 73 ± 26 ng/ml and $\text{AUC}_{0-168\text{ h}}$ was 7649 ± 1566 vs. 7059 ± 3325 h*ng/ml following 25 $\mu\text{g/kg}$ SC dosing in rats; for PEG40-EPO, C_{\max} was 85 ± 42 vs. 84 ± 26 ng/ml and $\text{AUC}_{0-168\text{ h}}$ was 5013 ± 3635 vs. 5193 ± 1493 h*ng/ml following 36 $\mu\text{g/kg}$ SC dosing in rats. These results suggested that ^{125}I -labeling did not significantly change the PK properties of PEG30-EPO and PEG40-EPO, and that the drug levels calculated from post-TCA radioactivity can generally be considered as representative of those determined by immunoassay. We also determined the systemic bioavailability of PEG30-EPO and PEG40-EPO following SC administration using unlabeled compounds in intact control SD rats, and they were 38% and 30%, respectively (Table 1). In addition, our PK studies with unlabeled compounds had demonstrated that the clearance of PEG40-EPO is linear between 4-36 $\mu\text{g/kg}$ in rats (2.0-2.2 ml/kg/h). The clearance of PEG30-EPO (CERA) was also reported to be approximately linear between 2.5-25

DMD/2011/043604

μg/kg in rats and 3-7.5 μg/kg in dogs (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000739/WC500033669.pdf).

The choice of SC injection site is very important for studying lymphatic contribution in rat thoracic LDC models. For all described LDC rat studies, we used lower hind leg (just below the knee) as the SC injection site to evaluate the contribution of lymphatic system since this is the only SC site that most of the lymph fluid would drain through the thoracic LDC site (Tilney, 1971). ¹²⁵I-labeled PEG30-EPO was dosed SC in the lateral left lower hind leg in the thoracic LDC rats (n=6) as well as sham operated control ones (n=3). PEG30-EPO levels (ng-eq/ml) in the serum were calculated from post-TCA radioactivity levels and the serum concentration-time profiles were shown in Fig 1A. Both C_{max} and serum exposure (AUC_{0-168 h}) of PEG30-EPO were significantly reduced (~70%) in the thoracic LDC rats compared to the sham operated control ones (Fig 1A). The serum and lymph concentration-time profiles from the thoracic LDC rats were compared in Fig 1B. During the absorption or ascending phase of serum concentration profiles (< 24 h), the drug levels were significantly higher in the lymph than that in the serum. Even though no IV study was conducted in LDC animals, the comparison between lymph and serum concentration-time profiles demonstrated that the drug exposure in lymph preceding that in systemic. This result clearly indicated that the observed lymph radioactivity at early time points (< 24 h) mostly came from SC absorption, not distribution.

We also collected radioactivity in the lymph over the 7-day study period, and the results are summarized in Table 1. When the total radioactivity (pre-TCA) was examined, about 31% (31.1±3.7%) of the dose was recovered in the thoracic duct lymph. When the post-TCA radioactivity (protein-associated) was counted, about 24% (23.8±3.5%) of the dose was recovered. The profiles of post-TCA cumulative lymph recovery in individual animals are also

DMD/2011/043604

shown in Fig 1C. Based on the post-TCA lymphatic recovery, the lymph contribution was estimated to be ~60-70% of what was available systemically in intact animals (F of ~38%). This estimate is consistent with the observed ~70% reduction of serum exposure and consequently the reduced F (to 10%) of PEG30-EPO in the thoracic LDC rats.

As shown in Table 1, nearly 80% of dosed radioactivity was recovered, primarily in urine and minimally in feces, in intact control animals. This result indicates that at least 80% of this protein drug was absorbed from the injection site. The total radioactivity recovery (lymph + excreta) in the LDC animals was comparably high (~72%), with nearly 40% of the dose recovered in urine and ~30% of dose in the lymph (Table 1). Considering that the total radioactivity recoveries were significantly higher than the observed F of ~38% in intact animals, these results suggest potential catabolism of PEG30-EPO following SC administration prior to reaching systemic circulation.

¹²⁵I-labeled PEG40-EPO, a PEGylated EPO produced in-house, was also examined in the rat thoracic LDC model (n=3). Similar to the observation with PEG30-EPO, ~70% reduction in C_{\max} and serum exposure ($AUC_{0-168\text{ h}}$) of PEG40-EPO in the thoracic LDC rats was observed (Fig 2A). Similarly, the post-TCA radioactivity levels were also significantly higher in the lymph than in the serum during the absorption phase (< 24 h), supporting that the observed lymph radioactivity at early time points came from SC absorption, not distribution (Fig 2B). Additionally, significant amount of dosed radioactivity (about 20% as pre-TCA and up to 17% as post-TCA) was recovered in the lymph over the studied period, which represents ~60% of the F value of ~30% (Table 1, Fig 2C).

Catabolism following SC administration in rats

DMD/2011/043604

We next examined potential catabolic activities following SC administration as possible causes for the significantly lower than expected F , based on the total radioactivity recovered in rats. We first assessed potential catabolic activity at the SC injection site using freshly prepared rat SC tissue homogenate from naïve animals. As shown in Fig 3A, distinctive degradation products were observed for both PEG30-EPO and PEG40-EPO following the 24 h incubation, suggesting that there were potential catabolism of these molecules at the SC injection site. The observed catabolic activity appeared to be specific to the SC tissue, as similar experiments with freshly prepared rat serum or lymph did not reveal any catabolic activity when incubated with PEG30-EPO and PEG40-EPO (data not shown). PEG40-EPO appeared to have slightly more breakdown fragments than PEG30-EPO, but no apparent degradation was seen for vast majority (90-95%) of both compounds over the 24 h incubation period (Fig 3A). Multiple SC tissue homogenate preparation methods had been evaluated, and the one used here exhibited the highest catabolic activity. Despite our efforts to avoid it, potential loss of catabolic activity during the SC tissue homogenization and/or incubation processes could not be ruled out.

We also investigated potential catabolism during the lymphatic transport as another possible mechanism for the relatively low F in rats. Dr. Charman had presented evidence of catabolism during lymphatic transport for hGH (Charman et al., 2000), but it has remained the only such example. In this study, we first examined the lymph collected from the thoracic LDC rats following SC administration. Interestingly, considerable amounts of non-TCA precipitable, dose-related radioactivity were found, especially during early time points (i.e. < 8 h) (Fig 3B). The presence of non-TCA precipitable radioactivity in the lymph suggested potential catabolism during lymphatic transport, since these non-TCA precipitable, small fragments were not expected to be absorbed via the lymphatic system if they were generated at the SC site. It was

DMD/2011/043604

also interesting to note that the %TCA for PEG30-EPO was considerably higher than that of PEG40-EPO at early time points (<8 h, $p < 0.01$) (Fig 3B), which suggested that the extent of catabolism during lymphatic transport could be compound-dependent.

Consistent with the previous report for hGH (Charman et al., 2000), we did not find any catabolic activity when freshly prepared rat lymph was incubated with PEG30-EPO and PEG40-EPO (data not shown). To investigate the potential source of catabolism during lymphatic transport, we prepared lymph node cell suspensions as described in Materials and Methods. Lymph node cell suspensions instead of lymph node homogenate was used because we anticipated that the catabolic activity likely came from live phagocytic cells residing in the lymph nodes. As shown in Fig 3C, profound catabolic activity was observed when lymph node cell suspensions were incubated with 125 I-labeled PEG30-EPO and PEG40-EPO under cell culture conditions: the amounts of protein corresponding to the original product were significantly reduced following the 24 h incubation with 2×10^6 live cells, and there was almost none left following the incubation with 17×10^6 live cells. Again, PEG40-EPO appeared to disappear slightly faster than PEG30-EPO (Fig 3C). No distinctive degradant was observed following the incubation with lymph node cell suspensions, which was different from the SC tissue homogenates.

SC absorption in "fat rats"

We next examined whether SC absorption in "fat rats" was similar to that in regular rats, because adipose tissue had been associated with poor lymphatic drainage (Ryan, 1995; Swartz, 2001) and body weight is a common negative covariate for F following SC administration in humans (Macdougall et al., 1991; Silva et al., 2006; Olsson-Gisleskog et al., 2007).

DMD/2011/043604

A comparison of the PK profiles of PEG40-EPO in "fat rats" and regular rats following IV and SC administration is shown in Fig 4. Since the "fat rats" had ~2-fold higher body weights than their normal counterparts, they received approximately two times more of the drug when a body weight-normalized dose (36 µg/kg) was given. Consistent with the finding that obese rats have comparable blood volume and lower blood volume-to-body weight ratio than regular rats (Schreihofer et al., 2005), the serum exposure of PEG40-EPO following IV administration was ~2-fold higher in "fat rats" than that in regular rats (Fig 4). However, PEG40-EPO exhibited ~2-fold lower exposure in the "fat rats" following SC administration, despite that ~2-fold more of the drug was administered (Fig 4). Overall PEG40-EPO exhibited significantly lower F in "fat rats" when compared to regular rats (6% vs. 25%).

The F of PEG40-EPO was also determined in "fat rats" following IV and SC administration, and the F was found to be ~19% (data not shown), which was significantly lower than the reported F of 31-45% in regular rats (http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-_Scientific_Discussion/human/000739/WC500033669.pdf) though the difference between fat rats and regular rats appeared to be less for PEG30-EPO (~2-fold) than that for PEG40-EPO (~4-fold).

SC absorption of PEG40-EPO in dogs

We also conducted limited studies in Beagle dogs to determine whether the SC absorption mechanism is conserved across species. First, ¹²⁵I-labeled PEG40-EPO was used to study the contribution of the lymphatic system in SC absorption using a thoracic LDC model in Beagle dogs. The results obtained in LDC dogs were similar to those obtained in LDC rats, despite

DMD/2011/043604

limited number of animals being used (one following SC administration, and one following IV administration) (Fig 5).

Following SC administration, the serum exposure and C_{\max} of PEG40-EPO were reduced significantly in the thoracic LDC dog, as compared to the control dogs (Fig 5A). Additionally, the post-TCA radioactivity levels in the lymph were significantly higher than those in serum, especially during the absorption phase (Fig 5B). Overall, 20% of the dosed radioactivity (post-TCA) was recovered in the thoracic duct lymph over the 7-day study period after SC administration.

In the thoracic LDC dog which received an IV dose, the serum PK profile only appeared to deviate significantly from that of the control animals post 72 h (Fig 5C). In contrast to all the SC studies in LDC animals, the post-TCA radioactivity levels in lymph was considerably lower than that in serum at all early time points (Fig 5D), consistent with the expectation that the lymph radioactivity came from distribution following IV administration. In this LDC dog, much lower radioactivity recovery (~6% after IV vs. 20% after SC) was recovered in the lymph, presumably through distribution. Although it was difficult to quantify the relative difference in the lymph recovery following the IV vs. SC doses, as we examined only one LDC dog in each arm, the lymph recovery results nevertheless are in line with the differences observed in the plasma profiles between the two administration routes and suggested that the lymphatic system played an important role in the SC absorption, not just solely in redistribution, of this macromolecule in dogs.

We also examined the impact of physical activity, another physiological factor pertinent to lymphatic transport, on the SC absorption in dogs, because physical activity is known to have a direct impact on the lymph flow rate (Downey et al., 2008; Swartz, 2001) and it is possible to

DMD/2011/043604

identify dogs with apparent differences in physical activities (see Materials and Methods). Five dogs with different (high or low) physical activity levels were used in the study. They were dosed SC with ^{125}I -labeled PEG30-EPO (1x high, 2x low) or PEG40-EPO (1x high, 1x low), ~10 cm in front of the tails. This SC injection site was selected to facilitate monitoring of the disappearance of radioactivity from the injection site with minimal interference of the signals from internal organs such as the thyroid. As shown in Figs 6A and 6B, dogs with higher levels of physical activity exhibited a faster disappearance of radioactivity from the injection site for both compounds, and correspondingly significantly faster appearance of radioactivity in systemic circulation and higher C_{max} and serum exposure than the dogs with lower physical activity levels (Figs 6C and 6D). Even though we have yet to establish an objective method to quantify the physical activity levels, these results clearly demonstrated the impact of physical activity on SC absorption in this species.

DMD/2011/043604

Discussion

Using the thoracic LDC models established in-house and PEGylated EPOs as model compounds, we showed that these macromolecules were mostly absorbed via the lymphatic system following SC administration in rats and dogs. The thoracic LDC animals exhibited ~70% lower serum exposure compared to the control animals and up to 70% of the systemically available drugs was recovered in the lymph. Moreover, following SC administration lymph exposure was found to precede systemic exposure in lymph cannulated animals, indicating that the radioactivity in the lymph during the ascending phase mostly came from absorption, not distribution. These results agreed well with previous reports in the sheep lymph cannulation model (Supersaxo et al., 1990; Porter and Charman, 2000; Porter et al., 2001). Taken together, our data and previous reports suggest that the role of the lymphatic system in SC absorption is likely conserved in these preclinical species.

However, our results contradicted a few previous reports, which suggested minimal lymphatic contribution to SC absorption in rats and rabbits (Bocci et al., 1986; Bocci et al., 1988; Kojima et al., 1988; Kagan et al., 2007). Though factors like the success of lymph duct cannulation models or the selection of model compounds, e.g. PEGylated vs. non-PEGylate proteins, could have contributed to the observed differences, one likely explanation for the discrepancy is the choice of SC injection sites. In smaller sized laboratory animals, the selection of SC injection site is important for studying lymphatic contribution. Given the size of a rat, technically it is only feasible to cannulate the thoracic duct at a site just above the cisterna chyli (Ionac M, 2003; Kagan et al., 2007; Kaminskas et al., 2009). The lymphatic drainage routes in adult laboratory rats have been mapped in detail by Dr. Tilney (Tilney, 1971). In contrast to the conventional wisdom, most of the commonly used SC injection sites, including what was used in those

DMD/2011/043604

previous reports (thigh), drained via the brachial, inguinal or axillary lymph nodes, which eventually enter systemic circulation through the subclavian duct, bypassing the thoracic LDC site. The only SC injection site that would drain mostly through the thoracic LDC site is the lower hind leg region (Tilney, 1971).

Using the lower hind leg area as SC injection site, we found that the cumulative recovery of dose in the thoracic duct lymph was much higher than what had been reported previously, up to 28% of the dose, or up to 70% of the systemically available dose. Even when the lower hind leg region was used as the SC injection site, a fraction of the dose was still expected to travel down the inguinal lymph nodes -subclavian duct path (Tilney, 1971). Therefore, the ~10% of drugs remaining systemically available in the thoracic LDC rats (Figs 1A and 2A) did not necessarily enter systemic circulation via blood capillaries, but could also have been absorbed via a lymphatic route that bypassed the thoracic LDC site.

Interestingly, the only previous work that reported recovery of significant percentage of SC dosed radioactivity in the thoracic duct lymph of rats also used the lower hind leg area as the SC injection site (Kaminskas et al., 2009). In larger animals such as sheep, it was possible to recover a significant amount of the SC dose in thoracic duct lymph even when injection sites that are other than lower hind legs were used (Kota et al., 2007). One possible reason is that in sheep, one can cannulate the thoracic duct at a position very close to where it enters systemic circulation (Kota et al., 2007). It could also be related to inter-species differences in lymphatic drainage pathways.

In this report, we present direct evidence for near complete absorption of two PEGylated proteins from the injection site following SC administration, and the catabolism at the SC injection site and during lymphatic transport as a likely cause for the limited SC bioavailability. The loss of

DMD/2011/043604

bioavailability following SC administration had mostly been empirically attributed to the loss/degradation at the injection site (Lee, 1988; Mrsny and Daugherty, 2009). Using rat SC tissue homogenates, we confirmed that there indeed was catabolic activity in the SC tissue, at least for the model compounds we examined. Additionally, a previous report had demonstrated significant loss of bioavailability during lymphatic transport of hGH in the sheep lymph-cannulation model, where 61.7% of drug was recovered in the peripheral lymph while only 8.6% of it was recovered in the central lymph (Charman et al., 2000). Here we observed considerable amount of non-TCA precipitable small fragments in the thoracic duct lymph following SC administration, which indicated catabolism during lymphatic transport, as small fragments generated at SC site are expected to be taken up by the blood capillary directly (Porter and Charman, 2000). In our study, we also found high catabolic activity with lymph node cell suspensions, suggesting that the lymph node is a potentially site for loss of bioavailability during lymphatic transport. Lymph nodes are part of our immune system designed to filter and trap foreign particles (Gonzalez et al., 2010). Although our finding is not entirely surprising, to the best of our knowledge, this is the first demonstration of such phenomenon. Interestingly, we encountered high variability in the catabolic activity for our lymph node cell suspension preparations (data not shown). It remains to be determined whether this was due to the technical variability or intrinsic variability of the catabolic activities of lymph nodes.

The differences we observed between PEG30-EPO and PEG40-EPO are interesting, as they suggested that relatively minor differences in molecular characteristics such as glycosylation or the size of PEG, may have an impact on SC absorption/stability. Admittedly, the differences observed here are relatively small and we have no reason to believe that they would be clinically important.

DMD/2011/043604

Many physiological factors such as age, body weight and injection sites have been reported as covariates for SC bioavailability in humans (Macdougall et al, 1991; Chan et al., 2003; Silva et al., 2006; Fishbane et al., 2007; Olsson-Gisleskog et al., 2007; Kakkar et al., 2011). In this investigation, we show that physiological factors such as fat content and physical activity can have a profound impact on SC absorption of PEGylated proteins in preclinical species. The impacts we observed were consistent with their expected impact on the lymphatic transport, i.e. factors associated with faster lymphatic transport are associated with faster absorption and higher bioavailability, and vice versa. In addition to the results presented here, we also found that factors like dose, dosing volume/dosing concentration and injection site, all could affect SC absorption of a macromolecule (data not shown). For all the covariates of SC absorption we evaluated, a common theme is that faster absorption usually leads to higher bioavailability. A recent study on SC absorption of rituximab in rats reported similar findings (Kagan et al., 2012). Though a slower absorption itself should not be the reason for lower bioavailability, it is conceivable that the rate of transport from the injection site and then through the lymphatic system determines the extent of catabolism prior to systemic exposure. For the purpose of comparing bioavailability, these results illustrated the importance of controlling these potential covariants of SC absorption in preclinical and/or clinical studies, as the magnitude of their impact can be significant. Given our observations, we should also actively seek preclinical models more reflective of the intended clinical settings in order to better assess the impact of a particular physiological factor for the compound of interest.

Human PK of biologics drugs following IV administration can usually be predicted with reasonable confidence using allometric scaling (Mordenti et al., 1991; Mahmood, 2004; Wang and Prueksaritanont, 2010), but it is much more challenging to predict their SC absorption

DMD/2011/043604

process in humans. There are fundamental differences in SC tissue structure between preclinical species and humans (Magnusson et al., 2001). Therefore, predicting human PK following SC administration likely will rely on mechanistic understanding of the SC absorption process, rather than empirical scaling methods. For example, in vitro tools such as the SC tissue homogenate and lymph node cell suspensions should be very useful in assessing catabolic activity which could play a major role in determining bioavailability of a given macromolecule compound. These systems not necessarily can be used to quantitatively assessing bioavailability, but rather for comparison purposes and rank-ordering of compounds. They are also one way to address potential inter-species differences in bioavailability associated with inter-species difference in catabolic stability. Interestingly, our results suggest that the impact of physiological factors on SC absorption can be compound-dependent, i.e. the intrinsic "stability" of a molecule can affect its sensitivity to such physiological features. We believe that an appropriate use of preclinical models and in vitro tools should facilitate mechanistic understanding and aid human PK prediction of bioavailability and potential variability. In combination with conventional PK studies in preclinical species, the tools presented here can provide useful information for candidate selection, human PK prediction, clinical study design and risk mitigation for given biological drug candidates.

DMD/2011/043604

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DMD/2011/043604

Authorship Contributions

Participated in research design: W. Wang, Chen, Liu, Hamuro, and Prueksaritanont

Conducted experiments: Chen, Shen, Cunningham, Hong, Fauty, Michel, B. Wang, Adreani, Nunes, Johnson, and Zou

Contributed new reagents or analytic tools: Chen, Yin, and Groff

Performed data analysis: W. Wang, and Chen

Wrote or contributed to the writing of the manuscript: W. Wang, and Prueksaritanont

DMD/2011/043604

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DMD/2011/043604

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DMD/2011/043604

Footnotes

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DMD/2011/043604

Legends for Figure

Figure 1. (A) Mean (SD) serum concentrations (ng-eq/ml, post-TCA) versus time profiles of PEG30-EPO following SC administration in the thoracic LDC rats (n=6) and sham operated control ones (n=3). (B) Mean (SD) serum and lymph concentrations (ng-eq/ml, post-TCA) versus time profiles in the thoracic LDC rats (n=6). (C) Cumulative recovery of dosed radioactivity (post-TCA) in lymph collected from the thoracic LDC rats (mean and individual data).

Figure 2. (A) Mean (SD) serum concentrations (ng-eq/ml, post-TCA) versus time profiles of PEG40-EPO following SC administration in the thoracic LDC rats (n=3) and sham operated control ones (n=3). (B) Mean (SD) serum and lymph concentrations (ng-eq/ml, post-TCA) versus time profiles in the thoracic LDC rats (n=3). (C) Cumulative recovery of dosed radioactivity (post-TCA) in lymph collected from the thoracic LDC rats (mean and individual data).

Figure 3. (A) In vitro stability of PEG30-EPO and PEG40-EPO in rat SC tissue homogenates for 24 h. (B) %TCA of dose related radioactivity in lymph collected from the thoracic LDC rats. (C) In vitro stability of PEG30-EPO and PEG40-EPO in rat lymph node cell suspensions.

Figure 4. Mean (SD) serum concentration (immunoassay, ng/ml) versus time profiles of PEG40-EPO following IV (n=3) and SC (n=6) administration in regular and fat rats.

Figure 5. (A) Serum concentrations (ng-eq/ml, post-TCA) versus time profiles of PEG40-EPO following SC administration in the thoracic LDC (n=1) and control (n=3, mean) dogs. (B) Serum and lymph concentrations (ng-eq/ml, post-TCA) versus time profiles in the thoracic LDC dog following SC administration. (C) Serum concentrations (ng-eq/ml, post-TCA) versus time profiles of PEG40-EPO following IV administration in the thoracic LDC (n=1) and control (n=3,

DMD/2011/043604

mean) dogs. (D) Serum and lymph concentrations (ng-eq/ml, post-TCA) versus time profiles in the thoracic LDC dog following IV administration.

Figure 6. Disappearance of dosed radioactivity from SC injection sites in dogs with different physical activity levels following SC administration (10 μ g/kg, \sim 10 μ Ci/kg): (A) PEG30-EPO (B) PEG40-EPO. Serum concentration (post-TCA, ng-eq/ml) versus time profiles: (C) PEG30-EPO (D) PEG40-EPO. The dog physical activity levels (High or Low) were as labeled in the graph.

DMD/2011/043604

Tables

Table 1. PK parameters of PEG30-EPO and PEG40-EPO in the thoracic LDC and control SD rats following SC administration

	PEG30-EPO(CERA)		PEG40-EPO	
Parameter	LDC (n=6)	Control (n=3)	LDC (n=3)	Control (n=3)
AUC _{0-168 h} (h*ng/mL)	2221 ± 1208	8356 ± 1446	1188 ± 213	5013 ± 3635
C _{max} (ng/mL)	37.5 ± 17.8	113.7 ± 30.0	26.6 ± 6.0	85.1 ± 41.9
Bioavailability (F, %)	10%	38%	10%	30%
Cumulative recovery_ lymph, post TCA (%dose)	23.8 ± 3.5	--	13.7 ± 4.8	--
Cumulative recovery_ lymph, pre-TCA (%dose)	31.1 ± 3.7	--	21.1 ± 4.5	--
Cumulative recovery_ urine, pre-TCA (%dose)	38.2 ± 4.2	75.7 ± 12.5	ND	ND
Cumulative recovery_ feces, pre-TCA (%dose)	1.6	2.2	ND	ND
Cumulative recovery_ Total, pre-TCA (%dose)	71.9	77.9	ND	ND

ND= Not Determined

Figure 1

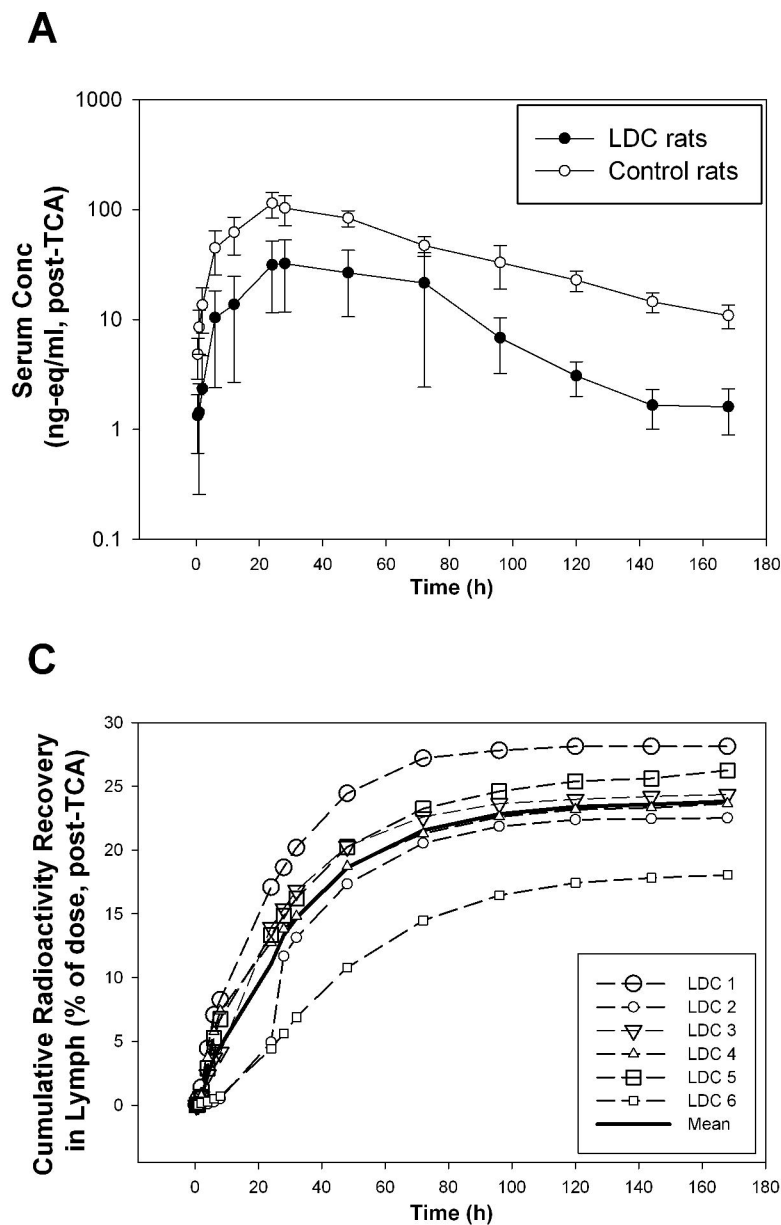


Figure 2

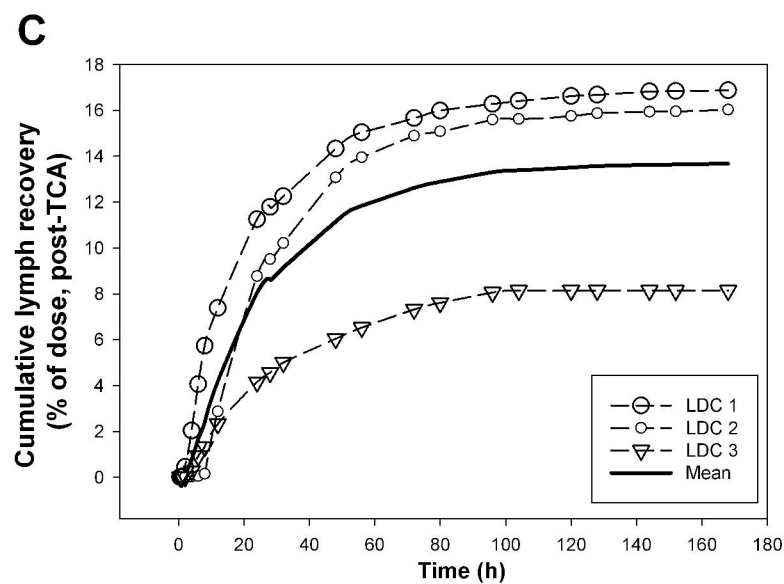
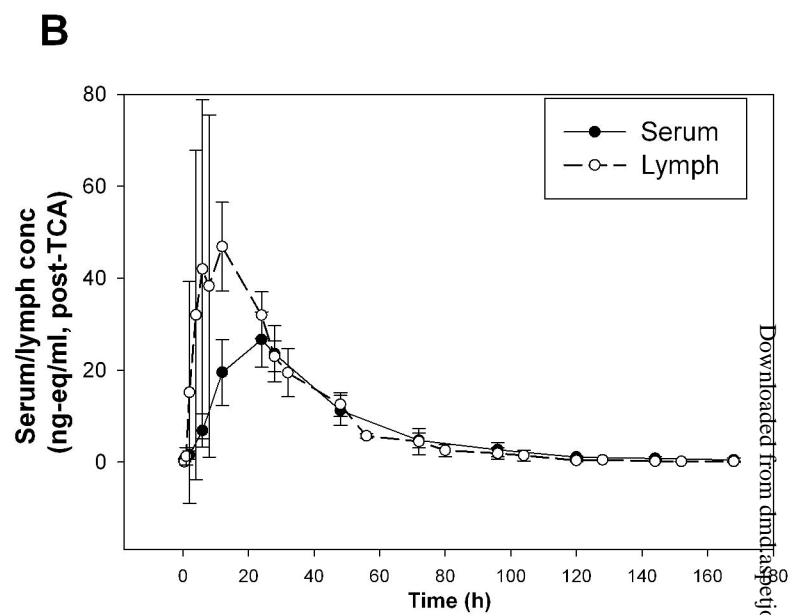
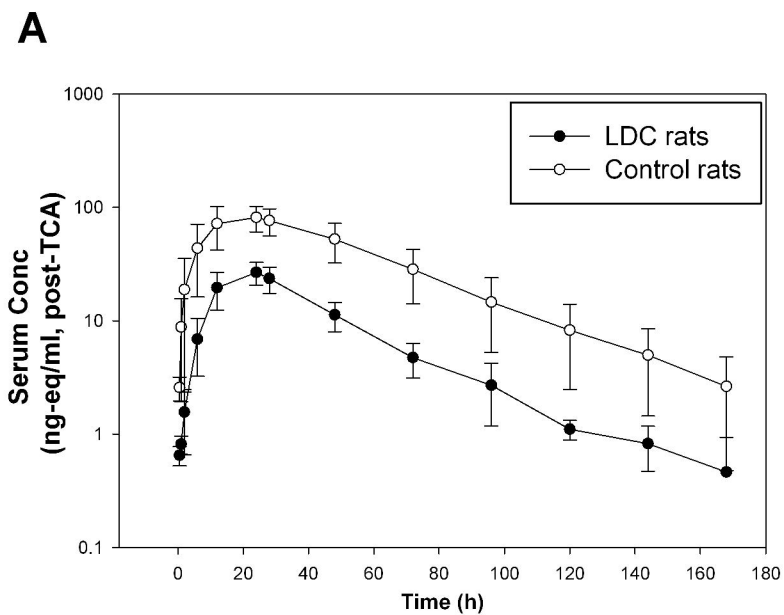
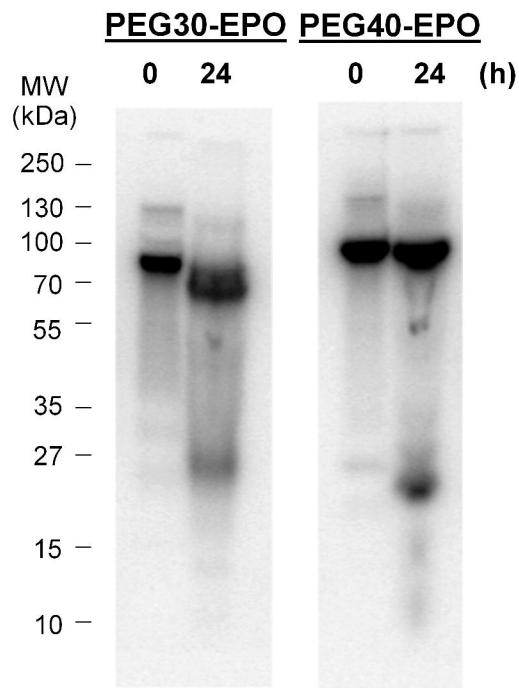
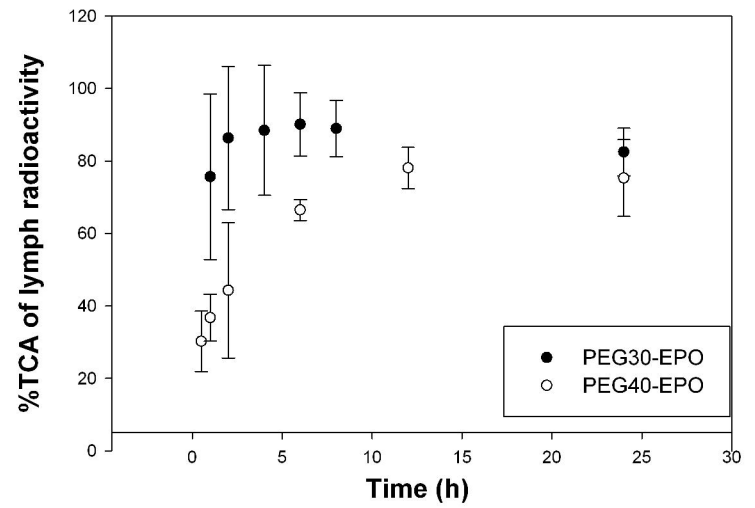


Figure 3

A



B



C

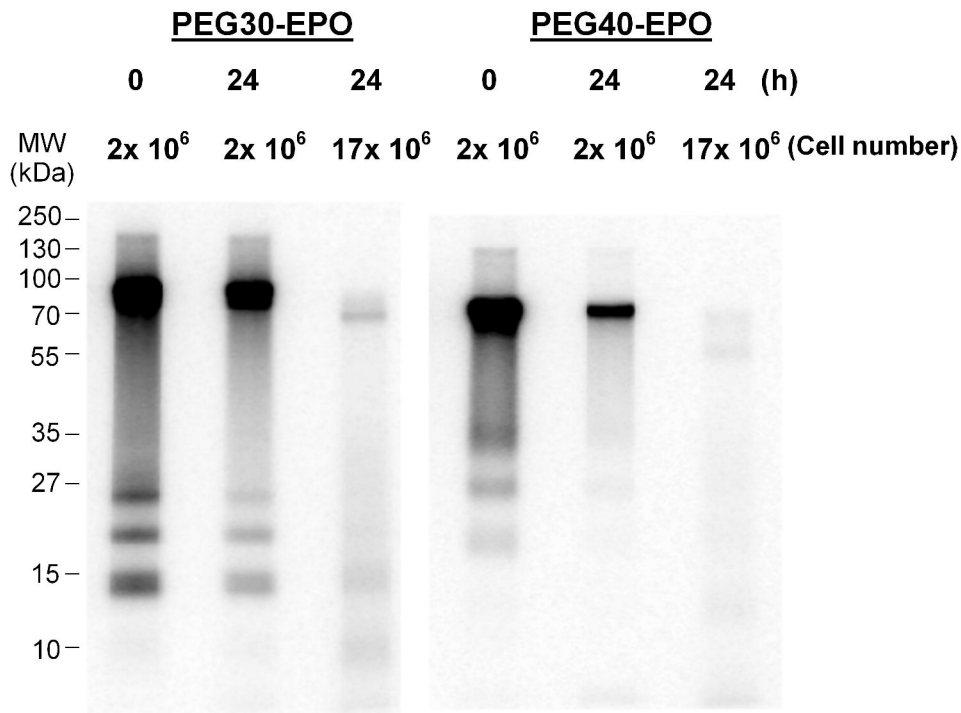


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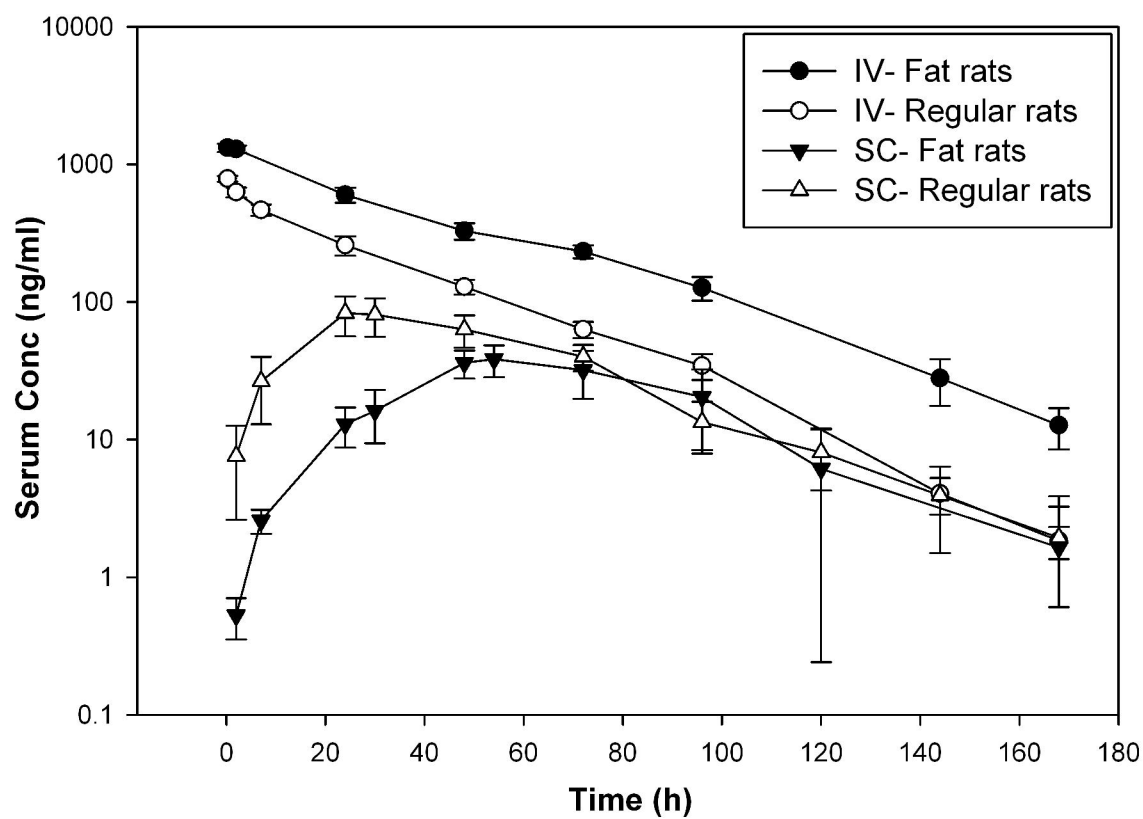


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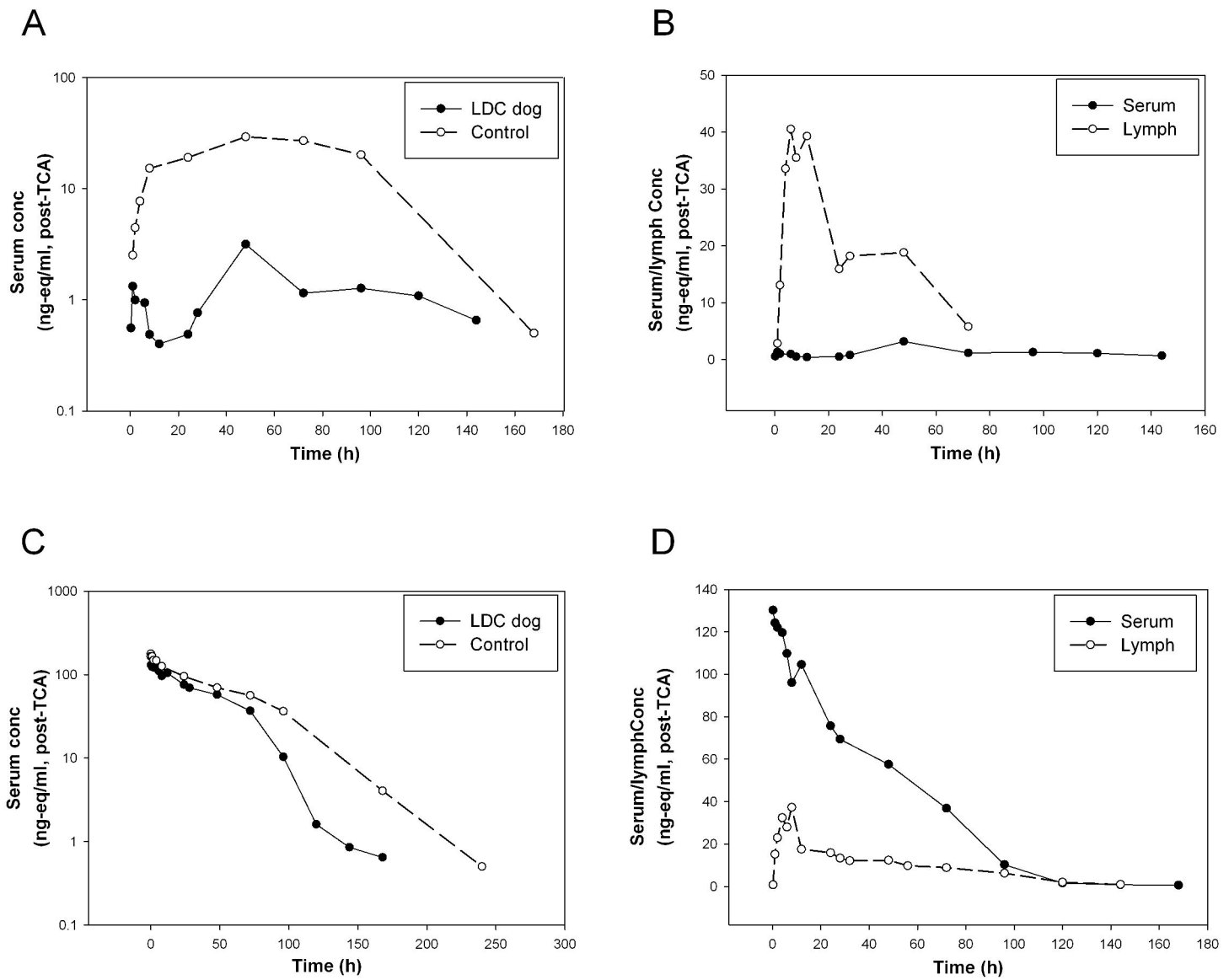
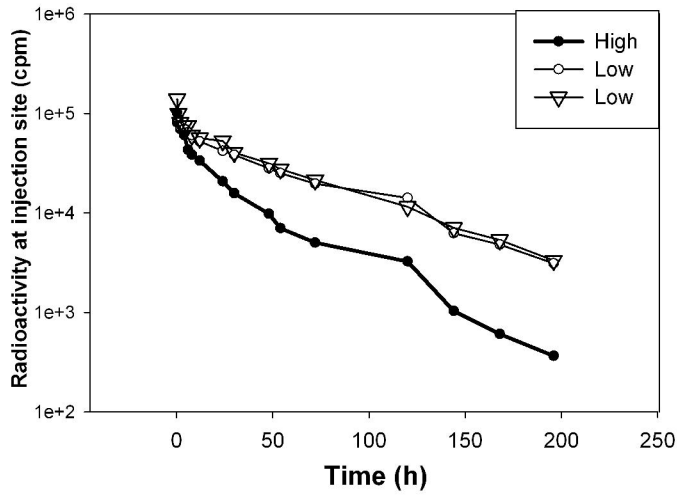
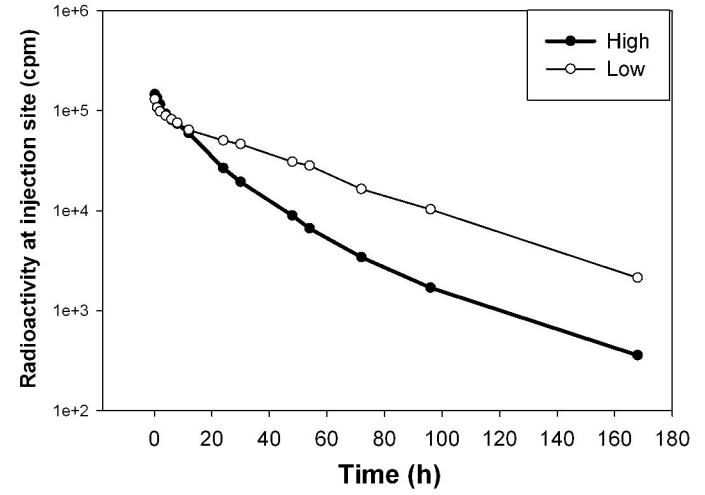


Figure 6

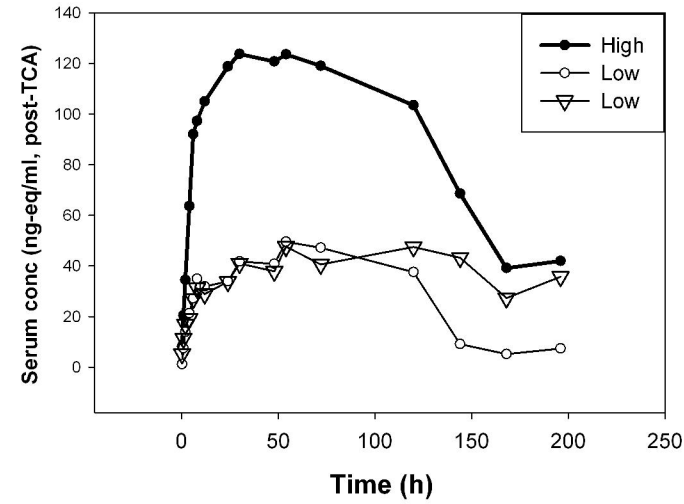
A



B



C



D

