

# Changes in Erythropoietin Pharmacokinetics following Busulfan-Induced Bone Marrow Ablation in Sheep: Evidence for Bone Marrow as a Major Erythropoietin Elimination Pathway

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## ABSTRACT

The contribution of the bone marrow to in vivo erythropoietin (EPO) elimination was evaluated by determining EPO pharmacokinetic (PK) parameters in five adult sheep in a paired manner before and after chemotherapy-induced marrow ablation. After busulfan-induced bone marrow ablation, EPO PK demonstrated progressive decreases in plasma clearance (CL), elimination half-life [ $t_{1/2}(\beta)$ ], and volume of distribution at steady state ( $V_{ss}$ ) with concomitant increases in mean residence time (MRT). Eight days after beginning busulfan treatment, there were no further changes in CL,  $t_{1/2}(\beta)$ , MRT, and  $V_{ss}$ . Only 20% of baseline CL remained by day 8. The volume of distribution ( $V_c$ ) and distribution half-life [ $t_{1/2}(\alpha)$ ], in contrast, remained unchanged from baseline. White blood cell counts and reticulo-

cytes gradually declined after the start of marrow ablation. Examination of bone marrow core biopsy samples obtained on day 10 revealed less than 10% of baseline marrow cellularity. No colony-forming unit erythroid (CFU-E) colonies were found after 6 days of incubation for bone marrow aspirates drawn at days 8 and 13 following busulfan treatment, whereas pre-busulfan aspirates yielded 29 CFU-E colonies per  $10^5$  cells in CFU-E cultures. Treatment of a sheep with 5-fluorouracil showed changes in PK parameters that were similar to the results from treatment with busulfan. The present study indicates that the bone marrow significantly contributes to the elimination of EPO in vivo.

The metabolic fate of erythropoietin (EPO), a heavily glycosylated protein and the primary hormone for erythrocyte production, is still controversial. Although the kidneys and liver are no longer considered the major organs responsible for in vivo EPO elimination (MacDougall et al., 1991; Widness et al., 1996b; Yoon et al., 1997), studies have shown conflicting results regarding the contribution of the bone marrow to in vivo EPO elimination. In vitro studies have demonstrated that EPO is degraded when incubated in the presence of erythroid progenitors having ligand-specific receptors (Mufson and Gesner, 1987; Sawyer et al., 1987). Patients with hypoplastic anemias tend to have higher serum EPO concentrations than patients with other anemias (Hammond et al., 1968; de Klerk et al., 1981; Jelkmann and Wiedemann, 1990). In a recent study in humans, erythroid progen-

itor mass was shown to be a determinant of serum EPO concentration at a given Hb level (Cazzola et al., 1998). In rats, the bone marrow and spleen—both important erythropoietic tissues in this species—have been shown to be actively involved in the elimination of  $^{125}\text{I}$ -rhEPO (Kato et al., 1997). Moreover, the tissue-uptake clearance of  $^{125}\text{I}$ -rhEPO has been shown to be directly related to the number of colony-forming unit erythroids (CFU-Es) present (Kato et al., 1999). In contrast to these findings, several other studies in rodents failed to show differences in the in vivo elimination of EPO with either hypoplasia or hyperplasia of the bone marrow (Naets and Wittek, 1969; Piroso et al., 1991; Lezon et al., 1998).

Because of these discrepancies regarding the role of erythroid progenitors in the bone marrow as a major site of EPO elimination, we sought to evaluate the bone marrow's contribution to the in vivo elimination of EPO. To do so, we compared EPO PK before and after busulfan-induced bone marrow ablation in sheep. Because of the nonlinear PK behavior

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**ABBREVIATIONS:** EPO, erythropoietin; rhEPO, recombinant human erythropoietin; PK, pharmacokinetic; CL, plasma clearance;  $t_{1/2}(\beta)$ , elimination half-life;  $t_{1/2}(\alpha)$ , distribution half-life; MRT, mean residence time;  $V_c$ , initial volume of distribution;  $V_{ss}$ , volume of distribution at steady state; 5-FU, 5-fluorouracil; CFU-Es, colony-forming unit erythroids; BFU-Es, burst-forming unit erythroids.

of EPO (Flaharty et al., 1990; Veng-Pedersen et al., 1995; Kato et al., 1997), tracer doses of biologically active  $^{125}\text{I}$ -rhEPO were used in this work. The sheep was selected because of its similarity in EPO PK with the human and because its size allows accurate PK determination by repeated blood sampling (Mladenovic et al., 1985; Widness et al., 1992, 1996a). We hypothesized that if the bone marrow is of importance in the in vivo elimination of EPO, attenuated elimination of  $^{125}\text{I}$ -rhEPO would be observed after the ablation at the time when the bone marrow cellularity is significantly reduced.

## Materials and Methods

**Study Protocol.** All procedures with animals received prior approval from the local University Animal Care and Use Committee. Animals were housed in an indoor, light- and temperature-controlled environment and were maintained in good health throughout the study period.

Jugular venous catheters were surgically placed a day before the first PK study. Each animal underwent four to five tracer  $^{125}\text{I}$ -rhEPO i.v. bolus PK studies (i.e., one to two baseline studies before busulfan treatment and three to four studies between days 1 and 10 after the commencement of busulfan bone marrow ablation).

The chemotherapy regimen was chosen to ensure ablation of the marrow without jeopardizing the animals' condition. Busulfan was administered orally with the aid of a pill gun twice a day in a dose of 11 mg/(kg-day) for 3 consecutive days. Ampicillin (1 g b.i.d.) was administered daily for the first 3 postoperative days and again daily after beginning chemotherapy. Animals were clinically monitored for adverse effects of chemotherapy such as weight loss, hair loss, blood in urine or stools, fever, unusual bleeding or bruising, and loss of appetite. Busulfan plasma concentration was measured in selected samples as previously described (Bleyzac et al., 2000).

To test a different chemotherapy regimen, 5-fluorouracil (5-FU) was administered to a sheep using the i.v. dosage regimen of 600, 750, 750, 750, and 750 mg on days 0, 2, 4, 8, and 9, respectively. The rate of infusion was 20 mg/min.

White blood cell counts and red blood cell counts were monitored daily using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). Reticulocyte counts and hematocrit levels were also monitored. Whole-blood pH, blood gases, and base excess were measured using an IL-1304 blood gas analyzer (Instrumentation Laboratories, Lexington, MA) corrected to normal body temperature for sheep, i.e., 39°C. Bone marrow core biopsies taken from the humerus were performed to examine cellularity before and after the ablation. To assess CFU-Es and burst-forming unit erythroids (BFU-Es), samples from the bone marrow before and after busulfan treatment were cultured as previously described (Clapp et al., 1991, 1995). Colonies derived from CFU-Es were counted after 6 days of incubation, whereas BFU-E colonies were counted after 9 days of incubation.

**EPO Pharmacokinetic Studies.**  $^{125}\text{I}$ -rhEPO (0.56–1.69  $\times 10^6$  cpm/kg) equivalent to 0.04 to 0.12 U/kg EPO was administered intravenously over less than 30 s. Ten to 15 blood samples were drawn over the ensuing 7- to 8-h period. Blood samples were centrifuged, and the supernatant plasma was kept on wet ice until analysis.  $^{125}\text{I}$ -rhEPO plasma concentration was measured by an immunoprecipitation assay method as previously described (Widness et al., 1992). To minimize erythrocyte loss and avoid the development of anemias, the red blood cells were re-infused following whole blood centrifugation. All the PK studies were performed at approximately the same time of day to minimize diurnal variation in EPO PK.

**Pharmacokinetic Data Analyses.** The EPO plasma concentration profile following the single intravenous bolus dose of  $^{125}\text{I}$ -rhEPO was best described by a biexponential disposition function [ $C(t) = Ae^{-\alpha t} + Be^{-\beta t}$ , where  $\alpha > \beta > 0$ ]. Curve fitting was performed using the general nonlinear regression program FUNFIT (Veng-Pedersen,

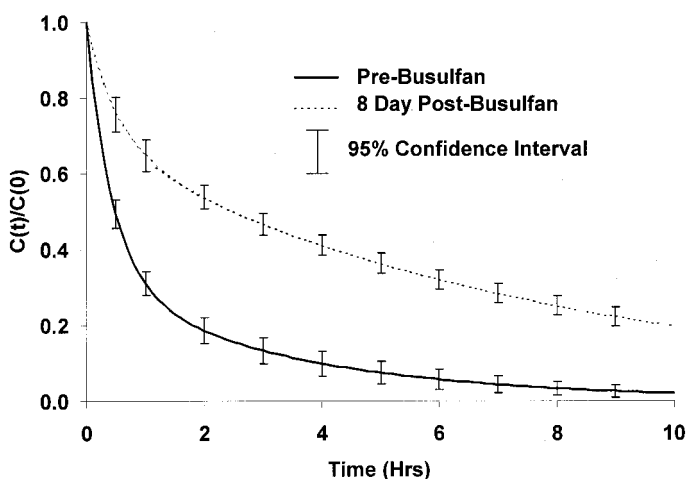
1977). Various PK parameters were calculated using the computer program PERDIS (Veng-Pedersen and Gillespie, 1987). Paired  $t$  tests were performed using SAS/STAT PROC MEANS (SAS Institute Inc., Cary, NC). The significance level used was 0.05.

## Results

Elimination of  $^{125}\text{I}$ -rhEPO was significantly decreased after busulfan-induced bone marrow ablation in all five study animals (Fig. 1, Table 1). Plasma EPO clearance (CL) was reduced to 20% of its pre-ablation value 8 days after busulfan treatment. Elimination half-life [ $t_{1/2}(\beta)$ ], mean residence time (MRT), and volume of distribution at steady state ( $V_{ss}$ ) were also significantly changed ( $p < 0.05$ ), whereas initial volume of distribution [ $V_c = \text{dose}/C(0)$ ] and distribution half-life [ $t_{1/2}(\alpha)$ ] remained unaffected by busulfan treatment. The EPO concentration-time profiles progressively shifted upward after busulfan administration, with no further changes evident 8 days after the start of busulfan treatment (Fig. 2). Plasma EPO CL, MRT,  $t_{1/2}(\beta)$ , and  $V_{ss}$  also showed progressive changes during the first 8 days of busulfan treatment, after which no further changes were observed (Table 2).

Baseline EPO concentrations, independent of the progress of busulfan treatment, ranged from 18.2 to 86.5 mU/ml (Table 3). During the study period, no major adverse clinical effects of busulfan treatment were recognized in all but one study animal, which experienced slight weight loss of <10% from day 4 until day 12 after beginning busulfan treatment. Hemoglobin level, pH,  $pO_2$ , and  $pCO_2$  remained normal throughout the protocol. Bone marrow cellularity decreased markedly in the post-ablated period (Fig. 3). White blood cell counts and reticulocyte counts decreased gradually during the course of the protocol, as shown in Table 3.

No CFU-E colonies were found after 6 days of incubation for bone marrow aspirates drawn at day 8 and day 13 following busulfan treatment. In contrast, pre-busulfan aspirates yielded 29 CFU-E colonies per  $10^5$  cells in CFU-E cultures. Similarly, when those samples were incubated for 9 days, 29, 3, and 0 colonies per  $10^5$  cells in BFU-E culture were observed for the samples drawn on days -1, 8, and 13, respectively. From a concentration-time profile after a dose of busulfan was given, an area under the curve value of 91



**Fig. 1.** Mean plasma  $^{125}\text{I}$ -rhEPO elimination ( $n = 5$ ) pre- and post-ablation. Data were presented as  $^{125}\text{I}$ -rhEPO plasma concentration normalized by the initial plasma concentration after  $^{125}\text{I}$ -rhEPO administration.

TABLE 1  
Comparison of various PK parameters in pre- and post-ablation ( $n = 5$ )

	Pre-Ablation		Post-Ablation (Day 8)		Pre-Post		<i>p</i> Value
	Mean	S.D.	Mean	S.D.	Mean	S.E.M.	
Clearance (ml/kg · h)	40.1	7.4	9.4	2.4	30.6	4.0	0.0015
$V_d$ (ml/kg)	55.4	8.5	52.9	9.9	2.5	1.6	0.1985
$V_{ss}$ (ml/kg)	109.7	21.3	74.7	17.8	34.9	4.3	0.0013
MRT (h)	2.8	0.9	8.0	1.0	-5.2	0.7	0.0014
$t_{1/2}(\alpha)$ (h)	0.3	0.1	0.4	0.1	-0.1	0.1	0.3739
$t_{1/2}(\beta)$ (h)	2.4	0.6	5.7	0.8	-3.3	0.5	0.0021

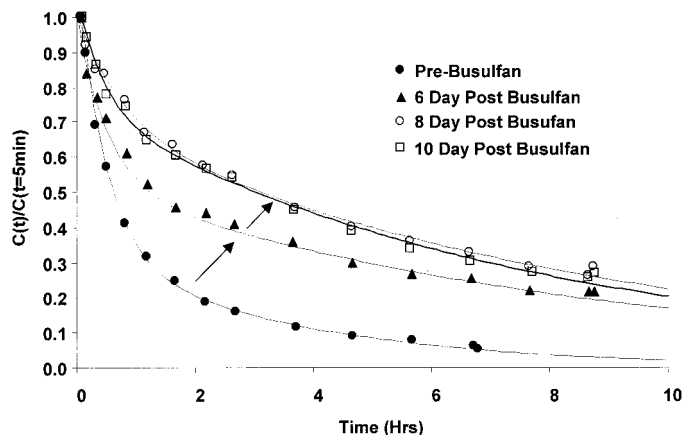


Fig. 2. Representative plasma  $^{125}\text{I}$ -rhEPO elimination profiles in a sheep with progressive changes after busulfan treatment. The effect of busulfan-induced marrow ablation on EPO PK did not change after day 8.

$\mu\text{g/ml} \cdot \text{h}$  was estimated, which is comparable to that of a human at the same dose assuming linear pharmacokinetics (Bleyzac et al., 2000).

For the single adult sheep treated with 5-FU, the changes in EPO PK parameters and concentration-time profiles showed the same temporal ablation patterns that were observed with busulfan (Fig. 4). EPO clearance was reduced to 42% of its pre-ablation value from 20.3 to 8.6 ml/kg · h, and the terminal half-life doubled 10 days after the initiation of 5-FU treatment.

## Discussion

Our finding that  $^{125}\text{I}$ -rhEPO PK was significantly affected by busulfan-induced bone marrow ablation provides strong evidence for the importance of the bone marrow in the in vivo elimination of EPO. The current study design has the following distinct advantages over most previous studies: paired testing in the same animals, administration of tracer amounts of biologically intact labeled EPO, measurement of the labeled EPO using a specific assay method, verification of the marrow ablation by bone marrow biopsies, and determination of complete PK concentration-time profiles allowing accurate estimation of various PK parameters.

Statistically significant changes in CL,  $t_{1/2}(\beta)$ , MRT, and  $V_{ss}$ , but not in  $t_{1/2}(\alpha)$  or in  $V_c$ , indicate that busulfan treatment affected the elimination phase ( $\beta$  phase) of  $^{125}\text{I}$ -rhEPO PK, but not the distribution phase ( $\alpha$  phase). Our finding that  $V_c$  approximated the plasma volume is consistent with findings from other studies. Moreover, the significantly larger  $V_{ss}$  values relative to  $V_c$  suggest slow extravascular transport of EPO. The interesting finding that  $V_{ss}$ , but not  $V_c$ , was reduced by busulfan treatment suggests that bone marrow

erythroid progenitors are of importance in the distribution and binding of EPO in peripheral tissue.

Changes in plasma  $^{125}\text{I}$ -rhEPO levels during the distribution phase, which are determined by the initial  $V_c$  and  $t_{1/2}(\alpha)$ , reflect primarily the movement of drug within—rather than loss from—the body. Once the drug distribution has been established, changes in plasma concentrations are primarily determined by drug elimination. Thus, in the present study, the substantial changes in CL,  $t_{1/2}(\beta)$ , and MRT following busulfan-induced marrow ablation in sheep provide strong evidence that the bone marrow plays a major role in EPO elimination.

The findings of the present study contradict previous studies reporting no significant differences in EPO PK under conditions of bone marrow hypoplasia or hyperplasia (Naets and Wittek, 1969; Piroso et al., 1991; Lezon et al., 1998). Definitive conclusions could not be drawn from those studies for several reasons. First, there may be species differences in linear versus nonlinear EPO PK behavior (Flaharty et al., 1990; Widness et al., 1996b; Kato et al., 1997). In rats, the linear pathway(s) may be more dominant, whereas EPO metabolism in humans is more dependent on nonlinear pathway(s). Second, the present study demonstrates that EPO PK changes occur in a progressive fashion following busulfan treatment before reaching a plateau 8 days after the start of chemotherapy. Therefore, our results show that the proper timing of EPO PK determination is critical to observe significant differences in EPO PK with cytostatic drugs.

The consistency in the result from an antimetabolite, 5-FU, with that of busulfan-induced ablation suggests that the PK changes by hypoplasia are not dependent on the specific chemotherapeutic mechanism of busulfan, which is an alkylating agent. Previous studies that failed to show PK changes with chemotherapy did not provide clear evidence of bone marrow suppression (Naets and Wittek, 1969; Piroso et al., 1991; Lezon et al., 1998). We speculate that EPO PK is closely related to bone marrow cellularity based on the observation that EPO PK was substantially changed at the time of reduced bone marrow cellularity, although a serial bone marrow cellularity assessment at various degrees of ablation was not performed due to technical difficulties.

Although the total plasma clearance of EPO was significantly reduced after bone marrow ablation, some EPO elimination remains, accounting for approximately 20% of the total pre-ablation elimination. Since the contribution of the liver and kidneys to in vivo intact EPO elimination is minimal (MacDougall et al., 1991; Widness et al., 1996b), the question arises regarding the location and nature of the remaining pathway(s). Possible candidates are nonpharmacologic EPO receptors (“silent receptors”) located throughout the body or receptors with unknown pharmacologic roles,

TABLE 2  
Progressive changes in PK parameters following busulfan treatment

Post-Busulfan Day	n	$t_{1/2}$ ( $\beta$ )		MRT		CL		$V_{ss}$	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
		h		h		ml/kg · h		ml/kg	
0	5	2.4	0.6	2.8	0.9	40.1	7.4	109.7	21.3
4–5	2	3.2	0.8	3.9	1.7	27.0	8.4	99.2	12.7
6	3	7.6	1.3	10.2	1.6	10.2	2.1	102.5	16.6
7–8	5	5.7	0.8	8.0	1.0	9.4	2.4	74.7	17.8
10–12	4	5.5	0.3	8.0	0.3	9.6	0.7	73.8	8.4

TABLE 3  
Hematologic parameters and plasma EPO concentration during ablation procedure

Day	WBC Count ( $\times 10^3/\mu\text{l}$ ) (n = 5)		Hb (n = 5)		Reticulocytes (% RBC) (n = 4)		Marrow Cellularity (n = 3) <sup>a</sup>	EPO (n = 3)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.		Mean	S.D.
			g/dl				%	mU/ml	
0	8.3	2.1	10.1	1.5	0.43	0.42	87	37.7	25.4
4	6.6	1.9	10.0	0.9	0.27	0.10		23.5	0.1
8	4.2	0.7	10.0	1.3	0.29	0.07		21.8	3.5
10	5.4	1.1	10.0	1.1	0.21	0.09		23.4	4.9
12	4.5	1.0	9.8	1.2	0.16	0.08	7	43.8	37.1

WBC, white blood cell; RBC, red blood cells.

<sup>a</sup> Values are means.

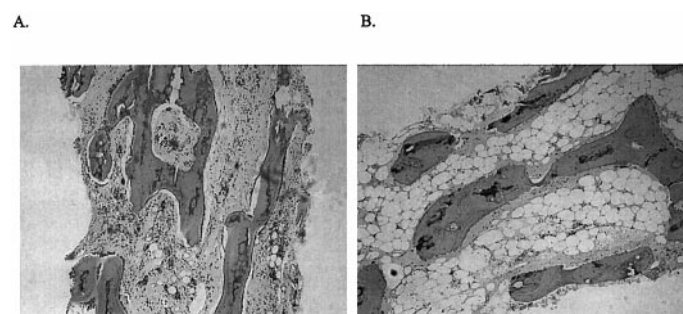


Fig. 3. Representative bone marrow core biopsies before (A) and after (B) bone marrow ablation. Cellularity was markedly reduced after busulfan treatment.

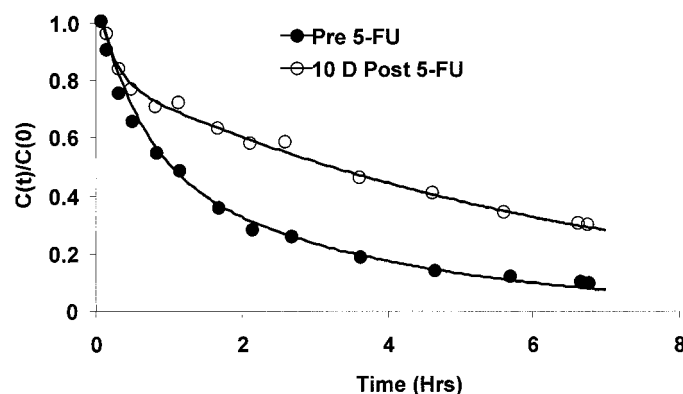


Fig. 4. PK profiles of  $^{125}\text{I}$ -rhEPO before and after 5-FU treatment in a sheep.

and/or enzymatic degradation in blood. The last was previously ruled out due to the stability of EPO in whole blood at room temperature (Kendall et al., 1991; Widness et al., 1996b). However, we observed significant breakdown of EPO in plasma at 37°C, but not in phosphate buffer (N. M. Schmidt, R. L. Schmidt, and J. A. Widness, unpublished data). Although the nonpharmacologic receptors are believed

to be important in some stage of human development (Juul et al., 1998), very little is known about their activity.

In summary, this study provides clear evidence that bone marrow plays a major role in the in vivo elimination of EPO. The remaining minor pathway(s) after the ablation is still in question and calls for further investigations.

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