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J Clin Invest. 1993;91(1):251-256. https://doi.org/10.1172/JCI116178.

Research Article

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Increased Plasma Viscosity as a Reason for Inappropriate Erythropoietin Formation

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Abstract

The aim of this study was to examine whether altered plasma viscosity could contribute to the inappropriately low production rate of erythropoietin (EPO) observed in patients suffering from hypergammaglobulinemias associated with multiple myeloma or Waldenström's disease. We found that the EPO formation in response to anemia in these patients was inversely related to plasma viscosity. A similar inverse relationship between plasma viscosity and EPO production was seen in rats in which EPO formation had been stimulated by exchange transfusion and the plasma viscosity of which was thereby altered by using exchange solutions of different composition to alter plasma viscosity and thus whole blood viscosity independently from hematocrit. Raising the gammaglobulin concentration to ~ 40 mg/ml plasma in the rats almost totally blunted the rise in serum EPO levels despite a fall of the hematocrit to 20%. Determination of renal EPO mRNA levels by RNase protection revealed that the reductions in serum EPO levels at higher plasma viscosities were paralleled by reductions in renal EPO mRNA levels.

Taken together, our findings suggest that plasma viscosity may be a significant inhibitory modulator of anemia-induced EPO formation. The increased plasma viscosity in patients with hypergammaglobulinemias may therefore contribute to the inappropriate EPO production, which is a major reason for the anemia developing in these patients. (*J. Clin. Invest.* 1993. 91:251–256.) Key words: viscosity • erythropoietin • paraproteinemias • gamma-globulins • dextrans

Introduction

Multiple myeloma and macroglobulinemia Waldenström are frequently associated with anemia (1). The pathogenesis of this type of anemia has not yet been unequivocally clarified but may involve bone marrow displacement and/or insufficient stimulation of erythropoiesis. Recent studies have provided evidence that erythropoietin (EPO)[1] levels in those patients are inappropriately low and that this type of anemia can be markedly improved by the application of recombinant human EPO (2, 3). In the adult EPO is predominantly produced by the kidneys, and its production rate is mainly determined by the hemoglobin concentration and the arterial oxygen tension (4). The reasons why patients suffering from hypergammaglobulinemias elaborate inappropriately low amounts of erythropoietin are unknown. Reduced renal function will contribute but does not provide a complete explanation. Another possibility is that the altered blood rheology, namely, the increased plasma viscosity in those patients could be involved. Since at present no information exists about a possible influence of blood viscosity on EPO formation it appeared reasonable to us to examine whether alterations of plasma viscosity can have an influence on the production of erythropoietin.

To this end we have examined the correlation between EPO production and plasma viscosity or renal function in anemic patients suffering from hypergammaglobulinemias. Moreover, we investigated the influence of plasma viscosity on EPO formation in normal rats, in which EPO formation had been stimulated by exchange transfusion.

Our findings indicate that in anemic patients with hypergammaglobulinemias insufficient EPO production is correlated with the increase of plasma viscosity rather than with restriction of renal function. Moreover, we observed an inhibitory effect of increasing plasma viscosity on EPO formation in anemic rats.

Methods

Patients. Patients with documented monoclonal paraproteinemia (Waldenström's macroglobulinemia or multiple myeloma), without major impairment of renal function (serum creatinine < 150 μmol/liter, creatinine clearance > 30 ml/min) and without evidence of chronic inflammatory disease (no fever and leukocyte count ≤ 10,000/μl with essentially normal distribution) were studied. Blood anticoagulated with K_2-EDTA was used. Hematocrit was measured with a microcentrifuge, the platelet count was determined with an electronic particle counter. Plasma was obtained by a first centrifugation at 1,500 *g* for 10 min, followed by gentle aspiration of plasma and a second 10-min centrifugation of the plasma at 1,500 *g*. Plasma viscosity was determined in a Couette-type viscometer (model LS30; Contraves, Zürich, Switzerland) at 37°C and shear rates of 10.2 and 87 s^{-1} and the arithmetic mean was calculated. Plasma samples were deep frozen at −20°C for EPO analysis (see below).

Blood exchange experiments in rats. Male Wistar and Sprague-Dawley rats (body weight 180–350 g) were anesthetized with 10 mg Nembutal intraperitoneally. When required small doses of the anesthetic were given repeatedly during the experiment. The anesthetized animals were placed on a heating table. A rectal temperature probe connected to a thermostat held the core temperature of the animal at 37.0±0.5°C by regulating the heating of the table. An arterial catheter (either femoral or carotid) and a venous catheter (either femoral or jugular) were inserted avoiding as far as possible any blood loss. Blood pressure was monitored by a Statham transducer connected to the arterial catheter. Blood exchange was carried out with one of four different

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Received for publication 24 March 1992 and in revised form 20 July 1992.

1. *Abbreviations used in this paper:* EPO, erythropoietin; hct, hematocrit.

J. Clin. Invest.

0021-9738/93/01/0251/06 $2.00
Volume 91, January 1993, 251–256

solutions: (*a*) rat plasma prepared from donor rats; (*b*) Ringer's solution (130 mmol/liter Na, 4 mmol/liter K, 1.5 mmol/liter Ca, 109 mmol/liter Cl, 28 mmol/liter lactate, 10 g/liter glucose); (*c*) Ringer's solution supplemented with graded doses of rat or bovine gammaglobulin (G 2885 and G 7516; Sigma Chemical Co., St. Louis, MO); or (*d*) Ringer's solution containing 260 g/liter dextran 40 (mol wt ~ 40,000; Pharmacia AB, Uppsala, Sweden).

The plasma exchange was started by gently aspirating 2 ml of blood into a syringe flushed with sodium heparin (10 U/ml), which was used for the determinations of blood gases, plasma viscosity, hematocrit, and EPO. After immediate replacement of this volume with exchange solution, blood exchange was continued over the next 10–20 min by simultaneous blood withdrawal on the arterial side and infusion of the exchange solution on the venous side. After the completion of the exchange (time: zero) an infusion of the same solution as used for the exchange was started with a flow rate of 0.6 ml/h to replace usual fluid loss by perspiration and diuresis. 4 h after the completion of the exchange (time: 4 h) 2 ml of heparinized blood was again aspirated for the measurement of hematocrit, plasma viscosity, EPO, and arterial blood gas analysis.

At the end of the experiment the animals were killed by cervical dislocation or an overdose of nembutal. The kidneys were removed and weighed. One kidney was snap frozen in liquid nitrogen and kept at −70°C for EPO mRNA analysis. The other kidney was cut into thin slices, fixed in Bouin's solution, and later embedded in methacrylate. Semi-thin sections were prepared and examined by a pathologist who was unaware of the experimental procedures.

The hematocrit level was determined with a microcentrifuge. Plasma was obtained by centrifuging the blood for 10 min at 1,500 *g*. Plasma viscosity was measured as described above. Aliquots of the plasma were deep frozen for the determination of EPO.

Determination of serum EPO. Serum EPO concentrations were measured by radioimmunoassay as described (5). For human samples human recombinant EPO was used as standard, for rat samples a rat EPO standard was used for the standard curve.

Determination of EPO mRNA. Kidneys from rats subjected to different treatments were homogenized in guanidine thiocyanate (4 mol/liter) containing sarcosyl (0.5%), EDTA (10 mmol/liter), sodium citrate (25 mmol/liter), and mercaptoethanol (700 mmol/liter). Total RNA was purified by centrifugation for 20 h at 33,000 rpm on a cesium chloride gradient (5.7 mol/liter CsCl and 100 mmol/liter EDTA) and EPO mRNA was measured by RNAse protection as described (6), using a ^{32}P-labeled EPO probe, which was a PstI/SacI fragment of the rat EPO gene, containing 132 bp of exon V and approximately 300 bp of the adjoining intron. In brief, 100 μg of total RNA was hybridized to 0.7×10^6 cpm of radiolabeled probe in 80% formamide/40 mmol/liter piperazine-*N,N'*-bis(2-ethane sulfonic acid), pH 6.4/400 mmol/liter NaCl/liter mmol/liter EDTA at 60°C overnight, and RNase digestion was carried out at 20°C for 30 min. Protected fragments were separated by electrophoresis on a denaturing 10% polyacrylamide gel. Autoradiography of the dried gel was performed at −70°C.

Statistics. Student's unpaired *t* test was used for comparison of groups and analysis of variance to test the significance of linear and exponential regressions. $P < 0.05$ was considered significant.

Results

16 patients with monoclonal paraproteinemia were studied, some of them repeatedly during ambulatory care. They are summarized in Table I. All the patients were anemic (hematocrit 17–38%) but most of them had normal platelet counts. The EPO levels ranged from 15 to 246 mU/ml. The three patients with platelet counts $< 100 \times 10^9$/liter (patients 7, 8, and 15) were those with the highest EPO levels. This suggests a general depression of the bone marrow function by other factors with an ensuing strong stimulation of EPO secretion. Indeed, in patient 7 a very severe bone marrow infiltration of 70–100% was found, in the two others a marked infiltration of 60–80% was combined with vitamin B_{12}-deficiency (Vitamin B_{12}: 19 and 131 pg/ml, respectively, normal values 150–630 pg/ml).

At first sight plasma EPO levels appeared to be relatively low when compared with the degree of anemia and it was therefore of interest to see whether the inappropriately low EPO production correlated with other clinical parameters, in particular plasma viscosity and serum creatinine concentrations. EPO production and plasma EPO levels, however, are normally strongly dependent on the hematocrit and the hematocrit varied in a wide range among the patients under study. To allow a correlation between the reduction of EPO production and other clinical parameters it was necessary therefore to standardize EPO production in relation to the degree of anemia in those patients. Since a number of studies have shown that a negative semilogarithmic correlation exists between plasma EPO concentrations and the hematocrit value in nonrenal anemias (8–10) we considered the value $(\log EPO_{patient} - \log EPO_{normal})/(hct_{patient} - hct_{normal})$ as a standardized parameter which we termed "EPO response." Normal EPO concentration of healthy adults (mean hematocrit 45%) average 17 mU/ml when measured with the radioimmunoassay used for this study (10). For nonrenal anemias average values for the EPO response are in the range of 6 to 10 (8–10). The EPO response for the individual patients in this study was set into relation with the plasma viscosity or with creatinine clearance as an indirect indicator for renal function. In patients with more than one measurement (patients 1–4 and 9) mean values were used. It appeared that the EPO response was correlated with both plasma viscosity and creatinine clearance values (Fig. 1). The best fit between plasma viscosity and EPO-response was obtained with an exponential inverse correlation: EPO response $= 4.4 \times$ plasma viscosity$(cp)^{-1.13}$ ($r = -0.54$; $P < 0.01$). The best fit between EPO response and creatinine clearance was a linear correlation: EPO response $= -0.37 + 0.05 \times$ creatinine clearance ($r = 0.62$, $P < 0.02$).

The question arose as to whether there was a causal link between EPO response and plasma viscosity. To analyze this we have performed a series of experiments with anesthetized rats. In these animals EPO production was stimulated by an acute reduction of the hemoglobin concentration to half normal values induced by volume exchange transfusion (Table II). Volume exchange was performed with solutions of different viscosities such as Ringer's solution, rat donor plasma, or Ringer's solution supplemented with graded doses of bovine gammaglobulin. 4 h after conclusion of the volume exchange plasma EPO levels were measured. As shown in Fig. 2 the increase of plasma EPO levels reflecting EPO release into the circulation was dependent on the solution used for volume exchange. Highest EPO levels were measured with Ringer's solution followed by plasma and Ringer's solution supplemented with gammaglobulin. A sham exchange experiment (replacement with whole blood) did not elevate the EPO level markedly (44 mU/ml).

As with the patients we have calculated the EPO response $(\log EPO_{4\ h\ after\ exch} - \log EPO_{before\ exch})/(hct_{4\ h\ after\ exch} - hct_{before\ exch})$ for the individual rats and have correlated this parameter with the plasma viscosity. As shown in Fig. 3 the EPO response of the rats was inversely correlated with the plasma viscosity in a very similar fashion as with the patients

Table I. Patients with Waldenström's Disease and Multiple Myeloma

Pat No.	Sex	Age	Diagnosis	Serum creatinine	Creatinine clearance	Platelet count	Plasma viscosity*	Hematocrit	EPO
				μmol/liter	*ml/min*	*×10⁹/liter*	*cp*	*%*	*mU/liter*
1	f	74	Waldenström (IgM Kappa)	75	45‡	246	2.4	34.5	18
							1.6	32.5	17
2	f	79	Myeloma (IgG Lambda)	109	35‡	181	4.0	20.5	21
							2.3	22.5	84
3	m	76	Waldenström (IgM Kappa)	133	50‡	128	10.4	22.5	17
							7.4	23.5	17
							7.2	24.0	16
							6.0	16.5	40
							7.8	19.5	17
4	m	54	Waldenström (IgM Kappa)	106	66‡	147	2.8	33.5	21
							9.8	36.5	15
							8.2	35.5	18
							9.2	37.0	21
							6.8	37.0	20
5	f	58	Myeloma (IgA Lambda)	87	60‡	—	1.9	31.5	23
6	m	65	Waldenström (IgM Kappa)	89	72‡	470	3.9	38.0	30
7	m	41	Waldenström (IgM Kappa)	91	110	16	1.5	24.5	208
8	f	63	Myeloma (IgG Kappa)	66	103	89	1.4	26.5	246
9	f	77	Waldenström (IgM Kappa)	107	41‡	—	2.4	36.0	23
							1.4	30.5	18
10	m	83	Waldenström (IgM Kappa)	98	69	263	2.8	25.5	36
11	m	71	Myeloma (IgG Kappa)	117	52‡	110	1.5	24.0	145
12	f	68	Waldenström (IgM Kappa)	80	67‡	174	1.9	29.0	46
13	f	80	Myeloma (IgG Kappa)	87	36‡	128	1.4	31.0	41
14	f	78	Waldenström (IgM Kappa)	125	32	421	1.4	31.0	51
15	m	64	Waldenström (IgM Lambda)	90	71	35	2.2	17.0	180
16	f	74	Myeloma (Light chains)	67	97	430	1.7	25.0	99

* Normal range: 1.1–1.4 cp. ‡ Calculated according to Cockcroft and Gault (7).

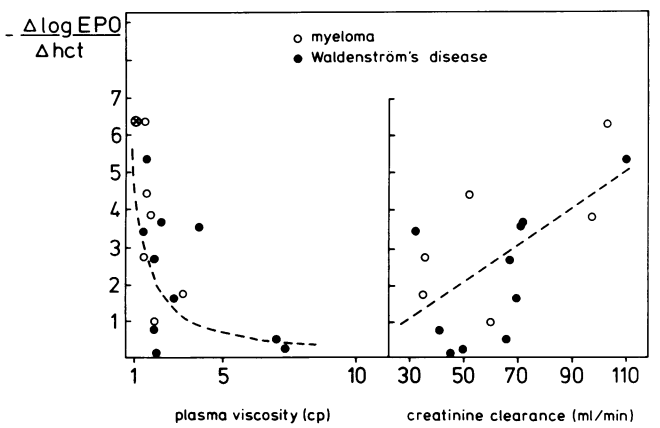

Figure 1. (*Left panel*) Relationship between plasma viscosity and EPO response in patients suffering from multiple myelomas and Waldenström's disease. EPO response is defined as ($\log EPO_{patient} - \log EPO_{normal}$)/($hct_{patient} - hct_{normal}$). The dashed line indicates the regression line. The symbol with a cross represents a value for nonrenal anemias as estimated from reference 10. (*Right panel*) Relationship between creatinine clearance and EPO response in patients suffering from multiple myelomas and Waldenström's disease. The dashed line indicates the regression line.

suffering from hypergammaglobulinemias. The reason for using bovine gammaglobulin for the exchange experiments resulted from the relatively poor availability of rat gammaglobulin. To test for the possibility that bovine gammaglobulin might have had nonspecific effects on EPO production in the rats, three animals were exchanged with Ringer's solution supplemented with 60 mg/ml rat gammaglobulin. In these animals hematocrit fell from 42.5±1.8% before to 22.0±1.8% 4 h after exchange. Serum EPO levels were 21±4 and 55±3 mU/ml (mean±SD) before and 4 h after exchange, respectively. These values are very similar to those obtained with bovine gammaglobulin, suggesting that a possible incompatibility was not the reason for the dose-dependent attenuation of EPO formation by bovine gammaglobulin in the rats. Also other parameters that might interfere with renal EPO formation such as changes of the arterial oxygen (Table II) or carbon dioxide tensions or changes of hemodynamics were not different among the different groups.

Since the plasma viscosity is causally linked with plasma concentration of gammaglobulins, it could not be distinguished whether the attenuation of the EPO response was dependent on the increase of viscosity or due to another effect induced by an increased level of gammaglobulins. We there-

Table II. Exchange Transfusion Experiments in Rats

Exchange with	Hct (%)		Systolic blood pressure		Arterial Po_2		Body weight
	Before exchange	4 h after	Before exchange	4 h after	Before exchange	4 h after	
			mmHg		*mmHg*		*g*
Ringer's	39.4±4.2	23.2±3.1	170.1±7.3	100.7±20.3	90.7±10.1	109.7±6.3	202.0±4.8
Plasma	35.3±14.8	21.5±4.1	174.0±18.2	116.3±17.0	86.7±4.7	112.2±9.4	217.6±23.3
Ringer's + 30 g/liter gammaglobulin	41.6±4.9	20.7±2.0	160.8±22.0	109.2±11.6	80.8±7.3	96.2±9.8	223.8±15.1
Ringer's + 60 g/liter gammaglobulin	41.2±1.9	19.8±2.1	144.2±13.5	96.0±7.4	77.9±7.9	111.5±7.1	189.4±26.5

fore attempted to increase plasma viscosity without increasing the plasma concentration of gammaglobulin. To this end a volume exchange transfusion with Ringer's solution supplemented with 260 g/liter dextran 40 was performed. Exchange transfusion with this solution led to a similar drop in hematocrit as with Ringer's solution alone; the plasma viscosity, however, increased from 1.13±0.07 cp before to 1.75±0.13 cp after exchange. The rise of plasma EPO levels induced by the reduction of the hemoglobin concentration was markedly attenuated in the presence of dextran (Fig. 4).

Histological examinations of the kidneys by light microscopy did not reveal obvious differences among the four groups. (Two experiments with Ringer's solution, two with plasma exchange, three with gammaglobulin [60 mg/ml] and five experiments with dextran). Glomeruli and interstitium were normal in all specimens. Slight tubular necrosis or epithelial swelling was seen in one of five and slight vacuolization of tubular epithelia in four of five dextran-treated rats. Kidneys of rats exchanged with gammaglobulin occasionally showed tubular casts in the medullary region.

The synthesis of EPO by the kidney was assessed by measuring EPO mRNA concentrations in the kidneys 4 h after conclusion of the blood volume exchange. As shown in Fig. 5 renal EPO mRNA levels were diminished if volume exchange was performed with solutions which increased plasma viscosity. Moreover, it was apparent that the diminuation of renal EPO mRNA levels went in parallel with the attenuation of plasma EPO concentrations.

Discussion

Although it is well established that altered blood oxygen availability is the principal stimulus controlling EPO production, in

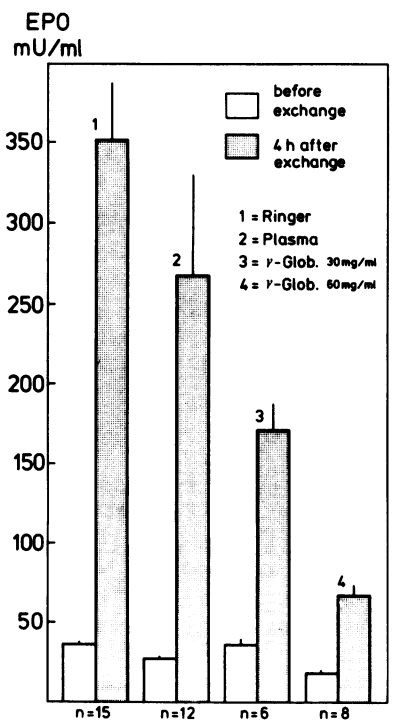

Figure 2. Plasma EPO concentrations in rats before and 4 h after exchange transfusion, which was performed with Ringer's solution, donor plasma, and Ringer's solution supplemented with bovine gammaglobulin. EPO values in animals exchanged with plasma or supplemented Ringer's solutions were significantly lower than those in animals exchanged with pure Ringer's solution.

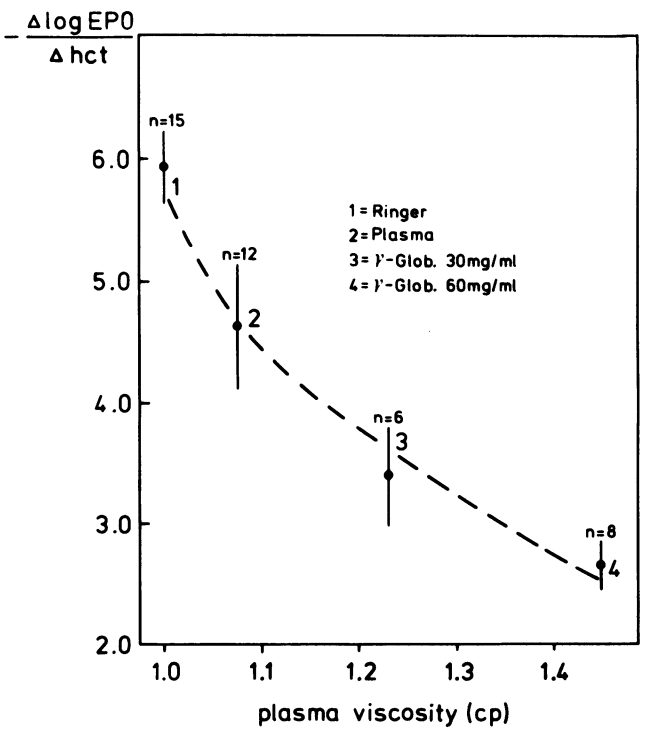

Figure 3. Relationship between EPO response and plasma viscosity in rats 4 h after exchange transfusion.

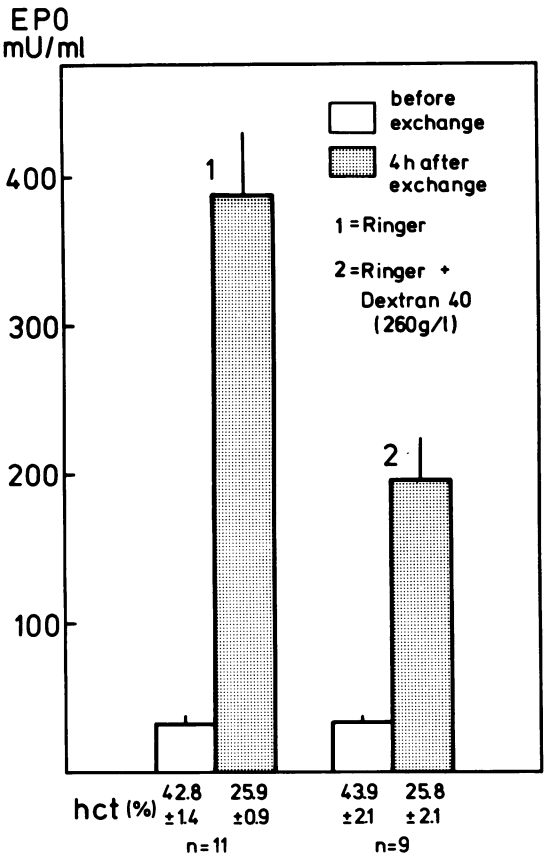

Figure 4. Plasma EPO concentration in rats before and 4 h after exchange transfusion with Ringer's solution or Ringer's solution supplemented with dextran 40 (260 g/liter). Dextran supplementation caused a significant reduction in EPO values.

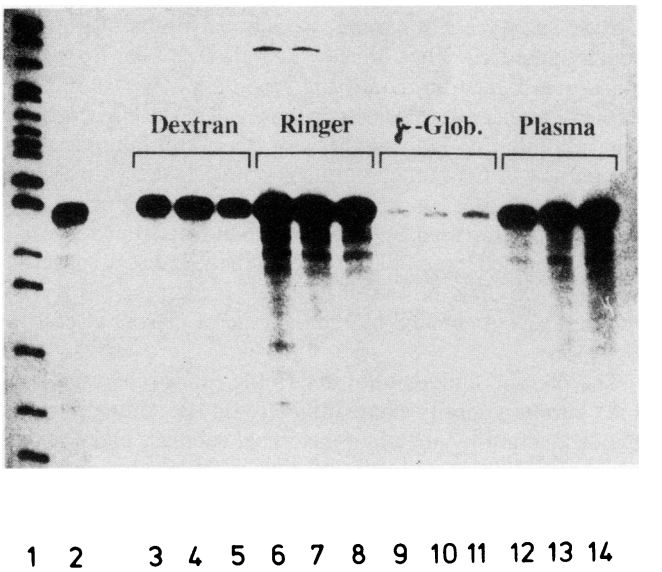

Figure 5. RNase protection assays of renal EPO mRNA in rats exchanged with Ringer's solution (lanes *6–8*), rat plasma (lanes *12–14*), Ringer's supplemented with 60 mg/ml gammaglobulin (lanes *9–11*), or Ringer's supplemented with dextran 40 (260 g/liter) (lanes *3–5*). Lane *1:* size marker generated by HpaII digest of pBR 322 DNA; lane *2:* external rat EPO mRNA standard consisting of 5 μg total RNA from kidneys of severely anemic rats (hematocrit < 10%).

a number of situations EPO production is less than that expected for the reduction in hematocrit. This study aimed to examine if changes of plasma viscosity could have an influence on EPO production and could thus contribute to the inappropriate EPO production observed in patients with increased plasma viscosity (2, 3) due to monoclonal gammopathies such as multiple myeloma or Waldenström's disease or polyclonal gammopathies of severe degree occasionally seen, e.g., in rheumatoid arthritis (11, 12). In agreement with previous studies we found that serum EPO levels are in fact relatively low in patients with multiple myeloma or Waldenström's disease when set into proportion to the degree of anemia. This circumstance becomes more obvious if the EPO response, defined as the rise of serum EPO related to the degree of anemia, is compared with that of patients suffering from hyporegenerative anemias. Our findings show an inverse correlation between EPO response and plasma viscosity. A striking feature of this correlation is that comparably small changes of plasma viscosity correlate with marked changes of the EPO response (Fig. 1).

An impaired EPO response is characteristic for the anemia associated with chronic renal insufficiency. Since hypergammaglobulinemias may induce a restriction of renal function, it appeared reasonable to us to consider an impairment of renal function as a possible pathophysiological mechanism for the inappropriate EPO formation in hypergammaglobulinemic patients. The EPO response correlated with creatinine clearance, which is surprising because it has been shown that erythropoiesis is unaffected at creatinine clearance values above 30–40 ml/min and renal anemia is not found above this threshold value (13, 14). For this reason it is unlikely that a restriction of renal function is the primary and only cause for the impaired EPO formation. It was of interest therefore to see whether a rise of plasma gammaglobulins and a related rise of plasma viscosity per se is capable to attenuate the EPO response in otherwise normal organisms. To this end we have investigated whether an association exists between the gammaglobulin concentration or the plasma viscosity and the EPO response to anemia in normal rats. It turned out that the rise in plasma EPO levels in response to the same degree of anemia was inversely related to the gammaglobulin concentration. Since gammaglobulins are major determinants of plasma viscosity, the EPO response was, not surprisingly, also inversely correlated with plasma viscosity (Fig. 3). Interestingly, the correlation between plasma viscosity and EPO response was rather similar for hypergammaglobulinemic patients and the normal rats, suggesting that the impaired EPO response in patients may in fact be due to effects related to the increase of gammaglobulins.

To distinguish whether the attenuation of the EPO response in the hypergammaglobulinemic rats was linked to the alteration of plasma viscosity or to other effects related to hypergammaglobulinemia, in additional experiments plasma viscosity was increased by dextran. Again this maneuver led to a marked attenuation of the EPO response (Fig. 4) and we therefore suggest that the plasma viscosity could be a factor in determining EPO production, and could therefore account for the impaired EPO response in hypergammaglobulinemic patients. Nonetheless it was obvious that raising plasma viscosity by dextran was less effective on EPO levels than a rise in plasma viscosity induced by infusion of gammaglobulins and we cannot explain this observation yet.

Since an increase of plasma viscosity may impair capillary circulation and may thus impede the release of EPO from the renocortical interstitium into the blood flow, we examined whether viscosity related effects on plasma EPO levels were due to an inhibition of EPO synthesis or due to an inhibition of EPO release. EPO production rate is primarily determined by the EPO mRNA levels within the EPO producing cells. We found that the impaired EPO response in the rats went in parallel with decreased levels of EPO mRNA in the kidneys, suggesting that it was the synthesis of EPO rather than the release of EPO that was attenuated by the increase of plasma viscosity (Fig. 5).

The present study cannot determine the mechanisms by which plasma viscosity exerts influence on the stimulation of EPO production by anemia. Since renal capillary endothelial cells are considered as likely candidates for producing EPO (15, 16), one could speculate that plasma viscosity exerts an influence on endothelial cells by changing the shear stress on the endothelial surface. It has been found for instance that the release of histamin (17), renin (18), endothelin (19), prostacyclin (20, 21), and endothelium-derived relaxing factor (22) are shear stress dependent, indicating that this parameter is capable to markedly modulate endothelial function. It is likely that mechanoreception, the most widely distributed sensory modality, is the mechanism involved (23).

The present data have clinical implications, since it has become common practice to administer recombinant EPO to patients with blunted EPO response. Clinicians should be aware that low EPO levels may represent a physiological response of the organism to impending hyperviscosity and that it may be dangerous to overcome this negative feedback control by exogenous EPO. In this context it is noteworthy that EPO levels are low in sickle cell anemia (24), a classical condition with increased whole blood viscosity.

Acknowledgments

The expert secretarial help provided by Hannelore Trommer and Aloisya Böck is gratefully acknowledged.

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