Circulating Blood Cells Modulate the Atherosclerotic Process in Apolipoprotein E–Deficient Mice

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The interaction of blood with the arterial tree may play an important role in the development of atherosclerotic lesions. The aims of this study were (1) to determine how anemia or increased hematocrit affect the development of atherosclerosis and (2) to find relationships between hematologic and hemorrheologic variables in apolipoprotein (apo) E–deficient mice. Forty-two mice were randomly divided into 3 groups of 14 mice each. There was no further manipulation in the control group. To induce anemia, the mice from one of the groups were repeatedly bled, drawing approximately 250 μL blood from each mouse twice a week. To increase the hematocrit levels in another group of mice, we injected 20 U recombinant human erythropoietin every other day. The development of lesions and the main variables involved in atherogenesis were compared among groups. Our results show that atherosclerosis was attenuated in the mice that were bled, and this was not accounted for by changes in plasma lipid levels, the distribution of lipoprotein particles, the body iron distribution, or oxidation parameters. Moreover, atherosclerosis was enhanced in the mice treated with the continuous administration of erythropoietin. To ascertain the relationship between hematocrit and whole blood viscosity, we measured both variables in pooled blood from 24 additional mice, which were manipulated to ensure a wide range of values. We found a direct and significant correlation between hematocrit and blood viscosity and between hematocrit and lesion size. Our data support in vivo the idea that hemorrheology has an important role in atherogenesis in this particular animal model.

THE PATHOGENESIS of atherosclerosis is complex and multifactorial. A possible factor involved in its development is the physical interaction of blood with the artery wall that may cause arterial injury and initiate a chronic, inflammatory response in the vascular wall.[1] Most of the accepted risk factors, such as lipid disorders, hypertension, smoking, and diabetes mellitus, are systemic. However, advanced atherosclerotic lesions do not affect the whole arterial tree equally: they preferentially develop at those places where blood flow is more turbulent, such as the arterial bifurcations. It is therefore conceivable that hemorrheology may play an important role in atherogenesis.[2,3]

In a previous study,[4] aimed at assessing whether modulating the hemorrheologic factors can affect the development of atherosclerosis in apolipoprotein (apo) E–deficient mice, we reduced their circulating blood mass with the intraperitoneal administration of phenylhydrazine (PHZ), a hemolytic agent. Our results showed a reduction in the progression of atherosclerosis in anemic mice. Unfortunately, we were unable to discount a possible and unknown secondary effect of PHZ, which made it necessary to undertake new studies to confirm our results and to investigate other mechanisms. Consequently, we have investigated the effect of anemia induced by repeated bleeding on the development of atherosclerosis in this animal model. Furthermore, we reasoned that by increasing the circulating blood mass and viscosity by the continuous administration of recombinant human erythropoietin (rHuEPO), we could obtain the opposite effects. Finally, as the actual measurement of blood viscosity in the experimental mice is not feasible, we have assessed the relationship between this parameter and the hematocrit in an effort to provide a simple and indirect measurement of hemorrheologic variables.

MATERIALS AND METHODS

Experimental Animals and Study Design

The mice were treated and the procedures were performed in accordance with the ethical standards of the Hospital Universitari de Sant Joan. The mice used were homozygous apo E–knockout hybrids of the C57BL/6J and 129 Ola strains.[5] They were housed in metabolic cages at 21°C to 23°C with a humidity level of 50% to 60% and a 12-hour light/dark cycle. The animals were fed a commercial mouse diet (BK Universal, Barcelona, Spain) until the beginning of the experiment, at 8 weeks of age. Forty-two mice were then randomly divided into 3 groups of 14 mice each. Relevant baseline values were obtained and the mice chow was supplemented with 20% (wt/wt) of palm fat (Unilever, Bilbao, Spain) and 0.10% (wt/wt) of cholesterol (Sigma Chemical Co, St Louis, MO) for the next 10 weeks, as previously described.[4] This diet was used to further increase plasma cholesterol concentration and accelerate lesion formation. In the designated control group, there was no further manipulation. To induce anemia, the mice from one of the groups were repeatedly bled, drawing approximately 250 μL blood from the retro-orbital venous plexus of each mouse twice a week. Conversely, to increase the hematocrit levels in another group of mice, we injected 20 U rHuEPO every other day.

To accurately measure blood viscosity, samples must be carefully obtained (at least 1 mL) and lack of contamination ensured (hair, fat, tissue, etc). Therefore, the actual measurement of hemorrheologic variables in the experimental mice, is not feasible. It is our hypothesis, however, that blood viscosity is closely related to hematologic variables, such as hematocrit, which are easy to measure. To test that, we used 24 mice, which were manipulated to increase the hematocrit levels with the injection of rHuEPO or to decrease the hematocrit with either bleeding or dilution, to ensure a wide range of values. If proper samples were obtained, blood from at least 4 mice was pooled and hematocrit and blood viscosity measured immediately. Data are presented for 55 measurements that were considered free of any methodologic artifact.

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Hematologic Examination and Hemorrheologic Variables

Complete blood cell counts were performed in an automatic cell counter (MD 10, Coulter Electronics, Miami, FL) and reticulocytes, stained with brilliant cresyl blue, were counted manually. To assess erythrocyte morphology, blood smears were made immediately after the blood sample had been collected and were stained with Giemsa. The blood viscosity was measured immediately at 37°C in a rotational cone and plate viscometer (Brookfield DVIII Plus, Stoughton, MA) at shear rates of 24, 51, 104, 150, 200, 250, 300, 350, and 450 s^{-1} as previously described.[6]

Lipid and Lipoprotein Measurement

Plasma cholesterol and triglyceride were measured as described.[7] High-density lipoprotein cholesterol was determined with a precipitation procedure.[8] To assess cholesterol distribution among lipoproteins, plasma was gel-filtered through a Superose 6HR column essentially as described.[9] Each fraction obtained from the column was assayed enzymatically for cholesterol and triglyceride concentrations.

Plasma Oxidizability and Related Plasma Chemistry

Plasma oxidizability was measured as previously described.[10] Briefly, heparin plasma (20 μL) was diluted with 2,950 μL phosphate-buffered saline (PBS). A total of 30 μL of a 5-mmol/L $CuCl_2$ solution was added to start oxidation. The susceptibility of plasma to oxidation was assessed by monitoring the changes in absorbance at 234 nm every 10 minutes for a period of 10 hours. The lag-phase, the maximal oxidation rate, and the maximal production of dienes were calculated as described.[11] Ferritin, iron, and albumin were measured as described.[12,13]

Quantitative Analysis of the Lesions and Macrophage Immunohistochemistry

Immediately after the injection of avertin (2,2,2-tribromoethanol) and desanguination through cardiac puncture, the vascular tree was perfused with 10% formaldehyde in PBS, pH 7.4. Ten-micrometer-thick sections were made sequentially at intervals of 20 μm throughout the aortic sinus. To quantify the atherosclerotic lesion area, the slides were stained with Sudan IV B and counterstained with hematoxylin and eosin. All the slides were reviewed and 4 sections in the region between the very proximal aorta and the point in the aortic sinus that contains 3 complete valve leaflets were used for morphometric evaluation.[14]

To further explore the content of macrophages, cryosections (n = 3 to 5 per mice) were stained with MOMA-2 monoclonal antibody (rat antimouse monocyte/macrophage; Biosource International, Camarillo, CA) followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase (Pharmingen, San Diego, CA). The signal was enhanced with the NEN Dupont Tyramide signal amplification kit and sections were counterstained for nuclei with SYTOX green (Molecular Probes, Eugene, OR).

Iron Hystochemistry and Determination of Tissue Iron Concentration

Iron deposits on tissue sections from the aortic sinus, liver, and spleen were examined by Perls' Prussian blue reaction as described[15] and quantified by a digitizing morphometry image-analysis system (LeicaQWin, Leica, Barcelona, Spain). Samples of each tissue were weighted, placed in a tube, mixed with 65% nitric acid (Suprapur, E.Merck, Darmstadt, Germany), and heated under pressure at 190°C to accomplish digestion. Samples were then diluted with deionized water, filtered, and the Fe measured in a computer-controlled sequential inductively coupled plasma spectrometer (Thermo Jarrell ASH, Franklin, MA, PolyScan 61E), as described.[16]

Table 1. Final Values of Body Weight and the Selected Biochemical Parameters for Each of the Experimental Groups

	Control	Bled	Epo-Treated
Body weight (g)	22.8 ± 0.9	23.5 ± 0.7	23.0 ± 1.1
Albumin (g/L)	21.5 ± 1.3	23.0 ± 0.7	23.1 ± 1.4
HDL cholesterol (mmol/L)	2.4 ± 0.3	2.7 ± 0.3	2.6 ± 0.4
Cholesterol (mmol/L)	28.6 ± 2.5	31.5 ± 1.7	41.2 ± 2.3*
Triglyceride (mmol/L)	1.9 ± 0.2	3.0 ± 0.3*	2.1 ± 0.3
Iron (μmol/L)	59.6 ± 1.9	60.0 ± 2.1	64 ± 2.2
Ferritin (μg/L)	138 ± 8	118 ± 6	155 ± 12†
Lag-phase (min)	218 ± 4	289 ± 16	201 ± 8
Maximal oxidation rate (mol diene/mol chol × min) × 10^4	7.1 ± 0.5	8.3 ± 0.4	6.9 ± 0.6
Maximal diene production (mol diene/mol chol) × 10^4	1,084 ± 54	1,126 ± 38	1,102 ± 54

NOTE. Results are expressed as mean ± SEM; n = 14 for each group.

*$P < .01$ with respect to controls, †$P < .05$ respect to bled animals.

Statistical Analyses

Statistical assessment was performed using SPSS/PC + 9.0 (SPSS, Chicago, IL). Values are expressed as mean ± SEM. Results were compared nonparametrically between groups by the Mann-Whitney *U* test. The Wilcoxon test was used to compare final and baseline values within groups. Linear regression was used to relate hematocrits and blood viscosity.

RESULTS

Body Weights and Nutritional Status

There were no apparent differences among groups in terms of mobility, behavior, or food consumption. In all groups, the body weight increment throughout the study was approximately 25%. There were no differences in final plasma albumin concentration (Table 1).

Hematologic and Hemorrheologic Variables

There were no changes in baseline values among groups, and in controls, there were no significant differences with respect to final values. The bled animals showed a 15% to 30% reduction in their hematocrit and number of red blood cells (Table 2). The mice responded to anemia by increasing erythropoiesis, which was evidenct by a considerable increment in the circulating reticulocytes and in the mean corpuscular volume of erythrocytes. The increment in circulating reticulocytes was maximal after 1 week of bleeding and remained steady throughout the study. Platelets decreased by approximately 15% in bled mice, but the number of leukocytes remained unchanged. As expected, EPO-treated animals showed, as a group, a significant increase in hematocrit, circulating red blood cells, mean corpuscular volume, and reticulocytes count (Table 2). The effect was observed in all animals during the first 3 weeks of treatment, but during the remaining 7 weeks, the effect was not so evident, and hematocrit values showed a trend towards lower values in 8 animals. We interpreted these data as a possible presence of antibodies directed against rHuEPO. Therefore, consistent results are difficult to achieve in this model.

Table 2. Final Values of Hematologic Variables

	Control	Bled	Epo-Treated
Hematocrit (%)	46.6 ± 0.5	35.8 ± 0.7*‡	61.4 ± 2.7
Red blood cells ($\times 10^{12}$/L)	9.3 ± 0.1	7.4 ± 0.2*‡	14.1 ± 0.8
Mean corpuscular volume (fL)	51.0 ± 0.1	58.2 ± 0.5*	59.4 ± 0.6*
Mean corpuscular hemoglobin (pg)	16.2 ± 0.1	18.6 ± 0.2*	17.0 ± 0.3
Leukocytes ($\times 10^9$/L)	3.7 ± 0.2	3.4 ± 0.4	4.5 ± 0.2
Platelets ($\times 10^9$/L)	820 ± 42	681 ± 68*	904 ± 53
Reticulocytes ($\times 10^9$/L)	204 ± 14	951 ± 66†‡	643 ± 233*

NOTE. Results are expressed as mean ± SEM; n = 14 for each group.

*$P < .05$; †$P < .001$ with respect to controls, ‡$P < .005$ respect to Epo-treated group.

Table 3. Tissue Distribution of Iron in the Experimental Animals

Tissue	Animals	Iron Concentration (μmol/g dry weight)
Aorta		
	Control	1.44 ± 0.07
	Bled	1.29 ± 0.97*
	PHZ-treated	1.35 ± 0.94
	Epo-treated	1.37 ± 0.58
Spleen		
	Control	46.2 ± 2.5
	Bled	19.8 ± 1.2*
	PHZ-treated	62.9 ± 3.3†
	Epo-treated	54.5 ± 3.2
Liver		
	Control	5.50 ± 0.16
	Bled	3.65 ± 0.15*
	PHZ-treated	6.21 ± 0.19†
	Epo-treated	5.72 ± 0.14

NOTE. Data presented as mean ± SEM for n = 5 to 13 mice.

*Significant difference *v* all other groups *(P* < .0001); †significant difference *v* control group *(P* < .0001).

We also found a linear association between the hematocrit and blood viscosity at a shear rate of 300 s^{-1} (Fig 1A; $R^2 = .613$, $P < .001$). There was also a direct ($R^2 = .465$) and significant ($P < .001$) correlation between lesion size and hematocrit when all experimental animals (n = 42) were considered (Fig 1B). Interestingly, there was no correlation between lesion size and any of the other hematologic variables considered.

Tissue Iron Distribution

Tissue iron was examined in the liver, spleen, and aortas. The availability of samples made it possible to perform this examination in the mice treated with PHZ from our previous report.[4] Hystochemically, the control and EPO-treated mice showed no iron in the liver, moderate iron deposits in the spleen, and few iron deposits in the aorta. The anemic animals had different features: the PHZ-treated animals had moderate iron deposits in the liver and abundant deposits in the spleen. On the other hand, the bled animals had no iron in the liver or in the spleen. However, in both cases, there was little iron in the aortic wall, and the quantities present were similar to those in controls and EPO-treated animals (Table 3). Plasma iron concentrations did not detect these changes (Table 1). Plasma ferritin concentration was lower in bled animals than in the Epo-treated groups, but there were no significant changes with respect to the control group.

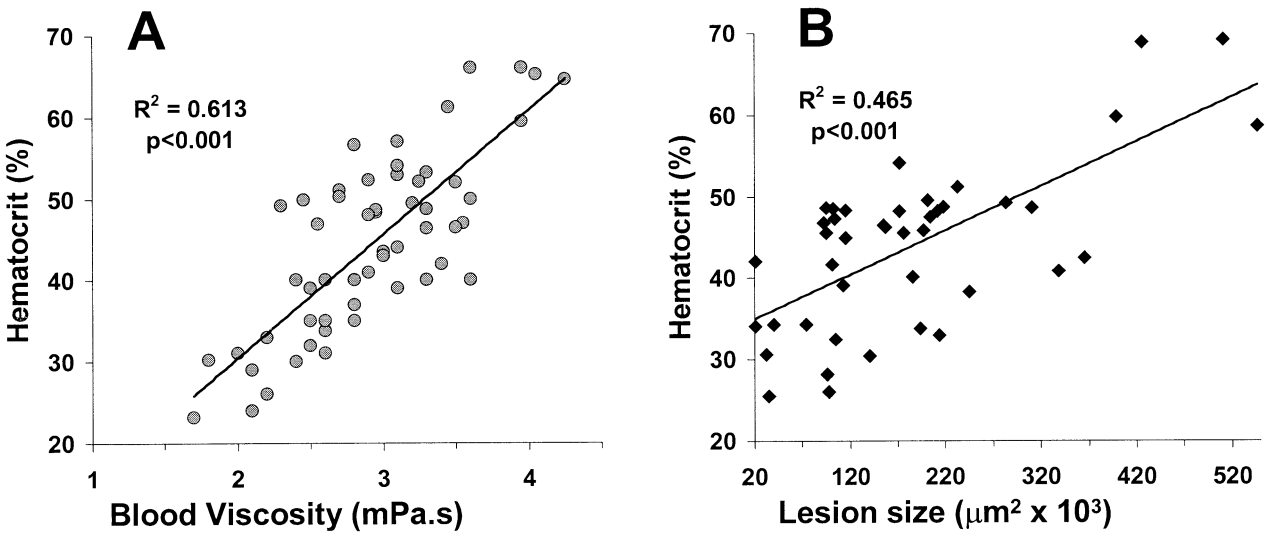

Fig 1. (A) Relationship between hematocrit and whole blood viscosity at a shear rate of 300 s^{-1}. Mice were manipulated to increase or decrease the hematocrit to ensure a wide range of values. If proper samples were obtained, blood from at least 4 mice was pooled and hematocrit and blood viscosity measured immediately. Data are presented for 55 measurements that were considered free of any methodologic artifact. (B) Relationship between hematocrit and lesion size. Data are presented for all the experimental animals (n = 42), controls, bled, and EPO-treated.

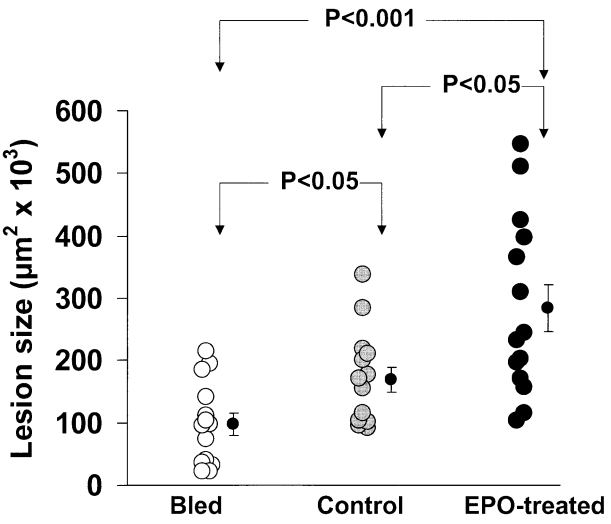

Fig 2. Distribution of the individual sizes of aortic lesions according to the experimental group.

Effect on Lipids and Lipoprotein Profiles

No change was observed in high-density lipoprotein (HDL)-cholesterol concentration. There were no significant differences in the baseline concentrations of plasma cholesterol and triglyceride (data not shown). Both parameters increased significantly during the experiment. Plasma cholesterol concentration increased significantly in EPO-treated animals with respect to the other groups, possibly as a consequence of increased needs for enhanced cell formation. For unknown reasons, the plasma triglyceride increment was significantly higher in the anemic mice. The chromatographic lipoprotein profiles showed no significant quantitative or qualitative changes in low and very–low-density lipoprotein particles (data not shown).

Effects on Plasma Oxidizability and Other Related Parameters

At the end of the study, the lag-phase, the maximal oxidation rate, and the maximal diene production were not significantly different among groups, although the mean lag-phase was slightly increased in the mice under bleeding (Table 1). Lesion size was not correlated with any of these parameters.

Evaluation of Atherosclerotic Lesions

The size of the lesion area (Fig 2) measured in the aortic sinus of control mice (168,000 ± 20,000 μm^2) was significantly ($P < .05$) higher than that observed in the anemic group. (97,000 ± 18,000 μm^2) and significantly ($P < .05$) lower than that observed in EPO-treated animals (284,000 − 38,000 μm^2). Results were similar if expressed as percentage of occlusion, which takes into account the possible influence of different heart sizes and, therefore, data are not shown. Despite this difference in the lesion area, there was a considerable degree of overlapping and the qualitative analysis revealed similar MOMA-2 positive staining in all groups (39.8% ± 1.1% in bled animals, 43.7% ± 1.3% in controls, and 45.3 ± 1.6% in EPO-treated mice).

DISCUSSION

This study shows (1) that the production of anemia by continuously bleeding apo E–deficient mice fed a high-fat, high-cholesterol diet attenuates the development of atherosclerosis; (2) that the continuous administration of rHuEPO in these mice resulted in a higher hematocrit value, an increased lesion size, and an increased plasma cholesterol concentration; and (3) that there is a direct linear relationship between the hematocrit and blood viscosity in this animal model and a significant correlation between these variables and the lesion size.

Bleeding reduced the lesion area by about the same amount as the continuous administration of PHZ.[4] However, there are differences between the 2 models of anemia. The hematocrit in the bled mice decreased constantly throughout the study. In the mice treated by PHZ, however, it was reduced abruptly at first and less sharply as the study progressed.[4] There were also striking differences in the iron balance. These differences were the logical consequence of the 2 mechanisms by which anemia was induced: tissular deposits were consumed in the bled mice, but accumulated in the mice under hemolysis. It is noteworthy, however, that despite the consumption of the iron stores in the bled animals, their plasmatic iron levels remained unchanged. There were no differences in plasma ferritin concentration between controls and neither bled nor EPO-treated groups, but such differences were significant in the comparison between the 2 manipulated groups. There was a high final reticulocyte count in the anemic group, which demonstrates that iron stores were enough to maintain the erythropoietic response until the end of the study. On the other hand, the fact that 2 different mechanisms to produce anemia, which had opposite effects on the iron balance, improved atherogenesis, and the fact that the iron deposits in the aortic wall were similar in all groups, means that iron depletion, which has been described as reducing atherosclerosis in this animal model,[17] cannot be the main mechanism for protecting against atherosclerosis in our mice. Likewise, qualitative and quantitative changes in the oxidative status cannot have an important role in the process in our study.

As expected, the administration of rHuEPO resulted in an increased hematocrit. An increase in the lesion size was also observed, but a relationship cause-effect cannot be speculated, because there was a concomitant increase in plasma cholesterol concentration, for which we do not have an easy explanation. Moreover, these quantitative changes in plasma cholesterol were not accompanied by an altered plasma lipoprotein distribution, as assessed chromatographically, and they were not correlated with the lesion size.

We did show, however, that there is a direct relationship between hematocrit and blood viscosity and, more importantly, between hematocrit and lesion size if all animals are considered. Hence, some of the forces by which blood interacts with the artery wall and which, like shear stress, directly depend on blood viscosity, will diminish in anemic mice and increase in EPO-treated animals. The gradient of the shear stress may be more important than the absolute magnitude in affecting the development of atherosclerosis.[18] The changes in shear stress in the aortic sinus are radical and range from very high values during ventricular compression to negative values. In the anemic mice, the differences between the maximal and minimal

values of shear stress may be lower than in controls, and this results in a lower gradient of the shear stress. When the hematocrit is higher, however, the gradient of the shear stress would be higher and the deleterious effect in the endothelium, more intense.

A considerable number of studies have been performed to elucidate the mechanisms by which hemodynamic forces can modulate atherogenesis. The results show that many key processes are influenced by the hemorrheologic variables, including (1) synthesis and release of procoagulant and anticoagulant factors by endothelium[8-20]; (2) platelet adherence and reactivity[21] (3) expression of cellular adhesion molecules and monocyte migration[22,23]; (4) migration and proliferation of smooth muscle cells[24]; (5) incorporation of lipoproteins into the artery wall[25]; (6) modification of the endothelial structure and tone[26,27]; and (7) modulation of the inflammatory response.[28,29] Most of these studies have been performed in vitro. They were not designed, therefore, to study the quantitative changes in the development of atherosclerosis. In our studies, we have shown that the development of atherosclerosis can decrease or increase in an experimental model in which blood viscosity is diminished or increased. We consider that our data are complementary to the mechanistic studies and suggest that hemorrheology has an important role in atherogenesis. However, we recognize that we have not provided direct evidence and other possibilities should be considered, such as changes in blood pressure or in the activation of platelets.[30] Moreover, our model with the continuous administration of rHuEPO is accompanied by an increase in plasma cholesterol concentration, adding a further complication. Future studies should include a long-term expression of mice erythropoietin with the intramuscular injection of a plasmid DNA vector.[31]

However, the fact that modifications to the hematocrit of apo E–deficient mice lead to modifications in hemorrheology and in the lesion size suggests that this is a useful model on which to base in vivo studies of the modifications caused by changes in hemorrheology, their relative importance in atherogenesis, and the prevention of their harmful consequences.

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