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Hypocholesterolemia and dysregulated production of angiopoietin-like proteins in sickle cell anemia patients

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ABSTRACT

Angiopoietin-like proteins (ANGPTL) are responsible for inhibiting lipoprotein lipase activity, and ANGPTL3 and ANGPTL4 deficiencies have been shown to lower lipoprotein levels in animal models and in humans carrying loss-of-function mutations. Sickle cell anemia (SCA) is a hereditary hemolytic anemia characterized by vaso-occlusive crises and end-organ damage, which is curiously associated with hypocholesterolemia and a low incidence of atherosclerosis, whose underlying mechanisms are unclear. We hypothesized that ANGPTL3 and ANGPTL4 dysregulation is responsible for the hypolipidemic phenotype in SCA. We measured circulating concentrations of ANGPTL3 and ANGPTL4 and correlated them with hemolytic biomarkers and lipoproteins in 40 patients with SCA and 30 control individuals. The association between hemolysis and low cholesterol levels in SCA was confirmed along with surprisingly higher levels of ANGPTL3 and ANGPTL4 in SCA patients than in controls. ANGPTL3 correlated with hemolysis markers LDH and reticulocyte counts, while ANGPTL4 did not. Our data show a paradoxical increase in production of ANGPTL3 and ANGPTL4 in SCA, which would be expected to cause hyperlipidemia, due to increased inhibition of lipoprotein lipase. ANGPTL3, exclusively produced by the liver, correlated with hemolysis markers, suggesting a possible hepatic response to hemolysis. Further functional studies and replication in larger cohorts are warranted to investigate the dysregulation of lipid metabolism in SCA.

1. Introduction

It has been known for several decades that high levels of circulating cholesterol is one of the main risk factors for the development of atherosclerosis [1,2]. Therefore, there is an understandable interest in dissecting mechanisms that can be harnessed to reduce cholesterol [3].

Sickle cell anemia (SCA) is a homozygotic hereditary anemia characterized by a point mutation that causes the production of the variant hemoglobin S. While most cases occur in Sub-Saharan Africa, its incidence is highly heterogeneous across countries and within regions of the same country. In Brazil, the incidence of sickle cell disease (SCD) varies from 1 in 650 newborns in the Northeastern state of Bahia to 1 in 13,500 in the Southern state of Santa Catarina [4,5]. SCA is characterized by hemolytic anemia and vaso-occlusive crises with end-organ damage [5]. Despite the chronic endothelial dysfunction in SCA, patients are rarely reported to suffer myocardial infarction, stroke is not associated with atherosclerotic plaques, and SCA patients have repeatedly been reported to have hypocholesterolemia [6,7], whose underlying mechanism is still unclear.

Angiopoietin-like proteins (ANGPTLs) are a family of angiogenesis regulators unable to bind to receptors classically targeted by angiopoietins [8], and its members are numbered from ANGPTL1 to ANGPTL8. ANGPTL3 and ANPTL4 are particularly noteworthy because they are involved in lipid metabolism [9] as inhibitors of lipoprotein lipase (LPL), and can decrease the release of triglycerides into circulation [8]. A mouse knockout model of ANGPTL4 was shown to have low cholesterol levels, and patients with a loss-of-function mutation in ANGPTL3 present with familial combined hypolipidemia with increased lipoprotein lipase activity [10,11].

Since lipoprotein levels in SCA correlate with hemolysis markers [12], we hypothesized that hemolysis could be causing hypocholesterolemia by dysregulating ANGPTL production. We aimed to determine the concentrations of circulating ANGPTL3 and ANGPTL4 and correlate with hemolysis markers and lipoproteins circulating in patients with sickle cell anemia and healthy individuals.

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2. Material and methods

2.1. Subjects

This study is an extension of an earlier published study in which we showed different data from some individuals described here [12]. Briefly, adult patients with sickle cell anemia (SCA) and adult control individuals (AA) were invited to participate in the study at the Hematology and Hemotherapy Center of the University of Campinas - UNICAMP - Campinas, Brazil. All participants signed a written informed consent form as approved by the local ethics committee, (approval number 32051214.5.0000.5404). Sickle cell anemia was diagnosed by hemoglobin electrophoresis and confirmed by high-performance liquid chromatography (Variant II, Bio Rad). All patients were in steady state, as defined by no history of sickle cell pain crisis or blood transfusion in the last 3 months. We also excluded patients during pregnancy or under treatment with lipid-lowering drugs, such as statins. Control individuals were required to have normal complete blood counts, no laboratory evidence of hemoglobinopathy, and a cholesterol level lower than 200 mg/dL.

2.2. Hematological indices

We collected whole blood samples in EDTA (K3 EDTA - Greiner Bio-One) tubes for complete blood counts on an automated hematological analyzer (ADVIA 2120i, Siemens).

2.3. Biochemical analyses

Blood samples collected in tubes with gel separator were centrifuged at 1500*g* for 15 min. Using an automatic biochemical analyzer (Evolution Modular, Hitachi/Roche), we measured total cholesterol (CT), high density lipoprotein (HDL), lactate dehydrogenase and total bilirubin. Low density lipoprotein (LDL) was calculated by the Friedewald equation [13]. We determined serum ANGPTL3 and ANGPTL4 by commercially available ELISA kits (R&D Systems, USA).

2.4. Statistical analyses

Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA), *t* test, Mann-Whitney test, and Spearman correlation test were applied as appropriate. A P-value below 0.05 was considered statistically significant

3. Results

In this study, 30 control subjects (AA) and 40 with sickle cell anemia (SCA) were included. The demographic characteristics, hematologic parameters, hemolytic biomarkers, and lipid measurements are shown in Table 1. We found no significant differences in age or gender, but body mass index was significantly higher in the AA population.

Typical hemolytic markers used in clinical practice, such as LDH, bilirubin, and reticulocyte count were increased in SCA compared to AA ($P = 0.0001$), as expected. Concentrations of total cholesterol and its circulating fractions (LDL and HDL) were decreased compared to AA ($P \leq 0.001$).

ANGPTL3 and ANGPTL4 levels were both found to be significantly elevated in SCA when compared to those in AA (Fig. 1), but as shown in Table 2, only ANGPTL3 correlated with hemolytic biomarkers, and with HDL. ANGPTL4 did not correlate with hemolysis or lipids.

4. Discussion

In this study, we investigated ANGPTL3 and ANGPTL4 production and its association with lower levels of cholesterol found in patients with sickle cell anemia.

Table 1
Characteristics and laboratory parameters of the study population.

	Healthy Subjects (AA) (n = 30)	Sickle cell anemia (SCA) (n = 40)	P
Female gender, (%)	8 (27)	19 (46)	> 0.05
Age (range), y	35 (18–66)	36 (18–55)	> 0.05
BMI, kg/m^2	27 (22–39)	21 (15–28)*	0.0001
Hemoglobin, g/dL	15 (12–18)	9 (5–12)*	0.0001
Reticulocyte, $\times 10^3/\mu L$	98 (43–155)	353 (80–625)*	0.0001
Total Bilirubin, mg/dL	0.6 (0.4–1.7)	2.4 (0.7–7)*	0.0001
Lactate dehydrogenase, U/L	144 (95–201)	827 (317–1761)*	0.0001
Total cholesterol, mg/dL	174 (119–196)	117 (72–184)*	0.0001
LDL, mg/dL	97 (21–143)	59 (15–116)*	0.001
HDL, mg/dL	45 (30–66)	37 (20–78)*	0.001

BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein. *comparison with healthy subjects; *P*-values refer to analysis of variance (Mann-Whitney). All biochemical parameters measured in serum. Data show median and range.

Decreased cholesterol levels have been reported in patients with sickle cell anemia and the mechanism for this is not clear [14]. However, lipids in patients with sickle cell trait do not seem to be affected [15,16]; suggesting significant hemolysis is necessary to affect lipoprotein metabolism.

Contrary to our expectations, we found elevated levels of ANGPTL3 and ANGPTL4 in patients compared to control subjects. We also found that ANGPTL3 correlates positively with hemolytic markers and negatively with HDL, but ANGPTL4 does not correlate with either hemolysis or lipid levels.

Correlation between serum cholesterol concentrations and erythropoietic activity has been observed not only in SCA, but also in other hemolytic anemias [17]. Although it has been suggested that the pool of plasma cholesterol is consumed for new membrane synthesis by the increased demand in the production of erythrocytes generated by hemolysis [18], another study has shown that cholesterolemia correlates with hematocrit levels in patients with hypoproliferative anemias of different causes, varying along with changes secondary to blood transfusions, iron, or cobalamin supplementation in nutritional anemias [6]. Therefore, erythropoietic activity by itself cannot be regarded as the sole driver of cholesterol availability in circulation, which has led to our hypothesis that ANGPTL production could be dysregulated and influence circulating lipoproteins.

ANGPTL3 and ANGPTL4 are inhibitors of lipoprotein lipase, which is responsible for the hydrolysis of circulating lipoproteins [19] and thus, has become a pharmacological target in the treatment of dyslipidemia [20,21].

With an increase in ANGPTL3 and 4, greater inhibition of LPL is expected, with higher concentrations of circulating cholesterol, as already reported in metabolic diseases [22,23]. However, this is paradoxical to our finding of hypocholesterolemia in SCA patients. It is even more surprising that the correlation between ANGPTL3 and HDL is negative, highlighting that the relationship between LPL activity and cholesterol is disrupted in SCA. These data are strikingly different from those found in subjects with metabolic syndrome [24] and hepatic steatosis [25], in whom ANGPTL4 correlates negatively with LDL and HDL, while ANGPTL3 is correlates positively with lipoproteins.

ANGPTL4 can be synthesized by the liver, adipose tissue, brain, intestine, thyroid, kidney, and heart, but ANGPTL3 can be regarded as a hepatokine, since it is exclusively produced by the liver [8]. The correlation of ANGPTL3 with hemolysis markers and HDL suggests that the overproduction of ANGPTLs is at least partially a hepatic response to stimuli generated by hemolytic anemia. Nevertheless, our data do not allow us to determine the source of ANGPTL4. Elucidating the exact mechanisms that explain these abnormalities requires additional studies of the production of each individual ANGPTL. They may involve

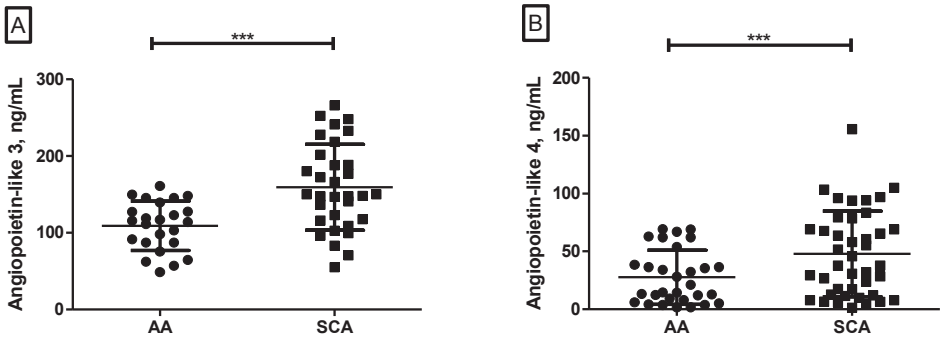

Fig. 1. Circulating angiopoietin-like protein 3 (A) and 4 (B) levels in serum of healthy individuals (AA) and patients with sickle cell anemia (SCA). ***P ≤ 0.0001, Mann-Whitney test.

Table 2
Correlations among angiopoietin-like proteins 3 and 4 levels and laboratory parameters.

Correlation with ANGPTL3	r_S	95% CI	P
Reticulocyte count	**0.4**	**0.08–0.6**	**0.008**
Lactate dehydrogenase	**0.4**	**0.1–0.6**	**0.006**
Total bilirubin	**0.5**	**0.2–0.7**	**0.0002**
Total cholesterol	−0.2	−0.5 to 0.06	> 0.05
Low density lipoprotein	−0.2	−0.5 to 0.03	> 0.05
High density lipoprotein	**−0.4**	**−0.6 to −0.1**	**0.006**
Correlation with ANGPTL4	r_S	95% CI	P
Reticulocyte	0.1	−0.1 to 0.4	> 0.05
Lactate dehydrogenase	0.2	−0.06 to 0.4	> 0.05
Total bilirubin	0.1	−0.1 to 0.4	> 0.05
Total cholesterol	−0.2	−0.4 to 0.08	> 0.05
Low density lipoprotein	−0.1	−0.3 to 0.2	> 0.05
High density lipoprotein	−0.2	−0.4 to 0.07	> 0.05

CI, confidence interval; r_S, correlation coefficient using Spearman correlation test.
Lines in bold represent statistically significant correlations found (P < 0.05).

tissue hypoxia due to anemia, nitric oxide depletion, or scavenging of heme and hemoglobin by haptoglobin and hemopexin, as well as the effects of hemolysis not only on hepatocytes, but also on Kupffer cells, hepatic endothelial cells, and other organs [26]. Fig. 2 illustrates the mechanisms involved in cholesterol production under low and high ANGPTL production, and what happens in sickle cell anemia patients.

Limitations of this study include the small number of patients studied, which may have limited the statistical power to detect more subtle correlations. We have not been able to control subject recruitment for other risk factors for dyslipidemia, such as obesity, fat-rich diets, or genetic predisposition for familial dyslipidemias. Our experimental design precludes drawing conclusions about causality, but it allows the generation of novel hypotheses that can be explored in future mechanistic studies on the pathophysiology lipid metabolism in hemolytic anemias.

In summary, we show that the production of ANGPTLs is increased in sickle cell anemia, with a paradoxical decrease in cholesterol. The regulation of lipid metabolism by ANGPTLs is complex, and functional studies of the pathways involved and the replication of these findings in larger cohorts of SCA patients are needed to increase our understanding of how hemolysis and circulating lipids interact.

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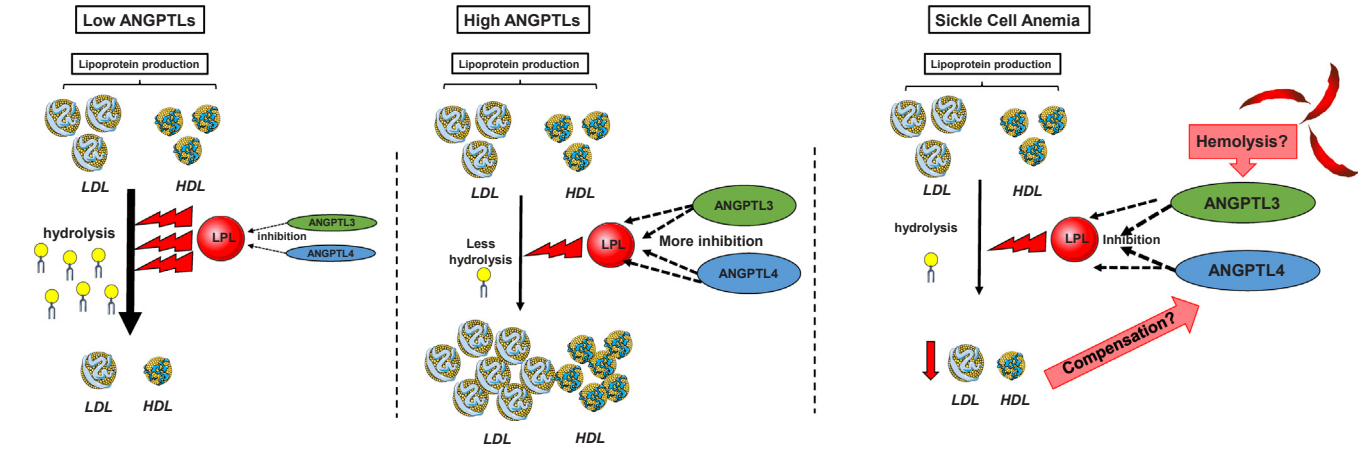

Fig. 2. Schematic representation of lipid metabolism under different conditions. Low angiopoietin-like protein (ANGPTL) production allows more lipoprotein lipase (LPL) activity and generates hypocholesterolemia (left panel). Elevated ANGPTLs will inhibit LPL, decreasing its hydrolytic activity, and increasing LDL and HDL (center panel). In sickle cell anemia (right panel), a paradoxical increase in AGPTLs is associated with low cholesterol. Hemolysis of sickling red blood cells generates byproducts that may stimulate hepatic production of ANGPLT3, and ANGPTL4 may be synthesized by peripheral tissue as a compensatory response to low cholesterol levels. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conflict of interest

The authors declare no conflict of interest.

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