Role of Circulating Cell-free DNA Levels in Patients With Severe Preeclampsia and HELLP Syndrome

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ABSTRACT

background

Increased plasma levels of circulating cell-free DNA (c-f DNA) have been recently described in diseases related to ischemia and/or hypoxia. Preeclampsia (PCL) is a hypertensive disorder of pregnancy, of unknown origin, where a defective placentation resulting in placental ischemia plays an important role. HELLP syndrome (haemolysis, elevated liver enzymes, and low platelet count) is the most serious form of PCL. The origin of the disease is unknown, and there are no markers to help us to make an early diagnosis of disease or to predict patients who are at risk of suffering serious complications.

methods

We measured circulating c-f DNA levels in a group of control pregnant women (n = 20), patients with mild PCL (n = 9), patients with severe PCL (n = 24), and patients with HELLP syndrome (n = 8).

results

Values of circulating c-f DNA were 333.59 ± 64.3 ng/ml in control subjects; 635.11 ± 111.7 ng/ml in patients with mild PCL; 1,264.63 ± 127.1 ng/ ml in patients with severe PCL, and 1,595.95 ± 269.8 ng/ml in patients with HELPP syndrome. ($P < 0.0001$). Values of c-f DNA >950 ng/ml had a sensitivity and specificity for detecting severe PCL and/or HELLLP syndrome of 0.71 and 0.93, respectively.

conclusions

As far as we know, this is the first report of increased c-f DNA levels in HELLP syndrome. In this preliminary report, we have observed a gradual and strong relation between c-f DNA levels and range of severity of PCL, with it the highest in patients with HELLP syndrome. Further studies are needed for evaluating the utility of this technique in hypertensive disorders of pregnancy and, particularly, in HELLP syndrome.

Keywords: blood pressure; HELLP syndrome; hypertension; hypertension in pregnancy; hypertensive disorders of pregnancy; preeclampsia

INTRODUCTION

It has recently been observed that circulating, cell-free (c-f) DNA levels rise in pathologies involving ischemia, such as acute coronary syndrome,1 ischemic heart failure,2 stroke,3 and mesenteric ischemia,4 as well as in patients who have suffered cardiac arrest outside the hospital.5 Similarly, increases in circulating c-f DNA have been documented in situations involving hypoxia, such as experimental acute pulmonary thromboembolism6 or obstructive sleep apnea/hypopnea syndrome.7

Preeclampsia (PCL) is a hypertensive disorder of pregnancy of unknown origin, where an ineffective placentation results in placental ischemia.8 Over many years, considerable effort has been invested in the search for risk factors or early markers of PCL. In this respect, it has been suggested that plasma circulating c-f DNA levels may represent a potential marker of PCL, probably related to the aforementioned placental ischemia.9–11 HELLP syndrome (haemolysis, elevated liver enzymes, and low platelet count) is a serious form of PCL that is mainly characterized by microangiopathic hemolytic anemia, thrombocytopenia, and hepatolysis. Its incidence is reported to be 0.1%–0.6% of all pregnancies, yet it rises to 4%–20% in women with PCL. HELLP syndrome is associated with 1% maternal mortality and a perinatal mortality of about 20%.12–16 However, several important complications, including disseminated intravascular coagulation, acute renal failure, subcapsular liver hematoma and liver rupture, heart failure, placental abruption, retinal detachment, intracranial hemorrhage, and many other neurological manifestations, have been observed in approximately 38%–44% of HELLP syndrome patients. The origin of the disease is unknown, and there are no markers to help us to an early diagnosis of disease or to predict patients who are at risk of suffering serious complications. As far as we know, there are no studies assessing circulating c-f DNA levels in this severe form of PCL.

METHODS

Subjects

Patients and controls were recruited at the Women's Hospital (University Hospital Virgen del Rocio, Seville, Spain). We studied a group of control pregnant women (n = 20), patients with mild PCL (n = 9), patients with severe PCL (n = 24), and patients with HELLP syndrome (n = 8). The diagnosis and degree of PCL severity were established according to the NICE clinical guidelines (http://www.nice.org.uk, issue date: August 2010), and HELLP syndrome was diagnosed according to the criteria described by Sibai *et al.*:17:platelet count ≤150,000/μl; elevated plasma transaminasa levels ≥70 U/L, including alanine-aminotransferase and aspartate-aminotransferase; increased anemia with indirect markers of hemolysis in the blood sample, such as schistocytes; lactic dehydrogenase
≥600 U/L; and/or bilirubin levels >1.2 mg/dl.

The exclusion criteria were patients suffering from any other kind of disease related to ischemia and/or hypoxia; patients receiving treatment with angiotensin-converting enzyme inhibitors, angiotensin receptor blockers or statins; coexistence of any inflammatory or autoimmune disease; evidence of any chronic illness that in the opinion of the investigator contraindicated inclusion in the study; diabetes mellitus; hypercholesterolemia; current tobacco consumption; and a body mass index >35 kg/m2. Before the study, the Human Investigation Review Committee at the Virgen del Rocio

University Hospital approved all the protocols, and all the participants provided their written informed consent before inclusion.

Circulating cell-free DNA measurement

The circulating c-f DNA was measured in peripheral blood (10 ml) collected from all the patients and control subjects in this study. The blood was drawn from the patients at the time of diagnosis and at a routine visit between the 30th and the 36th weeks of pregnancy in the control group. The blood samples were centrifuged for 8 minutes at 3,500 rpm, and the serum was frozen at −80 °C for later DNA extraction. DNA was extracted automatically from the stored serum samples (400 µl) using a Compact MagnaPure Instrument and the nucleic acid isolation MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Basel, Switzerland), according to the Total Plasma NA 100 400 V3 1 protocol. The DNA was resuspended in a final volume of 50 µl of water, and the serum DNA template was amplified in a final volume of 20 µl using a real-time quantitative polymerase chain reaction (PCR) assay for the beta-globin gene on a Light-Cycler 480 Real- Time PCR instrument (Roche Diagnostics), according tothe manufacturer's instructions. The β-globin Taqman system uses the following primers: beta-globin-354F (5′-GTG CAC CTG ACT CCT GAG GAG A-3′); beta-globin-455R (5′-CCT TGA TAC CAA CCT GCC CAG-3′); and a duallabeled fluorescent probe beta-globin-402T (5′-(FAM) TCTGGC CAA GTT TCA ACT CTG CTC GCT (TAMRA)-3′).
Amplification was carried out over 48 cycles at 95 °C for 5 minutes and at 62 °C for 20 minutes, and the final size of the amplicon was 102 base pairs.

Statistical analysis

The Shapiro–Wilk test was used to assess the normality of the distribution, and comparisons were made by means of analysis of variance, applying the Dunnett test as a post hoc analysis. Categorical variables were analyzed with the $\chi 2$ test, and finally, all data were expressed as the mean • } SEM, with $P < 0.05$ considered significant. The SPSS 10.0 statistical package (SPSS, Chicago, IL) was used for all analyses.

RESULTS

The clinical characteristics of the mothers and newborns studied here were recorded (Table 1). As shown in Figure 1, values of c-f DNA were 333.59 • } 64.3 ng/ml for the control subjects; 635.11 • } 111.7 ng/ml for the patients with mild PCL; 1,264.63 • } 127.1 ng/ml for the patients with severe PCL; and 1,595.95 • } 269.8 for the patients with HELLP syndrome ($P < 0.0001$). The post hoc analysis showed that the c-f DNA values in women with HELLP syndrome were different from those in the control group ($P < 0.0001$) and those with mild PCL ($P = 0.001$), whereas they were not significantly different from those with severe PCL ($P = 0.12$). When patients with severe PCL and patients with HELLP syndrome were considered together, the significant difference in the c-f DNA measurements with respect to the controls was still $P < 0.0001$, and $P = 0.001$ in comparison with patients with mild PCL. Between c-f DNA levels and blood pressure ($P < 0.001$), platelet counts ($P < 0.01$), and the weight of newborns at birth ($P < 0.01$). In the HELLP syndrome group, c-f DNA levels were significantly related to platelet count ($P < 0.01$) and alanine aminotransferase ($P < 0.05$) values but not to

hemoglobin values (P = 0.09). Values of c-f DNA >950 ng/ ml had a sensitivity and specificity to detect HELLP syndrome of 0.88 and 0.69, respectively. Moreover, values of c-f DNA >950 ng/ml had a sensitivity and specificity to detect severe PCL and/or HELLLP syndrome of 0.71 and 0.93, respectively.

During the follow-up period (until at least 2 days after delivery), we observed that the status of some patients changed. Specifically, the diagnosis of 3 patients in the mild PCL group was changed to severe PCL and the diagnosis of 1 patient in the mild PCL group was changed to HELLP syndrome, whereas 1 patient in the severe PCL group was rediagnosed with HELLP syndrome. None of the subjects changed to or from the healthy control group. The mean c-f DNA level in the patients initially misdiagnosed with mild PCL who were assigned a more severe diagnosis was 1,087.5 • } 115.2 ng/ml, and the c-f DNA value of the patient incorrectly diagnosed with severe PCL rather than HELLP syndrome was 1,615.2 ng/ml. Therefore, all patients initially classified in the incorrect groups at the time of blood sampling had unusually high c-f DNA values for these groups, and all were rediagnosed with a more severe illness.

Discussion

Measuring circulMeasuring circulating c-f DNA in plasma is technically easy and not expensive. Recently several authors have demonstrated a potential clinical interest of this measurement in several ischemic and hypoxemic diseases,[1–7] and here we show the value of such measurements when diagnosing PCL and HELLP syndrome. PCL is a relatively common disease of pregnancy that can range from a mild form to a serious illness with potentially dramatic consequences. The presence of placental ischemia due to defective placentation seems to be a common characteristic of the disease. HELLP syndrome is the most serious form of PCL, and although the exact cause of HELLP is unknown, general activation of the coagulation cascade is considered the main underlying problem. This cascade provokes a microangiopathic hemolytic anemia and the consumption of platelets. Because the liver appears to be the main site of this process, downstream liver cells suffer severe ischemia, leading to periportal necrosis. [18] Because circulating c-f DNA levels appear to reflect the existence of ischemia, we hypothesized that this parameter could be elevated in PCL and that the extent of the increase could be related to the severity of the disease, whereby the highest levels of circulating c-f DNA would be associated with HELLP syndrome.

difficult, and it is a disease that may develop in just a few hours, requiring rapid medical attention. In many cases, the diagnosis of the syndrome is made in puerperium in what are otherwise normal pregnancies, and these appear to be the cases in which the disease is most severe.[13] Therefore, it is necessary for clinicians to have tools that can alert their suspicion of this disease in these potentially lethal cases, as well as the clinical or laboratory armory to combat it effectively.

In this study, we measured the circulating c-f DNA in a group of control pregnant women, a group of patients with mild or severe PCL, and in a group of patients with HELLP syndrome. In this preliminary report, we observed a strong relationship between this parameter and the severity of the disease, with it maximal in cases of severe PCL and, particularly, in patients with HELLP syndrome. Based on our data, we propose a cutoff point of 950 ng/ml circulating c-f DNA as being suspicious of severe illness because values of c-f DNA >950 ng/ml had a high sensitivity and specificity for detecting severe PCL and HELLP syndrome. In this study, we did not investigate the source of circulating

c-f DNA, although the favored explanation for the release of DNA fragments is currently that they are generated and released by apoptosis or some other form of cell death. In our study, pregnancy-associated complications that involve increased turnover of placental cells (PCL) or systemic ischemia (HELLP syndrome) might explain the presence of elevated levels of free circulating fetal and maternal DNA.

We also observed that 5 patients were incorrectly assigned a less-severe disease, which was corrected during the followup when they were rediagnosed. All of these incorrectly classified patients had unusually high c-f DNA values for the groups to which they were originally assigned, and interestingly, all of them were rediagnosed with a more severe illness. Hence, high c-f DNA might be considered an early markerof severe PC and HELLP syndrome. However, we recognize that our study design is not the most suitable to reach such conclusions, a limitation of our study, and that future prospective studies should be set up to address this issue. Finally, another possible limitation of our study was the

small number of patients in the HELLP group, which is in part likely to be due to the low incidence of the disease and consequent difficulties for recruitment. Therefore, larger studies will be needed to evaluate the utility of this parameter in hypertensive disorders of pregnancy and particularly in HELLP syndrome. Similarly, future studies will have to consider the utility of developing a method to perform qualitative PCR (positive if >950 ng/ml, negative otherwise) that could be used even at the patient's bedside for an earlier diagnosis and better control of PCL and HELLP.

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Table 1. Clinical characteristics of mothers and newborns

	Healthy controls	Mild PCL	Severe PCL	HELLP syndrome	*P* value
Mothers' characteristics					
No.	20	9	24	8	
Age, y	32.50±5.27	32.88±5.30	31.83±5.63	30.12±7.33	NS
Previous hypertension, no. (%)	0	1 (11.1)	3 (12.5)	0	NS
IGR, no. (%)	0	0	5 (20.8)	1 (12.5)	NS
Primiparas, no. (%)	9 (45)	7 (77.7)	19 (79.1)	7 (87.5)	NS
IVF, no. (%)	2 (10)	1 (11.1)	5 (20.8)	3 (37.5)	NS
Cesarean, no. (%)	4 (20)	4 (44.4)	17 (70.8)	6 (75.0)	NS
Multiple pregnancies, no. (%)	1 (5)	2 (22.2)	5 (20.8)	1 (12.5)	NS
Preterm delivery,[a] no. (%)	0	1 (11.1)	9 (37.5)	6 (75.0)	NS
Onset, wks	—	35.44±1.43	31.66±2.92	30.65±3.63	<0.005
BS collection, wks	32.2±1.0	35.4±1.4	31.7±3.0	30.6±3.6	NS
SBP, mm Hg	112±11	150±10	171±9	193±12	<0.001
DBP, mm Hg	71±7	99±10	114±15	124±10	<0.001
Proteinuria, mgr./24 h	—	2,450±325	3,454±654	3,880±810	<0.005
Newborns' characteristics					
No.	21	11	29	9	
Male sex, no. (%)	7 (33.3)	6 (54.5)	12 (41.37)	4 (50.0)	NS
Gestational age at delivery, wks	39.99±1.15	37.47±0.87	33.40±2.43	31.8±3.66	<0.001
Birth weight, g	3,229.6±813.8	2,778.1±523.3	1,970.9±570.0	1,639.7±855.6	<0.001

Data are mean ± SEM unless otherwise noted.

Abbreviations: BS, blood sample; DBP: diastolic blood pressure; IGR, intrauterine growth retardation, IVF, *in vitro* fertilization; PCL, pre-eclampsia; SBP, systolic blood pressure.

[a]Less than 37 weeks.

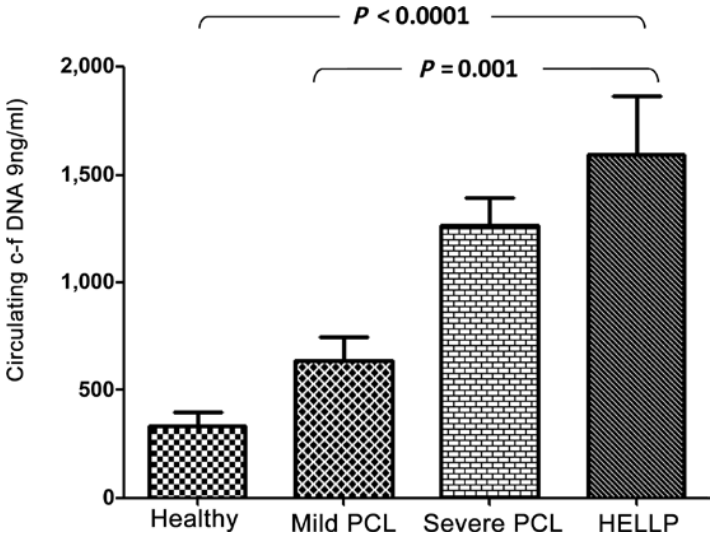

Figure 1. Circulating cell free DNA levels in the studied subjects. Abbreviation: PCL, preeclampsia.

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