Systemic lupus erythematosus: pathogenesis, diagnosis, and treatment

Wilhelmus, S.

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Microchimerism in Peripheral Blood of Patients with Systemic Lupus Erythematosus during and after Pregnancy

Suzanne Wilhelmus, Malu Zandbergen, Juan D.N. Diaz de Pool, Ron Wolterbeek, Hans J. Baelde, Clara Kolster-Bijdevaate, Kitty W. Bloemenkamp, Jan A. Bruijn, Ingeborg M. Bajema

Abstract

Objectives

Microchimerism has been shown to be increased in peripheral blood of women with systemic lupus erythematosus (SLE) many years after pregnancy. We hypothesized that either SLE patients accrue more microchimerism during pregnancy or clear chimeric cells less efficiently after pregnancy. Therefore, we studied the kinetics of microchimerism in peripheral blood from 30 weeks of gestation until six months postpartum in SLE patients and control subjects.

Methods

Peripheral blood was drawn from six pregnant SLE patients and eleven control subjects at 30 weeks of gestation, just after delivery, and one week, six weeks, three months and six months postpartum. Quantitative PCR for insertion-deletion polymorphisms and null alleles was used to detect microchimerism in peripheral blood mononuclear cells and granulocytes. Disease activity was monitored.

Results

SLE patients had a significantly higher median number of fetal chimeric cells in the granulocyte fraction just after delivery than control subjects (7.5 gEq/10^6 versus 0 gEq/10^6, respectively; P=0.02). At three and six months postpartum neither patients nor control subjects had detectable microchimerism. A relationship between microchimerism and disease activity was not found.

Conclusions

Although just after delivery SLE patients have more microchimerism than control subjects do, this difference cannot be demonstrated thereafter. Interestingly, many years after pregnancy SLE patients have been shown to have more microchimerism than control subjects, shedding new light on the dynamics of microchimerism during and after pregnancy.

Introduction

Microchimerism (Mc) refers to the presence in an individual of a small number of genetically distinct cells, originating from a different zygote. Transplantation of solid organs[1] or bone marrow,[2] blood transfusions,[3] and pregnancies[4] are possible sources of Mc, the latter being the most common.

Pregnancy can have an effect on the symptomatology of several autoimmune diseases, such as systemic lupus erythematosus (SLE). Pregnant SLE patients are more likely to experience a flare of disease activity than non-pregnant SLE patients.[5] Since Mc is known to be increased in pregnancy,[6] it is possible that Mc plays a role in these flares..

Indeed, there are also indications that Mc plays a role in disease development. SLE mainly affects women and has a peak incidence in the reproductive years.[7] Second, studies in mice demonstrated that injection of parental lymphocytes in their offspring, in selected parent-to-F1 combinations, leads to a graft-versus-host response and a lupus like disease.[8 9] Finally, we (manuscript submitted) and others[10 11] have shown an increase in Mc in peripheral blood of SLE patients compared to control subjects. The cause of this increase is unknown and since these chimeric cells are most likely derived from pregnancy, we hypothesized that either SLE patients accrue more Mc during pregnancy, or they clear chimeric cells less efficiently after pregnancy, or both.

Several studies investigated the kinetics of Mc during and after pregnancy in healthy individuals.[12-16] In these studies it was demonstrated that Mc tended to increase with gestational age and disappeared in the months postpartum. However, Mc has not been studied yet in pregnant patients with an autoimmune disease.

Therefore, our aim was to study the kinetics of Mc in peripheral blood mononuclear cells (PBMCs) and granulocytes from 30 weeks of gestation to six months postpartum in SLE patients and control subjects. Furthermore, we collected clinical data to study the relationship between disease activity and the amount of Mc detected.

Materials and methods

Ethics statement

This study was approved by the Medical Ethics Committee of the Leiden University Medical Center (LUMC) (P09.047). Informed consent was obtained from all participants. If DNA from an infant was required, the parents gave written consent on his/her behalf.

Patients and control subjects

Six pregnant SLE patients and 11 pregnant control subjects were studied. The pregnant SLE patients were recruited from the Obstetrics department at the LUMC. All included patients fulfilled at least four of the 1982 revised American College of Rheumatology Criteria for the classification of SLE.[17] The control group consisted of women without a history of autoimmune disease. Of both patients and control subjects peripheral blood samples were drawn at 30 weeks of gestation, just after delivery, and 1 week, 6 weeks, 3 months, and 6 months postpartum. Disease activity was monitored during the study period. To acquire DNA of the infant either umbilical cord blood was used or a buccal mouth swab from the infant was obtained. All subjects were asked to fill out a questionnaire including their age, ethnicity, reproductive history, history of blood transfusion, and medical history.

Isolation of peripheral blood subsets

Peripheral venous blood samples were drawn in sodium-heparine solution vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) and processed to isolate peripheral blood mononuclear cells (PBMCs) by Ficoll amidotrizoate (pharmacy LUMC) with density gradient centrifugation 1.077 g/mL. To remove the erythrocytes from the remaining granulocytes erythrolysis was applied. Until DNA extraction, samples were stored at -180°C in 10% dimethyl sulfoxide in fetal bovine serum.

DNA extraction

DNA was extracted from PBMCs and granulocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with modifications. Briefly, 40 µL of proteinase K was added to 5 x 10^6 cells suspended in 200 µL phosphate-buffered saline. After the addition of 400 µL AL buffer, the suspension was incubated for 30 min (PBMCs) or overnight (granulocytes) at 56 °C. The mixture was applied to the Mini spin column and centrifuged, after adding 200 µl of ethanol. Buffers AW1 and AW2 were used to wash the column. To elute the DNA 100 µl AE buffer was added and incubated at 70 °C for 10 minutes. For an optimal yield, the eluate was reapplied. DNA concentration was measured using Nanodrop (Thermo Scientific, Wilmington, DE). All DNA samples were stored at 4 °C until quantitative PCR (qPCR). DNA extraction from buccal sterile OmniSwabs (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) was performed with the same kit and according to the manufacturer's instruction.

Table 1. Primers

Marker name	Position	5' Primer 3'
S04b	F	CTG GTG CCC ACA GTT ACG CT
S04b	R	AGG ATG CGT GAC TGC TCC TC
S10b	F	TTA GAG CCA CAA GAG ACA ACC AG
S10b	R	TGG CTT CCT TGA GGT GGA AT
S11a	F	TAG GAT TCA ACC CTG GAA GC
S11a	R	CCA GCA TGC ACC TGA CTA ACA
FVII	F	CCC AAC TTA CAT TCC TAT ATC CT
FVII	R	GGG ACA GGA GAA AGG TCA
GSTT1	F	TCC TTA CTG GTC CTC ACA TCT C
GSTT1	R	TCC CAG CTC ACC GGA TCA T
SRY	F	TGG CGA TTA AGT CAA ATT CGC
SRY	R	CCC CCT AGT ACC CTG ACA ATG TAT T

F, forward; R, reverse.

Allele informativity and genotyping

For the detection of FMc a set of previously published insertion-deletion polymorphisms (indels) and null allelles was used.[18-20] For the quantification of FMc an informative difference between the patient or control and her infant was required. For genotyping qPCR was performed with the same protocol as described below, only with a DNA input of 20 ng. Of the published sets of indels and null alleles, six were informative in our study population: GSTT1, SRY (null alleles), and S04b, S10b, S11a and FVII (indels). The primer sequences are listed in Table 1.

Chimerism detection by qPCR

FMc was detected and quantified by qPCR. In all assays iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) was used, with 7.5 µM of each amplification primer. The amplification and melting conditions for all primers consisted of incubation at 96.5 °C for 10 min, followed by 44 cycles of 96.5 °C for 30 s and 60 °C for 1 min. The melting curve started at 65 °C for 5 s followed by 0.2 °C incremental increase, each lasting 5 s, to 95 °C. Amplification and melting data were collected by a Bio-Rad CFX96 detector and analyzed by Bio-Rad CFX Manager version 3.1.

Sanger sequencing of the amplification product was performed and compared with known genomic DNA sequences to ensure primer specificity. Serial dilutions of DNA positive for the indel or null allele in a background of DNA negative for the respective indel or null allele was tested to determine sensitivity. A sensitivity of one genome equivalent (gEq, based on 6.6 pg DNA content per cell) in 100 000 gEq was reached for all primer sets. For every patient and

control, for every time point during or after pregnancy, four aliquots containing 660 ng DNA (100 000 gEq) were tested, in both granulocytes and PBMCs. In order to quantify the chimeric cells and validate the assay on each plate a standard curve for the specific assay was included in each run. This standard curve consisted of 100, 10, and 1 gEq spiked DNA per 100 000 gEq background DNA. In addition, every sample was tested for the housekeeping gene GAPDH. Results were expressed as the gEq of chimeric cells per one million gEq (gEq/10^6). Negative controls consisted of either a water control or background DNA not carrying the indel or null allele tested. Negative controls were consistently negative across all experiments. If the specificity of the amplification product was questioned, the length of the PCR product was compared to that of the positive control using QIAxcel Advanced System (Qiagen) according to the manufacturer's protocol.

Anti-contamination procedures

Strict anti-contamination procedures were applied during blood work-up, DNA extraction and qPCR preparation. The isolation of PBMCs and granulocytes from peripheral blood was performed in a laminar flow cabinet. DNA decontamination reagent (Sigma-Aldrich, St. Louis, MO) was used to clean the cabinet where the DNA was extracted and the qPCR prepared. Furthermore, this cabinet was irradiated with UV light for one hour. All lab consumables were certified DNA free. We used aerosol-resistant pipette tips and clean gloves for all procedures. Eight-wells strips with individual lids were used for the qPCR experiments.

Statistical analysis

For comparison of categorical data, a Fisher's exact test was used (history of blood transfusion, presence of Mc at any time point during or after pregnancy, male fetus, mode of delivery). A Student's *t*-test was used to compare normally distributed data (age proband, gestational age at delivery). For comparison of non-normally distributed numerical data a Mann-Whitney U test was used (number of pregnancies, number of children, number of chimeric cells). A *P*-value ≤0.05 was considered statistically significant. All analyses were performed using SPSS Statistics 20.0 (IBM, Armonk, NY).

Results

Characteristics of patients and control subjects are shown in Table 2; no differences were found between the groups with respect to age, number of children and pregnancies at time

Table 2. Characteristics of SLE patients and control subjects

Parameter	SLE patients (n=6)	Control subjects (n=11)	*P*-value
Age (y)	31.0 ± 4.6	31.1 ± 3.5	0.97[a]
Number of children at time of pregnancy	0 (0.25)	0 (1)	0.38[b]
Number of pregnancies at time of pregnancy	1 (1)	1 (1)	0.72[b]
Blood transfusion (%)	16.7	0	0.35[c]
Gestational age at delivery (weeks)	37.5 ± 2.2	39.9 ± 1.2	0.01[a]
Male fetus	84%	46%	0.30[c]
Delivery mode			
Vaginal delivery	83%	100%	0.35[c]
Cesarean section	17%	0%	

Results are shown as mean ±SD or as median (interquartile range), unless otherwise specified. *P*-values were assessed with [a] Student's *t*-test, [b] Mann-Whitney U test, or [c] Fisher's exact test. SLE, systemic lupus erythematosus; y, years.

Table 3. Microchimerism at any time point during or after pregnancy in patients and control subjects

Parameter	SLE patients (n=6)	Control subjects (n=11)	*P*-value
Mc present in PBMCs or granulocytes (%)	72.7	83.3	1.0
Mc present in PBMCs (%)	54.5	66.7	1.0
Mc present in granulocytes (%)	45.5	83.3	0.30

P-values were assessed with Fisher's exact test. Mc, microchimerism; PBMCs, peripheral blood mononuclear cells; SLE, systemic lupus erythematosus.

of pregnancy, history of blood transfusion, sex of the infant and mode of delivery. However, patients delivered at an earlier gestational age than control subjects (average gestational age 37.5 versus 39.9 weeks, respectively). The majority of both patients and control subjects had detectable Mc at one time point during or after pregnancy (Table 3). Just after delivery, the median number of fetal chimeric cells in the granulocyte fraction was significantly higher in the patient group than in the control group (7.5 gEq/10^6 versus 0 gEq/10^6, respectively; *P*=0.02). This difference was not found in the PBMC fraction (2.5 gEq/10^6 in patients versus 0 gEq/10^6 in control subjects; *P*=0.13). Figure 1 shows the dynamics of the Mc detected during the study period. The demonstrated difference just after delivery disappeared quickly and was no longer present one week after delivery. In both patients and control subjects there was no detectable Mc at three and six months postpartum.

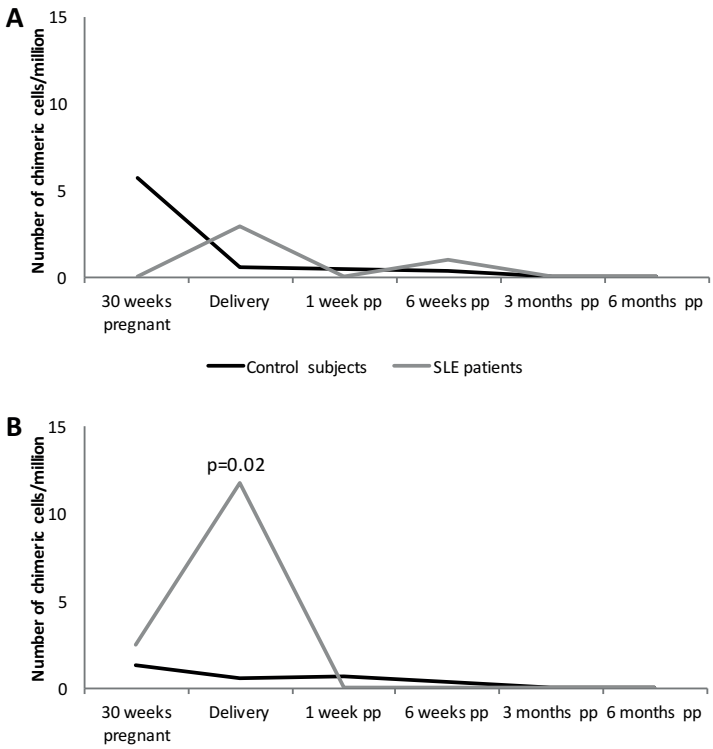

Figure 1. Microchimerism dynamics during and after pregnancy in patients and control subjects
Average number of chimeric cells per million peripheral blood mononuclear cells (panel A) and granulocytes (panel B). Just after delivery SLE patients have more chimeric cells in their granulocyte fraction than control subjects (*P*=0.02, Mann-Whitney U test). At other time points and in the PBMC fraction there are no statistical differences. Pp, postpartum; SLE, systemic lupus erythematosus.

Only one of the patients experienced an increase in disease activity during the course of the study; she had new onset arthritis in seven joints in the postpartum period. Both at the time of disease activity as well as thereafter, there was no detectable Mc.

All but one patient used anti-inflammatory or immunosuppressive medication (hydroxychloroquine, prednisone and/or azathioprine). Interestingly, the one patient without medication was also the only patient without detectable Mc at any of the time points. One of the patients and one of the control subjects developed preeclampsia. They showed Mc at one time point, only in the PBMC fraction, at 6 weeks and one week postpartum, respectively.

Discussion

Our study demonstrates that pregnant women with SLE had more chimeric cells circulating in their peripheral blood just after delivery than pregnant control subjects. Furthermore, these chimeric cells were mainly present in the granulocyte fraction rather than in the PBMCs. They quickly disappeared, and none of the patients and control subjects had detectable Mc three months after pregnancy.

We are the first to study Mc in pregnant SLE patients. The observed difference between patients and control subjects in the blood samples just after delivery was striking, and could bear important implications for the perceived role of Mc in the pathogenesis of SLE. It is particularly interesting that this difference was demonstrated in the granulocyte fraction. Neutrophils are capable of a form of cell death called NETosis (formation of neutrophil extracellular traps, or NETs) in which the neutrophils extrude their chromatin; an autoantigen in SLE.[21] It is conceivable that chimeric neutrophils undergoing NETosis are more likely to illicit an immune response than "regular" neutrophils undergoing NETosis, which may have an effect on the development of SLE or activity of the disease. Furthermore, defects in clearance of apoptotic debris in SLE (for review, see Rekvig *et al*.[22]) may lead to an increased exposure to 'chimeric' chromatin. We can only speculate about the cause of the observed difference. Because the difference was observed in the hours after delivery it may be that a minor feto-maternal hemorrhage is responsible for the increase in detectable Mc, although in this scenario, an increase of Mc in both PBMCs and granulocytes would be expected.

Our results concerning Mc in uncomplicated pregnancies are comparable to those obtained by Ariga *et al.*[15] and Adams Waldorf *et al.*.[14] Although we did find a difference between patients and control subjects in our study, we could not confirm our hypothesis that either SLE patients accrue more Mc during pregnancy or clear chimeric cells less efficiently after pregnancy: *i.e.*, at 30 weeks pregnancy we did not find a difference between patients and control subjects, and the higher level of Mc in SLE patients just after pregnancy was quickly cleared. The quick clearance of the Mc in the granulocytes was not unexpected, because they have a short half-life.[23] However, all Mc was cleared to the extent that three and six months after pregnancy neither patients nor control subjects showed any Mc. This result is striking since our previous study (manuscript submitted) showed that over 50% of women with SLE have detectable Mc more than 20 years after their last pregnancy. A previous study on Mc in SLE suggested that the number of chimeric cells may slowly increase over the years after pregnancy[10] in SLE patients but not in healthy control subjects. Thus, rather

than a decreased clearance of chimeric cells, it is possible that chimeric stem cells obtained during pregnancy are the supply for the higher number of chimeric cells many years after pregnancy, *i.e.*, that persistent Mc in stem cells generates *de novo* Mc in peripheral blood (and solid organs).

Our study has some limitations. First, the number of included SLE patients was small. However, all participants were prospectively followed in the same study protocol. Second, not all participants were primigravid. Nevertheless, because none of the participants had any detectable Mc after three and six months postpartum, it is unlikely that this influenced our results. Finally, all patients in our study were already diagnosed with SLE at inclusion. Strictly speaking, the results of this study are not suitable to draw any conclusions about the pathogenic role of Mc in SLE. In a recent study, however, it was shown that even before the diagnosis of SLE, SLE-associated pregnancy complications occur more frequently than in the general population, suggesting similarities between pregnancies before and after the diagnosis of SLE.[24]

In summary, we found that pregnant women with SLE have more peripheral blood Mc than pregnant control subjects just after delivery. This increase was mostly due to the increased presence of chimeric cells in the granulocyte fraction. Although both cause and consequence of this observation are speculative, it can be hypothesized that these chimeric cells modulate the disease through “chimeric” NETosis and defects in the clearance of apoptotic chimeric cells.

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