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Effect of preeclampsia on umbilical cord blood stem cells in relation to breast cancer susceptibility in the offspring

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Women born from a preeclamptic (PE) pregnancy are associated with a lower risk of breast cancer. Prenatal and early-life exposures are hypothesized to influence breast cancer susceptibility through their effect on stem cells. We examined stem cell populations in umbilical cord blood from PE pregnancies and compared with those from pregnancies without this condition. We isolated mononuclear cells from 58 PE and 197 normotensive (non-PE) umbilical cord blood samples and examined the different stem cell populations. Hematopoietic ($CD34^+$ and $CD34^+CD38^-$), endothelial ($CD34^+CD133^+$, $CD34^+VEGFR2^+$, $CD133^+VEGFR2^+$ and $CD34^+CD133^+VEGFR2^+$), and putative breast ($EpCAM^+$, $EpCAM^+CD49f^+$, $EpCAM^+CD49f^+CD117^+$, $CD49f^+CD24^+$, $CD24^+CD29^+$ and $CD24^+CD29^+CD49f^+$) stem/progenitor cell subpopulations were quantified by flow cytometry and compared between PE and non-PE samples. Hematopoietic $CD34^+$ cell counts were significantly lowered in PE compared with non-PE samples ($P = 0.039$, Kruskal–Wallis test). Levels of $CD34^+CD133^+$ endothelial progenitor cells were also lower in PE samples ($P = 0.032$, multiple regression analysis). $EpCAM^+$ and $EpCAM^+CD49f^+$ putative breast stem cell levels were significantly lowered in PE subjects (multiple regression analysis: $P = 0.038$ and 0.007, respectively). Stratifying by newborn gender, $EpCAM^+$ and $EpCAM^+CD49f^+$ stem cells were significantly lowered in PE samples of female, but not male, newborns. Umbilical cord blood samples from pregnancies complicated by preeclampsia thus had significantly lower levels of hematopoietic, endothelial, and putative breast stem cells than non-PE controls. With a lowered breast cancer risk for offspring of a PE pregnancy, our findings provide support to the hypothesis that susceptibility to breast oncogenesis may be affected by conditions and processes during the prenatal period.

Introduction

Accumulating evidence suggest that prenatal factors influence the offspring's risk of developing breast cancer in later life (1–3). Among maternal, gestational and newborn characteristics, a strong inverse association with breast cancer risk has been found for prenatal exposure to preeclampsia (4,5). Two large studies based on Swedish birth records found that women born from a preeclamptic (PE) pregnancy had a lowered breast cancer risk of about 60%, with an estimated relative risk of 0.41 [95% confidence interval: 0.22–0.79 (6,7)]. This risk reduction is comparable with what can be achieved by prophylaxis with tamoxifen/raloxifene (8) or exercise (9). Mechanisms for the strong inverse association between preeclampsia and breast cancer risk are still unknown.

We postulate that prenatal and early-life exposures influence breast cancer susceptibility through their effect on stem cells, in accordance with the 'stem cell burden and susceptibility' hypothesis (10). The hypothesis puts forward that human cancer risk is influenced in part by the *in utero* environment where high levels of growth factors drive the proliferation of stem cell populations and increase the number or concentration of stem cells ('burden') resulting in a greater chance that these stem cells will be the targets for genetic and/or epigenetic alterations that make them 'susceptible' to carcinogenic transformation later in life (10,11). A reduced offspring breast cancer risk associated with preeclampsia would suggest a reduced number of stem cells in the *in utero* environment. Indeed, hematopoietic (12,13) and endothelial (14–16) stem cell populations have been reported to be reduced in umbilical cord blood from pregnancies complicated by preeclampsia.

We recently detected putative breast stem cells in umbilical cord blood samples obtained from normotensive (non-PE) pregnancies (17). The effect of preeclampsia on these stem cell populations is not known. To this end, we compared the number of putative breast stem cells as well as hematopoietic and endothelial stem/progenitor cell populations in umbilical cord blood samples obtained from PE and non-PE pregnancies.

Abbreviations: MNC, mononuclear cells; non-PE, non-preeclamptic; PE, preeclamptic.

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Materials and methods

Study subjects.

The study protocol was approved by the Institutional Review Boards of the University of Massachusetts Medical School, Worcester, MA and Tufts Medical Center, Boston MA. Study subjects were recruited from February 2007 to April 2012 among pregnant woman who delivered at the Tufts Medical Center. Written informed consent was obtained from all subjects. Subjects were >18 years old with a singleton pregnancy, negative for human immunodeficiency, hepatitis B and C viruses, and with a fetus free of structural anomalies by ultrasound examination. Infants were delivered according to standard obstetric practices. After birth, labor and delivery data were abstracted from medical records. preeclampsia was diagnosed when subjects had documented evidence of a sustained elevation in blood pressure of >140 mmHg systolic and/or 90 mmHg diastolic and significant proteinuria after 20 weeks of gestation. Significant proteinuria was defined as >300 mg per 24 h. All PE cases were verified by a postnatal review of their medical records.

Umbilical cord blood samples

Umbilical cord blood was collected at delivery from the umbilical vein into a sterile bag containing 35 ml of citrate phosphate dextrose anticoagulant (Fenwal, Lake Zurich, IL). Samples were processed for mononuclear cells (MNC) using a Ficoll-Paque (Stemcell Technologies, Vancouver, Canada) density gradient within 24 h of birth as described previously (17).

Flow cytometry

Flow cytometric analyses were carried out as described previously (17). Briefly, 1×10^6 umbilical cord blood-derived MNC were incubated for 30 min on ice in the dark with the following fluorochrome-conjugated antibodies: anti-CD34-fluorescein isothiocyanate (FITC) (Clone 581; BD BioSciences Pharmingen, San Diego, CA), anti-CD38 phycoerythrin (PhE) (Clone HIT2; BD BioSciences Pharmingen), anti-CD133 allophycocyanin (APC) (Clone AC133; Miltenyi Biotechnology, Auburn, CA), anti-VEGFR-2 PhE (Clone 89106; Research & Diagnostics systems, Inc, Minneapolis, MN), anti-EpCAM FITC (Clone VU-1D9; STEMCELL Technologies, Vancouver, Canada), anti-CD49f PhE (Clone GoH3; BD BioSciences Pharmingen), anti-CD117 APC (Clone YB5. B8; BD BioSciences Pharmingen), anti-CD24 FITC (Clone SN3; Antibodies-online, Aachan, Germany), anti-CD29 APC (Clone MAR4; BD BioSciences Pharmingen), or the combination of these antibodies. Samples with vehicle in place of primary antibody served as negative controls. Cells were washed, fixed with 4% paraformaldehyde, and analyzed using a FACSCalibur flow cytometer (BD Biosciences Immunocytometry Systems, San Jose, CA). Hematopoietic

[CD34$^+$and CD34$^+$CD38$^-$ (18)], endothelial [CD34$^+$CD133$^+$, CD34$^+$VEGFR2$^+$, CD133$^+$VEGFR2$^+$ and CD34$^+$CD133$^+$VEGFR2$^+$ (16,19,20)], and putative breast [EpCAM$^+$, EpCAM$^+$CD49f$^+$, EpCAM$^+$CD49f$^+$CD117$^+$, CD49f$^+$CD24$^+$, CD24$^+$CD29$^+$ and CD24$^+$CD29$^+$CD49f$^+$ (17)] stem/progenitor cell subpopulations were quantified from the gated MNC populations using the FlowJo software program (Tree Star, Ashland, OR). The number of cells was normalized to 10^3 MNC.

Statistics

Statistical analysis was conducted using STATA (version 12.0, Stata Corporation, College Station, TX). Descriptive categorical data were compared by chi-square test. Non-parametric Kruskal–Wallis test was used to compare stem/progenitor cell population of subjects with or without preeclampsia. Multiple regression analysis was performed to examine the association between natural log-transformed concentration of each stem cell measurement (dependent variable) and PE complication (independent binary variable, using non-PE pregnancy as a reference). In the multiple regression analysis, we adjusted for potential confounding factors from maternal and neonatal variables, including maternal age, parents' ethnicity, number of previous live births, gestational age, time at delivery (day or night), newborn birth weight, and gender. Maternal age, number of previous live births and birth weight were treated as continuous variables. The fitted coefficients from the regression analysis were exponentiated to obtain the estimated proportional change in outcome associated with each independent variable. A two-sided P value <0.05 was considered to be statistically significant.

Results

The study samples were comprised of umbilical cord blood obtained from 58 PE and 197 non-PE pregnancies. Table I shows the characteristics of the study subjects. The two groups were similar in terms of maternal age, ethnicity and fetal gender. Gestational age was significantly shorter and newborn birth weight was significantly lower in the PE group compared with the non-PE group ($P = 0.0001$, Kruskal–Wallis test). A significantly higher proportion of the PE samples (70%) were from nulliparous women compared with the non-PE samples (46%) ($P = 0.033$, chi-square test).

We quantified the percentages of umbilical cord blood-derived MNC with reported stem and progenitor cell markers of hematopoietic, endothelial and putative breast stem and progenitor cells. Flow cytometric analysis reports among normal samples have been presented (17). Levels of CD34$^+$ cells were significantly decreased in PE compared with non-PE samples (Table II), with mean values of 7.23 ± 7.41 and 8.30 ± 7.28 per 1000 MNC, respectively (13% lower levels in PE pregnancies; $P = 0.039$, Kruskal–Wallis test). Levels of endothelial progenitor cells were all lower in PE samples (Tables II and III), with significant difference observed for the CD34$^+$CD133$^+$ population ($P = 0.032$, multiple regression analysis).

Among putative breast stem cell sub-populations, EpCAM$^+$ and EpCAM$^+$CD49f$^+$ cells were significantly lowered in PE samples (Tables II and III). In univariate analysis (Table II), the mean value of EpCAM$^+$ sub-population was 3.10 ± 3.67 cells per 1000 MNC in non-PE samples and 1.90 ± 2.02 cells per 1000 MNC in PE samples (39% lower levels in PE pregnancies; $P = 0.0069$, Kruskal–Wallis test) while the mean value of EpCAM$^+$CD49F$^+$ sub-population was 1.62 ± 1.76 cells per 1000 MNC in non-PE versus 1.19 ± 1.52 cells per 1000 MNC in PE samples (27% lower levels in PE pregnancies; $P = 0.0034$, Kruskal–Wallis test). In multivariate analysis (Table III), levels of EpCAM$^+$- and EpCAM$^+$CD49f$^+$-marked stem cells continued to be significantly lowered in PE samples ($P = 0.038$ and 0.007, respectively). The results remained essentially unchanged after adjusting further for delivery methods or exposure during pregnancy to cigarette smoking, and no significant interaction between preeclampsia and gestation age was observed. When the analysis was conducted separately for samples from male and female newborns (Table IV), EpCAM$^+$ and EpCAM$^+$CD49f$^+$ cells were significantly lowered in samples from female newborns ($P = 0.044$ and 0.018, respectively), but not in samples from male newborns.

Discussion

Preeclampsia is a disorder characterized by hypertension and proteinuria during the second half of pregnancy. It complicates ~5–8% pregnancies and is an important cause of maternal and fetal morbidity and mortality (21,22). preeclampsia pregnancy has been associated with a lowered risk of maternal breast cancer risk (23). Moreover, offspring born from pregnancies complicated with preeclampsia also have a lower risk of developing breast cancer later in life (4,5). The influence of the *in utero* environment on subsequent risk of disease onset in adult life suggests that mitogens and stem cells may be involved, and this may include also breast stem cells (10,11,24).

Our findings on the effects of preeclampsia on lowered hematopoietic stem cells and endothelial progenitor cells numbers are

Table I. Summary of the maternal and newborn characteristics of subjects with or without preeclampsia

	Non-PE ($N = 197$)	PE ($N = 58$)	P values from Kruskal–Wallis or chi-square test[a]
	Mean ± SD or N (%)	Mean ± SD or N (%)	
Maternal age (years)	30.2 ± 6.2	30.4 ± 6.1	0.69
Parity			
First	82 (46.1)	35 (70)	**0.033**
Second	57 (32.0)	7 (14)	
Third	24 (13.5)	4 (8)	
Fourth and over	15 (8.4)	4 (8)	
Unknown	19	8	
Gestational age (weeks)	39.6 ± 1.1	36.1 ± 2.4	**0.0001**
Newborn gender			
Male	98 (49.8)	33 (56.9)	0.34
Female	99 (50.2)	25 (43.1)	
Parents' ethnicity			
Both Caucasian	111 (56.9)	30 (52.6)	0.22
Both African-American	33 (16.9)	9 (15.8)	
Both Asian	26 (13.3)	4 (7.0)	
Both Hispanic	18 (9.2)	10 (17.5)	
Other	7	4	
Unknown	2	1	
Newborn birth weight (g)	3374.9 ± 493.3	2605.9 ± 767.0	**0.0001**
Umbilical cord blood volume (ml)[a]	97.6 ± 28.5	81.9 ± 26.8	**0.0001**

For continuous variables mean ± standard deviation (SD); for categorical variables N (%).
[a]Kruskal–Wallis test was used for the comparisons between non-PE and PE samples on continuous variables (mother's age, gestation duration, newborn birth weight and umbilical cord blood volume) and chi-square test for categorical variables (parity, newborn gender and parents' ethnicity).
[b]Includes 35 ml of citrate phosphate dextrose anticoagulant.

Table II. Comparison of umbilical cord blood stem/progenitor cell counts between subjects with or without preeclampsia

Umbilical cord blood cell populations	Non-PE (N = 197[a])		PE (N = 58[a])		Kruskal–Wallis test
	N	Mean ± SD	N	Mean ± SD	P value
Initial total nucleated cells[b]	195	15.61 ± 5.06	57	12.60 ± 5.06	**0.0001**
Initial total MNC[b]	195	7.47 ± 2.85	57	7.29 ± 2.64	0.75
Hematopoietic stem cells					
$CD34^{+}$ [c]	196	8.30 ± 7.28	56	7.23 ± 7.41	**0.039**
$CD34^{+}CD38^{-}$ [c]	197	2.49 ± 2.37	57	2.79 ± 2.63	0.93
Endothelial progenitor cells					
$CD34^{+}CD133^{+}$ [c]	91	3.91 ± 3.71	48	3.64 ± 4.24	0.21
$CD34^{+}$ $VEGFR2^{+}$ [c]	91	0.49 ± 0.87	48	0.38 ± 0.52	0.59
$CD133^{+}VEGFR2^{+}$ [c]	91	0.23 ± 0.54	48	0.16 ± 0.32	0.98
$CD34^{+}CD133^{+}VEGFR2^{+}$ [c]	91	0.20 ± 0.51	48	0.14 ± 0.30	0.44
Breast stem cells					
$EpCAM^{+}$ [c]	141	3.10 ± 3.67	52	1.90 ± 2.02	**0.007**
$EpCAM^{+}CD49f^{+}$ [c]	138	1.62 ± 1.76	51	1.19 ± 1.52	**0.003**
$EpCAM^{+}CD49f^{+}CD117^{+}$ [c]	60	0.52 ± 1.07	44	0.40 ± 0.63	0.49
$CD49f^{+}CD24^{+}$ [c]	80	13.86 ± 11.73	45	13.11 ± 12.75	0.65
$CD24^{+}CD29^{+}$ [c]	83	8.22 ± 8.44	44	7.45 ± 6.69	0.72
$CD49f^{+}CD24^{+}CD29^{+}$ [c]	80	7.03 ± 7.69	44	6.06 ± 5.24	0.81

[a]Two non-PE and one PE samples had missing data on the initial total nucleated and mononuclear cells. Different samples had missing data on stem cell populations measured in the sorted mononuclear cells.
[b]Cell counts × 10^6 per milliliter of umbilical cord blood.
[c]Cell counts per 10^3 mononuclear cells.

Table III. Multiple regression analysis of the association between preeclampsia and umbilical cord blood stem/progenitor cell population

Umbilical cord blood cell populations	N	Difference (%)[a]	95 % CI	P value
Initial total nucleated cells	216	−16.32	−27.7 to −3.14	**0.017**
Initial total mononuclear cells	216	−10.39	−23.35 to 4.77	0.17
Hematopoietic stem cells				
$CD34^{+}$	216	−12.10	−42.42 to 34.19	0.55
$CD34^{+}CD38^{-}$	218	13.78	−22.37 to 66.77	0.51
Endothelial progenitor cells				
$CD34^{+}CD133^{+}$	118	−52.51	−75.91 to −6.39	**0.032**
$CD34^{+}$ $VEGFR2^{+}$	118	−11.27	−58.47 to 89.59	0.76
$CD133^{+}VEGFR2^{+}$	118	−33.91	−74.04 to 68.21	0.38
$CD34^{+}CD133^{+}VEGFR2^{+}$	118	−54.51	−83.84 to 28.08	0.13
Breast stem cells				
$EpCAM^{+}$	167	−41.48	−64.71 to −2.94	**0.038**
$EpCAM^{+}CD49f^{+}$	163	−51.93	−71.60 to −18.65	**0.007**
$EpCAM^{+}CD49f^{+}CD117^{+}$	88	−22.97	−67.75 to 83.99	0.55
$CD49f^{+}CD24^{+}$	106	−12.49	−57.01 to 78.15	0.71
$CD24^{+}CD29^{+}$	108	13.99	−43.68 to 130.70	0.71
$CD49f^{+}CD24^{+}CD29^{+}$	104	−12.65	−60.80 to 94.60	0.74

CI, confidence interval. The analysis controlled for clinical variables include mother's age, parents' ethnicity (both Caucasian versus other), number of previous live births, gestation durations, gender of baby, time at delivery (day or night) and birth weight. The analysis included samples with no missing data on the covariates.
[a]Proportional difference in stem cell numbers comparing PE with non-PE samples.

consistent with published reports. Preeclampsia has been found to be associated with a decreased level of $CD34^{+}$ hematopoietic stem cells in comparison with non-PE controls (12,13) and significantly lower levels of $CD34^{+}CD133^{+}$ endothelial progenitor cells in PE samples have been reported (16).

We also measured previously reported markers for putative breast stem/progenitor cells (25–29) in umbilical cord blood (17). We found significantly lower numbers of $EpCAM^{+}$ and $EpCAM^{+}CD49f^{+}$ mammary stem cells in the cord blood samples of PE pregnancies compared with the respective levels in samples collected from non-PE pregnancies. To the best of our knowledge, this is the first report that examined putative breast stem cell numbers in umbilical cord blood samples from pregnancies complicated by preeclampsia.

It is not immediately clear why the $EpCAM^{+}$ and $EpCAM^{+}CD49f^{+}$ populations are significantly lower in the cord blood of pregnancies complicated by preeclampsia. Breast epithelial progenitor cells carrying the EpCAM and CD49f markers can be bipotent progenitors, which give rise to luminal or myoepithelial lineages, and/or luminal progenitors (25). Interestingly, such progenitors may serve as precursors of different breast tumor subtypes such as basal-like, luminal A and luminal B tumors (29). Although chance cannot be ruled out, the preeclampsia-associated decrease in cell numbers that is specific only to female newborns further corroborates a potential role for these cells in susceptibility to breast carcinogenesis.

Alterations in the concentrations of circulating cytokines, hormones, and growth factors in the setting of preeclampsia (30) are likely to influence the observed decrease in the quantity of overall stem cell populations, including that of putative breast stem cells, in the cord blood compartment. A reported decrease in insulin-like growth factor-1 (IGF-1) levels in the cord blood plasma of PE pregnancies (31,32) may explain at least in part the link between breast cancer risk and the number of growth hormone/IGF-1 axis-regulated stem cells (18,33).

In the current analysis, we examined whether the relation between preeclampsia and breast stem cells corresponds to its relation with

Table IV. Multiple regression analysis of the association between preeclampsia and umbilical cord blood stem/progenitor cell population according to newborn gender

Umbilical cord blood cell populations	Female				Male			
	N	Difference (%)[a]	95 % CI	*P* value	*N*	Difference (%)[a]	95 % CI	*P* value
Initial total nucleated cells	106	6.67	−15.06 to 33.96	0.58	110	−26.23	−39.00 to 10.77	**0.002**
Initial total mononuclear cells	106	8.04	−15.21 to 37.67	0.53	110	−18.42	−33.67 to 0.34	0.054
Hematopoietic stem cells								
$CD34^+$	105	−14.31	−55.50 to 65.00	0.64	111	−10.53	−49.48 to 58.45	0.70
$CD34^+CD38^-$	107	13.40	−38.59 to 109.39	0.69	111	15.25	−30.40 to 90.84	0.58
Endothelial progenitor cells								
$CD34^+CD133^+$	56	−18.44	−75.02 to 166.24	0.73	62	−62.86	−85.06 to −7.67	**0.034**
$CD34^+$ $VEGFR2^+$	56	68.05	−58.45 to 579.70	0.46	62	−25.27	−71.00 to 92.62	0.54
$CD133^+VEGFR2^+$	56	108.84	−56.73 to 907.83	0.35	62	−60.37	−89.16 to 44.95	0.16
$CD34^+CD133^+VEGFR2^+$	56	−19.53	−86.14 to 367.33	0.81	62	−64.17	−91.56 to 52.14	0.16
Breast stem cells								
$EpCAM^+$	83	−55.77	−79.99 to 2.23	**0.044**	84	−32.34	−66.74 to 37.65	0.28
$EpCAM^+CD49f^+$	80	−64.92	−85.17 to 17.06	**0.018**	83	−41.14	−71.42 to 21.24	0.15
$EpCAM^+CD49f^+CD117^+$	43	−12.53	−80.57 to 293.70	0.86	45	−37.07	−80.02 to 98.25	0.42
$CD49f^+CD24^+$	52	14.33	−69.81 to 333.04	0.84	54	6.01	−51.17 to 130.14	0.88
$CD24^+CD29^+$	54	−19.46	−76.77 to 179.25	0.73	54	58.09	−36.70 to 294.80	0.32
$CD49f^+CD24^+CD29^+$	51	−18.21	−83.69 to 310.19	0.80	53	2.07	−59.16 to 155.10	0.96

CI, confidence interval. The analysis controlled for clinical variables include mother's age, parents' ethnicity (both Caucasian versus other), number of previous live births, gestation durations, time at delivery (day or night), and birth weight. The analysis included samples with no missing data on the covariates.
[a]Proportional difference in stem cell numbers comparing PE with non-PE samples.

breast cancer risk observed in epidemiologic studies. Our data showed that the effect of preeclampsia on putative breast stem cell populations in cord blood is more striking than those previously described for hematopoietic and endothelial progenitor cells. Intriguingly, evidence in support of an intrauterine origin of susceptibility to cancer in later life is stronger for breast cancer (1,3) compared with other cancer sites (34). Given the reduced breast cancer risk for offspring of a PE pregnancy, a lower breast stem cell concentration in umbilical cord blood samples from such pregnancies lends support to the "stem cell burden and susceptibility" hypothesis for the early-life influence in breast cancer susceptibility.

Our assays were updated during the study period to keep pace with increasing number of reported mammary stem cell markers, resulting in smaller samples sizes for newer markers. Further studies are therefore needed to replicate in new umbilical cord blood samples these and other markers that have been suggested for detecting mammary stem cells (10), and to assess the functional relevance of these cord blood stem cells, using for example an *in vivo* cell-lineage tracing model to genetically label these putative breast stem cells and track their developmental fate from the intrauterine environment until the development of breast cancer.

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