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Et al.

Association of Fetal Hormone Levels with Stem Cell Potential: Evidence for Early Life Roots of Human Cancer

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Abstract

Intrauterine and perinatal factors have been linked to risk of childhood leukemia, testicular cancer, and breast cancer in the offspring. The pool of stem cells in target tissue has been suggested as a critical factor linking early life exposures to cancer. We examined the relation between intrauterine hormone levels and measurements of stem cell potential in umbilical cord blood. Cord blood donors were 40 women, ages ≥18 years, who delivered, from August 2002 to June 2003, a singleton birth after a gestation of at least 37 weeks. We assayed plasma concentrations of estradiol, unconjugated estriol, testosterone, progesterone, prolactin, sex hormone binding globulin, insulin-like growth factor-I (IGF-I), and IGF binding protein-3. For stem cell potential, we measured concentrations of $CD34^+$ and $CD34^+CD38^-$ cells and granulocyte-macrophage colony-forming unit (CFU-GM). We applied linear regression analysis and controlled for maternal and neonatal characteristics. We found strong positive associations between IGF-I and stem cell measures, 1 SD increase in IGF-I being associated with a 41% increase in $CD34^+$ ($P = 0.008$), a 109% increase in $CD34^+CD38^-$ ($P = 0.005$), and a 94% increase in CFU-GM ($P = 0.01$). Similar associations were observed for IGF binding protein-3. Among steroid hormones, estriol and testosterone were significantly positively associated with $CD34^+$ and CFU-GM. These findings indicate that levels of growth factors and hormones are strongly associated with stem cell potential in human umbilical cord blood and point to a potential mechanism that may mediate the relationship between *in utero* exposure to hormones and cancer risk in the offspring. (Cancer Res 2005; 65(1): 358-63)

Introduction

It has long been established that many chemicals can lead to prenatal carcinogenesis in several animal species (1, 2). In humans, prenatal exposures to ionizing radiation have been shown to cause leukemia and other tumors in children (3, 4), whereas intrauterine exposure to diethylstilbestrol has been found to cause vaginal ad-enocarcinoma in young women (5) and possibly testicular cancer in men (6). Evidence linking endogenous, rather than exogenous, perinatal factors to cancer risk in the offspring is more tenuous. Animal studies have implicated intrauterine hormonal exposures and maternal diet in breast cancer risk of the female rat offspring (7, 8), whereas in humans most of the relevant evidence concerns also breast cancer and stems from the fairly consistent association between birth weight and the risk of this disease in the offspring (9–14). Positive associations of birth weight, however, have been reported with several other cancers in the offspring, including childhood leukemia (15, 16), prostate cancer (17, 18), and, indeed, cancer overall (19).

The indicated link between birth weight and cancer risk in the offspring, as well as the strong positive association of insulin-like growth factor-I (IGF-I) with birth weight (20–22) and the relation of IGF-I with the risk of several forms of adult cancer (23–25), brings forward a simple hypothesis that has been implicitly considered by several investigators searching for common conditions or processes that may underlie cancer risk (26–29). According to this hypothesis, articulated by Trichopoulos and Lipworth (30), growth hormones in intrauterine life, such as IGF-I and steroid hormones, are positively associated with total number of stem cells and, by extension, total number of replicating immature cells and eventually number of cells at risk for malignant transformation.

Among stem cells, those with hematopoietic potential are the only ones that are both identifiable and easily accessible in the cord blood. We report here the findings on the relation between measures of hematopoietic stem cell potential and plasma concentrations of estrogens, progesterone, prolactin, sex hormone binding globulin (SHBG), IGF-I, and IGF binding protein-3 (IGFBP-3) in cord blood.

Materials and Methods

Study Subjects. Subjects were participants in the American Red Cross (ARC) cord blood program that harvests and stores hematopoietic stem cells from umbilical cord blood for transplantation purposes. Eligible participants were pregnant women (ages ≥18 years) who delivered a singleton birth (gestational age ≥37 weeks) at the University of Massachusetts Memorial Medical Center and St. Vincent's Hospital. Those who reported drug use, blood diseases, cancer, AIDS, sexual diseases, or serious infectious diseases were excluded. Each participant signed a Human Subjects Committee–approved informed consent form before collection of cord blood. Among the participants in the ARC program, our study subjects were identified from those whose cord blood samples collected were not accepted for storage in the cord blood bank mainly due to insufficient

volume (<85 mL) or incomplete information on the label of the collection bag (e.g., missing exact time of delivery).

As a part of the protocol, extensive medical and family history data were obtained from the participants before delivery. Specifically, women were asked to self-report their age, race, education, number of prior pregnancies and live births, and complications during pregnancy in addition to detailed information of their family medical history. After delivery, a nurse collected and reported labor and delivery data to the ARC, including the weight and gestational age of the baby. We obtained information of these maternal and neonatal characteristics from the ARC database.

The study protocol was approved by the institutional review boards of the ARC, University of Massachusetts Medical School, University of Massachusetts Memorial Medical Center, and St. Vincent's Hospital.

Human Umbilical Cord Plasma and Stem Cell Samples. Umbilical cord blood was collected from infants delivered according to standard obstetric practices, while the placenta was still *in utero*. For infant births requiring cesarean section, cord blood was collected after the delivery of the placenta. Cord blood was drained from the umbilical vein using a 16-gauge needle and was collected in a plastic bag containing 35 mL citrate-phosphate-dextrose anticoagulant (Baxter Health Care, Deerfield, IL). After collection, cord blood samples were stored at room temperature and were processed within 24 hours from the time of blood collection.

Nucleated cell counts were measured in the whole cord blood sample with an automated Coulter STKS (Beckman-Coulter, Hialeah, FL). The cord blood samples were centrifuged at 20°C for 30 minutes at 1,400 rpm, and plasma from each sample was separated. Aliquots containing a total of 2 mL plasma in each cryovial were prepared and stored at −70°C until the time of analysis.

After withdrawing plasma, the remaining specimen was diluted 4-fold with PBS-EDTA solution (Dulbecco's PBS/5 mmol/L EDTA/2% FCS) and layered onto lymphocyte separation medium (ICN Biomedicals, Inc., Aurora, OH) density gradients to deplete RBC. Light-density (ρ < 1.077 g/mL) mononuclear cells (MNC) were collected, diluted with PBS-EDTA solution, and centrifuged at 1,200 rpm for 10 minutes. After erythrocyte lysis, the cell pellet was washed twice with PBS-EDTA solution and once with PBS solution (Dulbecco's PBS/2% FCS) and resuspended in PBS solution. For flow cytometric analysis, stem cell samples were prepared after dilution to a concentration between 1×10^6 and 2×10^6 cells per microliter.

Hormone Assays. Hormone assays were conducted at the ILAT Steroid RIA Laboratory, Department of Physiology, University of Massachusetts Medical School, and hormone levels were measured in umbilical cord plasma. Estradiol was measured by RIA using kits from Diagnostic Systems Laboratories, Inc. (Webster, TX). Testosterone was measured by solid-phase ^{125}I RIA using kits from Diagnostic Products Corp. (Los Angeles, CA). Unconjugated estriol, progesterone, prolactin, and SHBG were measured using chemiluminescent immunoassay methodologies from Diagnostic Products. IGF-I and IGFBP-3 were measured by immunoradiometric assay using kits obtained from Diagnostic Systems Laboratories.

Samples were assayed in three batches. For each assay run, two aliquots from each of the three in-house control samples formed by pooled sera were assayed. The interassay and intra-assay coefficient variations of the in-house controls were 12.5% and 4.3% for estradiol, 5.2% and 1.6% for testosterone, 6.3% and 6.3% for estriol, 9.5% and 5.9% for progesterone, 6.6% and 1.7% for prolactin, 9.1% and 4.8% for SHBG, 11.3% and 7.8% for IGF-I, and 6.3% and 12.7% for IGFBP-3, respectively. For each assay, control samples that accompanied the kit were also examined; all results were within the manufacturer's specifications.

Flow Cytometric Analysis of Stem Cell Populations. We measured currently recognized, standard populations of hematopoietic stem cells, notably CD34+ and CD34+CD38− (31, 32). Stem cell samples were incubated with appropriate cocktails of antibodies including anti-CD34 FITC and anti-CD38 phycoerythrin (Becton Dickinson, San Jose, CA) for 30 minutes on ice. This staining process allows simultaneous multicolor analysis to identify CD34+ and CD34+CD38− cell populations. Following wash with PBS solution to remove unbound antibodies, the cells were fixed in 10% paraformaldehyde, stored at 4°C overnight, and analyzed by a multilaser flow cytometer (FACSCalibur, Becton Dickinson). Results are expressed as density (concentration) of CD34+ and CD34+CD38− cells per 1,000 MNC.

CD34+ Cell Isolation and Colony-Forming Assay. Primitive progenitor cells have the ability to form colonies *in vitro* in semisolid and liquid

Table 1. Summary of the maternal and newborn characteristics and the laboratory measurements

Variables	*n* (mean ± SD or %)	Range
Subject characteristics		
Mother's age (y)	40 (29.6 ± 5.0)	18-39
Race of mother and biological father		
Both Caucasian	36 (90.0)	
Both African American	1 (2.5)	
Both Hispanic	1 (2.5)	
Caucasian, Hispanic	1 (2.5)	
Caucasian, Asian	1 (2.5)	
No. previous live births		
0	14 (35.0)	
1	13 (32.5)	
2	7 (17.5)	
3	6 (15.0)	
Gestation duration (wk)	40 (39.6 ± 1.4)	36.5-41.6
Gender of the baby		
Male	21 (53.9)	
Female	18 (46.2)	
Birth weight of the baby (g)	39 (3,313.1 ± 450.6)	2,500-4,570
Hormones		
Estradiol (ng/dL)	40 (792.4 ± 733.1)	40.6-3,271.0
Unconjugated estriol (ng/mL)	40 (281.8 ± 115.8)	18.1-516
Testosterone (ng/mL)	40 (1.4 ± 0.6)	0.3-3.4
SHBG (nmol/L)	40 (24.7 ± 9.0)	11.7-52.0
Progesterone (ng/mL)	40 (214.3 ± 110.0)	77.2-525.7
Prolactin (ng/mL)	40 (236.8 ± 108.4)	50.8-555.4
IGF-I (ng/mL)	40 (68.3 ± 40.3)	10.2-170
IGFBP-3 (ng/mL)	40 (1,040.2 ± 278.4)	640-1,644
Cord blood cell populations		
TNC (counts/μL), ×10⁷	39* (86.0 ± 47.3)	36.1-252
MNC (counts/μL), ×10⁷	39* (42.4 ± 19.7)	16.6-112.1
CD34+ cell density†	40 (34.5 ± 17.1)	4.9-77.6
CD34+CD38− cell density†/10³ MNC	40 (4.0 ± 5.9)	0-19.7
CFU-GM colony counts		
Mean in five plates	20 (56.1 ± 20.8)	19.2-88.4
Density in MNC/10³ MNC	20 (0.84 ± 0.47)	0.17-1.46

NOTE: One subject each had unknown information on birth weight and gender of baby.

*Total blood volume was missing for one sample.

†Cell density per 1,000 MNC.

Table 2. Spearman correlation coefficients (*Ps*) among cord blood cell populations

Variables	MNC	CD34$^+$	CD34$^+$CD38$^-$	CFU-GM
TNC	0.77 (<0.0001)	0.18 (0.27)	0.07 (0.69)	0.20 (0.42)
MNC		0.18 (0.27)	0.20 (0.23)	0.06 (0.82)
CD34$^+$			0.43 (0.005)	0.77 (<0.0001)
CD34$^+$CD38$^-$				0.49 (0.03)

NOTE: TNC (cell counts/μL of cord blood); MNC (cell counts/μL of cord blood); CD34$^+$, CD34$^+$CD38$^-$, and CFU-GM: density per 1,000 MNC.

cultures on stimulation with specific growth factors. The growth of colonies derived from granulocyte-macrophage colony-forming unit (CFU-GM) by *in vitro* colony-forming cell assay characterizes proliferative potential of stem cells (33, 34). Thus, we measured CFU-GM colonies by a semisolid culture assay among CD34$^+$ cells isolated from cord blood.

CD34$^+$ cells were isolated on magnetic-activated cell sorting columns using microbead-conjugated antibodies (MiniMACS system, Miltenyi Biotec, Bergisch Gladbach, Germany). To determine the number of CFU-GM colonies, 2,500 sorted cells were plated in 35-mm tissue culture plate containing 1.5 mL methylcellulose medium (MethoCult GF H4535, Stem Cell Technologies, Inc., Vancouver, British Columbia, Canada). Plates were incubated at 37°C in humidified 5% CO_2 for 14 days. Colonies that contained ≥40 cells were enumerated using a dissecting microscope (Olympus, Melville, NY) in five plates for each of 20 samples. Based on the mean of colony counts from five plates, results are expressed first as density of CFU-GM colony counts among the CD34$^+$ cells and then as number of CFU-GM per 1,000 MNC.

Statistical Analysis. Descriptive statistics on the characteristics of study population and laboratory data were calculated. Spearman rank correlation coefficients were estimated for bivariate analyses. Multivariate linear regression was used to examine the association between hormones (independent variable) and natural log-transformed measures of stem cell potential (dependent variable), adjusting for maternal and neonatal characteristics (mother's age, race of parents, number of live births, gestation duration, baby's gender and birth weight, and delivery time). Maternal age, gestational duration, and birth weight were treated as continuous variables. The fitted coefficients from the regression analyses were exponentiated to obtain the estimated proportional change in outcome associated with each independent variable. Statistical significance was set at 0.05 (two-sided). To conduct statistical analyses, the SAS program version 8.0 (SAS Institute, Cary, NC) was used.

Results

Among the cord blood samples collected by the ARC cord blood program from August 2002 to June 2003, we assayed 40 samples. With the exception of birth weight, the study samples were similar to all ARC donors on all maternal and newborn characteristics. Mean birth weight was lower in babies of the study donors than in those of the ARC donors overall (mean ± SD, 3,313 ± 451 versus 3,524 ± 464 g), because the study samples included cord blood not accepted for banking by the ARC for volumes that were insufficient for clinical purposes.

Summary statistics on maternal and newborn characteristics, plasma concentrations of hormones, and measures of stem cell potential in umbilical cord blood are presented in Table 1. The majority (90%) of the cord samples were from neonates of parents who were both Caucasian. One third of the samples were from the first live birth. Birth weight ranged from 2,500 to 4,570 g, with a slightly higher proportion (12.8%) of the subjects classified as small-for-gestation age (<10th percentile for gestational age) and only 1 (2.6%) subject as large-for-gestation age (>90th percentile) based on U.S. singleton births by race and gender (35). Slightly more than half (53.9%) of the samples were from male neonates. Mean plasma concentration of estradiol, unconjugated estriol, testosterone, progesterone, prolactin, and IGF-I were 792 ng/dL, 282 ng/mL, 1.4 ng/mL, 214 ng/mL, 237 ng/mL, and 68.3 ng/mL, respectively. Mean SHBG and IGFBP-3 concentrations were 25 nmol/L and 1,040 ng/mL, respectively. As measures of stem cell potential, mean density of CD34$^+$ and CD34$^+$CD38$^-$ per 1,000 MNC were 34.5 and 4.0, respectively. CFU-GM assays were successfully completed for 20 samples. Mean CFU-GM count in five plates was 56.1 and density of CFU-GM per 1,000 MNC was 0.84. Table 2 presents the results of mutual associations among total nucleated cell counts (TNC), MNC, and measures of stem cell potential. TNC and MNC were highly correlated, but neither TNC nor MNC were associated with stem cell potential as evaluated through densities of CD34$^+$, CD34$^+$CD38$^-$, and CFU-GM. The three measures of stem cell potential were, as expected, positively and significantly intercorrelated (Table 2).

Bivariate analysis of associations between plasma hormone levels and measures of stem cell potential is presented in Table 3. Among the studied endocrine compounds, estradiol, estriol, testosterone,

Table 3. Spearman correlation coefficients (*Ps*) between cord blood hormones and cell populations

Variables	TNC	MNC	Measures of stem cell potential, counts/1,000 MNC		
			CD34$^+$	CD34$^+$CD38$^-$	CFU-GM
Estradiol	0.24 (0.13)	0.20 (0.21)	0.30 (0.06)	0.47 (0.002)	0.63 (0.003)
Estriol	0.32 (0.05)	0.31 (0.06)	0.36 (0.02)	0.23 (0.15)	0.63 (0.003)
Testosterone	0.20 (0.23)	0.20 (0.21)	0.30 (0.06)	0.15 (0.35)	0.69 (0.0008)
SHBG	−0.03 (0.87)	−0.04 (0.80)	−0.08 (0.62)	0.30 (0.06)	0.27 (0.25)
Progesterone	0.20 (0.23)	0.14 (0.38)	0.15 (0.37)	0.34 (0.03)	0.39 (0.09)
Prolactin	0.35 (0.03)	0.32 (0.05)	−0.13 (0.43)	−0.16 (0.32)	0.34 (0.14)
IGF-I	0.24 (0.15)	−0.05 (0.75)	0.33 (0.04)	0.20 (0.21)	0.61 (0.004)
IGFBP-3	0.50 (0.001)	0.27 (0.10)	0.37 (0.02)	0.36 (0.02)	0.64 (0.002)

Table 4. Multiple linear regression analysis for the association between measures of stem cell potential and hormone levels in cord blood

Hormones	% Change (95% confidence interval)	P	% Change (95% confidence interval)	P	% Change (95% confidence interval)	P
	CD34$^+$ cells/10^3 MNC		CD34$^+$ CD38$^-$ cells/10^3 MNC		CFU-GM counts/10^3 MNC	
Estradiol	9.5 (−11.8 to 35.9)	0.40	38.0 (−9.8 to 111.2)	0.13	31.3 (−9.6 to 90.3)	0.14
Estriol	30.5 (8.8, 56.4)	0.006	34.4 (−10.4 to 101.4)	0.15	39.6 (1.3, 92.4)	0.04
Testosterone	22.6 (1.6, 47.9)	0.04	30.5 (−12.4 to 94.4)	0.18	37.2 (1.6, 85.1)	0.04
SHBG	−1.4 (−22.0 to 24.5)	0.90	53.1 (−1.8 to 138.7)	0.06	66.8 (8.4, 156.8)	0.02
Progesterone	7.4 (−14.2 to 34.3)	0.52	51.1 (−1.6 to 132.1)	0.06	11.2 (−24.9 to 64.7)	0.56
Prolactin	−4.0 (−25.2 to 23.3)	0.74	−21.1 (−52.1 to 29.9)	0.34	46.3 (−4.3 to 123.7)	0.07
IGF-I	40.9 (10.0, 80.5)	0.008	108.8 (27.6, 241.7)	0.005	93.6 (19.6, 213.4)	0.01
IGFBP-3	32.3 (5.0, 66.7)	0.02	97.3 (26.3, 208.1)	0.004	47.6 (−0.5 to 119.0)	0.05

NOTE: From multiple regression model, adjusting for mother's age, race of parents, number of live births, gestation duration, gender of baby, delivery time, and birth weight of baby. % Change: expected proportional change in dependent variable associated with 1 SD increase per independent variable. Ps for the estimated coefficients.

and the strongly intercorrelated IGF-I and IGFBP-3 ($r = 0.70$) seem to be associated with measures of stem cell potential. These results do not, however, take into account potential confounding influence of maternal and neonatal characteristics.

The associations between measures of stem cell potential and the studied hormones in cord blood after adjustment for maternal and neonatal characteristics are presented in Table 4. Statistically significant positive associations with all three measures of stem cell potential are evident with respect to IGF-I and these positive associations are reflected in similar associations with IGFBP-3. Weaker associations of stem cell potential are also noted with respect to estradiol, estriol, and testosterone, whereas for SHBG, progesterone, and prolactin the evidence for a positive association with stem cell potential is even weaker.

Discussion

In this study, we have found that IGF-I and IGFBP-3 are significant predictors of stem cell potential in cord blood. Cord blood estrogens and testosterone were also positively associated with stem cell potential, although the respective relations seemed to be weaker than those of IGF-I and IGFBP-3. We interpret these findings as indicating that growth hormones during the perinatal period tend to increase the number of stem cells and, by extension, the total number of replicating immature cells susceptible to malignant transformation. Thus, the findings of this study, which explores an issue that has not been evaluated previously in humans, point to potential mechanisms that may underlie prenatal origin of cancer risk at several sites in the offspring (36).

The working hypothesis for this research is that the size of the pool of stem cells, which is largely determined during the perinatal period, is associated with cancer risk (26–28, 37–39). Methodologic and ethical complexities do not allow a direct testing of the postulated mechanism in human subjects, as it is difficult to examine the effects of fetal exposure to pregnancy hormones on stem cell quantity and on the subsequent occurrence of cancer in the offspring. It is also not feasible in a study in humans to use specimens

from fetal organs to study the association between hormone levels and stem cell quantity. To overcome these constraints, we have exploited the unique accessibility of cord blood as a human fetal tissue to examine the relation between intrauterine hormone levels and measurements of stem cell potential.

Levels of various hormones in the cord blood have been examined in earlier research (40, 41). Among the hormones examined by Shibata et al. (40), the geometric mean was 662 ng/dL for estradiol, 65.0 ng/mL for IGF-I, and 696 ng/mL for IGFBP-3 in the cord blood plasma from 57 Caucasian subjects. In another study, the mean was 26 ng/dL for testosterone, 12,420 pg/mL for estradiol, and 249 ng/mL for estriol in the cord blood sera from 86 subjects of different races (~60% Caucasian; ref. 41). Our results on estradiol and IGF-I were very similar to that found in Shibata et al. (40), but our findings on testosterone, estradiol, and estriol were not directly comparable with that in Troisi et al. (41) due to the difference in type of samples (plasma versus sera) and in racial composition of the subjects.

Although hematopoietic stem cells may not share all properties of organ-specific stem cells, they are by definition largely undifferentiated. CD34 antigen is an integral membrane glycoprotein of 90 to 120 kDa and is considered as one of the defining hallmarks of hematopoietic stem cells (42). However, the CD34$^+$ population is heterogeneous and includes differentiating cells, committed progenitor cells, early multipotent progenitors, and stem cells, whereas CD34$^+$CD38$^-$ and CFU-GM cells are more primitive subpopulations (31, 33, 34, 42). It is of interest to note that the regression coefficients linking cord blood estrogens, testosterone, and IGF-I to stem cell potential tend to be smaller for CD34$^+$ cells that include more differentiated cells.

We consider it important that the strongest associations with cord blood stem cell potential were found with IGF-I and its strongly correlated binding protein IGFBP-3. IGFs and their binding proteins are recognized as important factors in carcinogenesis (43). IGF-I and IGFBP-3 have been shown in a recent meta-analysis to be associated with increased risks of non-smoking-related cancers in epidemiologic studies (44). It may also be of importance that IGF-I in the fetus is regulated by metabolic factors rather than being significantly influenced by growth hormone as in

adult life (45), that IGF-I cannot cross the placenta (46), and that cord blood levels of IGFBP-3 are positively correlated with those of IGF-I (47).

In addition to questions concerning the degree of similarity between blood and organ-specific stem cells and to considerations regarding sample size, particularly with respect to CFU-GM, our study has another limitation stemming from the cross-sectional ascertainment of hormone levels and cell populations. Experimental evidence, however, points to quiescent stem cells proliferating on stimulation by IGF-I, estrogens, and growth-enhancing hormones (48–50), so that the directionality of the positive associations between hormone levels and stem cell potential can reasonably be inferred. Results from the Dutch Famine Study suggest that caloric restriction in the third rather than the earlier trimesters reduces birth weight (51). Thus, it is possible that hormones and growth factors in late pregnancy influence a subject's cancer risk through their effect on birth weight, whereas organogenesis interlinked with stem cell potential in early gestation might involve a distinct pathway. A positive correlation between birth weight and CD34$^+$ cell density (Spearman correlation $\gamma = 0.12$) was observed in this study, but sample size constraint did not allow informative analyses that

adjusted properly for covariates in the relation between stem cell measurements and birth weight.

In conclusion, we have found evidence that IGF-I and IGFBP-3 and, to a lesser extent, estradiol, estriol, and testosterone in the umbilical cord are positively associated with measures of hematopoietic stem cell potential. These findings support a possible link between increased *in utero* exposure to IGF-I, estrogens, and testosterone and increased proliferative potential of stem cells and suggest a plausible biological mechanism underlying the early life roots of human cancer. The number of samples in the current analysis is relatively small, however, and the results should be replicated in studies with larger samples.

Acknowledgments

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