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REGULAR ARTICLE

Epigenetic modulation at birth – altered DNA-methylation in white blood cells after Caesarean section

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Abstract

Aim: Delivery by C-section (CS) has been associated with increased risk for allergy, diabetes and leukaemia. Whereas the underlying cause is unknown, epigenetic change of the genome has been suggested as a candidate molecular mechanism for perinatal contributions to later disease risk. We hypothesized that mode of delivery affects epigenetic activity in newborn infants.

Methods: A total of 37 newborn infants were included. Spontaneous vaginal delivery (VD) occurred in 21, and 16 infants were delivered by elective CS. Blood was sampled from the umbilical cord and 3–5 days after birth. DNA-methylation was analyzed in leucocytes.

Results: Infants born by CS exhibited higher DNA-methylation in leucocytes compared with that of those born by VD ($p < 0.001$). After VD, newborn infants exhibited stable levels of DNA-methylation, as evidenced by comparing cord blood values with those 3–5 days after birth ($p = 0.55$). On postnatal days 3–5, DNA-methylation had decreased in the CS group ($p = 0.01$) and was no longer significantly different from that of VD ($p = 0.10$).

Conclusion: DNA-methylation is higher in infants delivered by CS than in infants vaginally born. Although currently unknown how gene expression is affected, or whether epigenetic differences related to mode of delivery are long-lasting, our findings open a new area of clinical research with potentially important public health implications.

INTRODUCTION

The stress of being born exceeds that of any other critical life-event. It is fundamental for intact survival during the transition from foetal to neonatal life. The massive sympathoadrenal activation during labour (1) mobilizes fuel for the hypoxic journey through the birth canal and triggers lung-liquid resorbtion (2), thereby facilitating air-breathing immediately after birth. Labour also activates inflammatory defence systems (3) and the central nervous system in such way that the foetus is optimally prepared and adapted for life outside the womb.

Timing and magnitude of birth stress are altered if delivery is performed with CS. Infants delivered by elective CS before onset of labour lack the catecholamine surge seen after normal VD (1). As compared with normal birth, stress in infants delivered by CS is also immediate rather than gradually evolved as during labour. CS may therefore be maladaptive for the newborn infant and has been associated with increased short-term neonatal morbidity (4).

Recently, individuals born by CS have been reported to face an increased risk also for common diseases in later life, such as asthma and allergy (5–9), type 1 diabetes mellitus (10), childhood leukaemia (11,12) and testicular cancer (13). The underlying mechanisms for these associations are unclear. However, experimental evidence shows that adverse perinatal stress may permanently alter and exaggerate neuroendocrine and behavioural responses to stress in the adult offspring (14). Poor maternal engagement and separation from the offspring immediately after birth results in permanently increased stress sensitivity and altered behaviour in the offspring (15). At the heart of the mechanisms for these changes in phenotype, early and stable epigenetic modifications have been demonstrated (15).

Epigenetic states provide the basic mechanisms for the function of the genome (16–18) and they mediate adaptations to a dynamic environment. The sum of all epigenetic modifications control transcriptional permissiveness of genes so that those that are required for a particular time of differentiation or cell type are turned on, while others are silenced. Among various epigenetic control mechanisms, DNA-methylation is one of the best studied. A key property of DNA-methylation of genes is that it is able to retain its stability for the cell's lifetime and even through cell divisions. Accordingly, differences in genomic DNA-methylation around birth and alterations in cell memory could be molecular mechanisms for later differences in disease risk associated with mode of delivery.

Changes in regulation of gene expression as a result of environmental impact are likely to occur through more or less subtle alterations in the epigenetic patterns of many

genes/gene regions. It is therefore plausible that the profound change in internal and external conditions occurring at birth can reveal quite large changes in the global genomic epigenetic patterns. Such changes may affect loci and individual genes in long-term ways. We hypothesized that global DNA-methylation in newborn infants differs in relation to mode of delivery. As the diseases implicated with CS are immunological disorders and as white blood cells (WBC) have been described to function differently after CS as compared with that after normal birth (3), we studied global DNA-methylation in WBC.

METHODS

Subjects

We studied 37 (17 girls) healthy newborn infants born at term (gestational age 40 ± 2.6 week). Multiple pregnancy, maternal diabetes, hypertension, pre-eclampsia, medication during the index pregnancy, preterm delivery, neonatal asphyxia, malformations, chromosomal disorders or congenital infection were all exclusion criteria. The mean maternal age was 33 ± 4.7 years, 11 out of 37 mothers were primigravida, one pregnancy had resulted from in-vitro fertilization and one mother smoked during early pregnancy. All infants had normal birth weights (3699 ± 379 g). We studied the effects of different mode of delivery: spontaneous VD occurred in 21 infants, whereas the other 16 infants were delivered by elective CS under spinal analgesia and before start of labour. Characteristics of the study subjects are shown in Table 1. The regional ethical vetting board at Karolinska Institute in Stockholm approved the study and informed consent was obtained from all parents before delivery.

Blood sampling

Five millilitre of EDTA blood was sampled from the umbilical cord directly after delivery. At days 3 to 5 of postnatal age, 2 mL EDTA blood was sampled from a peripheral vein in conjunction with the metabolic screening test recommended for all infants born in Sweden.

DNA-methylation analyses

DNA from circulating WBC was extracted using commercially available reagents (Nucleon BACC3 kit; GE Healthcare Europe GmbH, Freiburg, Germany). DNA quantification was performed using the NanoDrop ND-1000 (NanoDrop Technologies Inc./Thermo Fisher Scientific Inc., Wilmington, DE, USA). Luminometric Methylation Assay was performed as described elsewhere in detail (19,20). Genomic DNA (200–500 ng) was cleaved with restriction enzymes *Hpa*II + *Eco*RI or restriction enzymes *Msp*I + *Eco*RI (New England Biolabs, Beverly, MA, USA) in two separate reactions. The digestion reactions were carried out in a 96-well format using a PSQ96TM MA system (Biotage AB, Uppsala, Sweden). Peak luminometric heights were calculated using the PSQ96TM MA software. The *Hpa*II/*Eco*RI and *Msp*I/*Eco*RI ratios were calculated as (dGTP + dCTP)/dATP for the respective reactions. The *Hpa*II/*Msp*I ratio was defined as (*Hpa*II/*Eco*RI)/(*Msp*I/*Eco*RI). A *Hpa*II/*Msp*I ratio of 1 corresponds to no DNA-methylation, whereas a ratio approaching 0 corresponds to complete DNA-methylation.

Table 1 Subject characteristics

	Vaginal delivery n = 21	Caesarean section n = 16
Maternal age, yrs	31.8 ± 3.5	34.5 ± 5.6
Pre-pregnancy BMI, kg/m^2	23.0 ± 3.1	24.0 ± 3.0
Parity, n	1.8 ± 0.7	2.0 ± 0.6
Gestational age, weeks	40.7 ± 3.3	38.9 ± 0.5
Birth weight, g	3723 ± 425	3668 ± 321
Girls/boys	9/12	8/8

Data are mean ± SD or proportions. There were no statistically significant differences between the two groups.

Folate and CRP measurements

Folate levels were determined as they might influence the level of methyl donors with consequences for DNA-methylation (21) and CRP levels were determined as systemic inflammation is known to affect DNA-methylation in adults (22). Red blood cell (RBC) folate levels were analyzed with fluoroimmunoassay. The CRP concentrations were analyzed in serum by a high sensitive method using particle-enhanced immunonephelometry. Both analyses were performed at the Department of Clinical Chemistry, Karolinska University Hospital, Stockholm, Sweden.

Statistical analyses

Data are expressed as mean values and standard deviation (normally distributed variables) or median values and range (skewed distribution). Statistical analysis was performed using rank sum tests (Mann–Whitney *U*-test and Wilcoxon signed-rank test) and associations were tested for by calculating Spearman's correlation coefficients. A p-value <0.05 was considered as statistically significant.

RESULTS

At birth, global DNA-methylation – which according to LUMA (19,20) analysis is inversely proportional to the WBC *Hpa*II/*Msp*I ratio – was significantly higher in infants delivered by elective CS as compared with those born by normal vaginal delivery ($p < 0.001$). Three to five days after birth, the difference in global DNA-methylation between the two groups was smaller and no longer reached statistical significance ($p = 0.10$). In normally delivered infants, global DNA-methylation did not change between birth and 3–5 days of postnatal age ($p = 0.55$). However, in infants delivered by CS, DNA-methylation decreased significantly over the same period of time ($p = 0.01$) (Table 2 and Fig. 1).

Neonatal DNA-methylation did not correlate to maternal risk factors (age, pre-pregnancy BMI, parity, maternal folate and CRP-levels), perinatal risk factors (gestational age, duration of delivery, duration of ruptured membranes) or infant risk factors (gender, birth weight, folate and

Table 2 Mode of delivery and levels of CRP, folate and global DNA-methylation (*Hpa*II/*Msp*I ratio) in white blood cells

	Vaginal delivery n = 21	Caesarean section n = 16	p-value
Cord blood			
CRP, mmol/L	0	0	–
Folate, nmol/L	640 (570–730)	600 (540–730)	0.6435
*Hpa*II/*Msp*I-ratio	0.29 (0.28–0.31)	0.24 (0.24–0.26)	0.0002
Peripheral blood days 3–5			
CRP, mmol/L	4 (0–9.5)	2 (1–4)	0.5459
Folate, nmol/L	720 (645–845)	760 (550–830)	0.9203
*Hpa*II/*Msp*I-ratio	0.29 (0.27–0.33)	0.27 (0.27–0.28)*	0.1030

A *Hpa*II/*Msp*I ratio of 1 corresponds to no DNA-methylation, whereas a ratio approaching 0 corresponds to complete DNA-methylation. Data are median and interquartile ranges. *p = 0.01 for difference between cord blood and peripheral blood days 3–5 in infants delivered by CS.

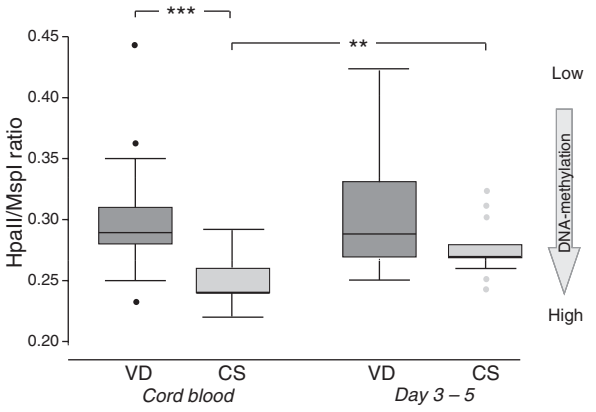

Figure 1 Mode of delivery and global DNA-methylation in healthy newborn infants. VD = normal vaginal delivery; CS = Caesarean section. DNA-methylation in white blood cells is expressed as *Hpa*II/*Msp*I-ratios. A *Hpa*II/*Msp*I ratio of 1 corresponds to no DNA-methylation, whereas a ratio approaching 0 corresponds to complete DNA-methylation.

CRP-levels) (p-values varying between 0.16 and 0.98). Accordingly, no multivariate analyses were performed.

DISCUSSION

These results demonstrate that DNA-methylation is more dynamic around birth than previously known. Epigenetic mechanisms may therefore play a role for gene-environment interactions not only in early embryonic life but also throughout foetal and neonatal development (23).

This is the first report of an association between elective CS and higher DNA-methylation at birth as compared with that in normal VD. We speculate that normal birth through VD is associated with global demethylation of DNA. In infants delivered by CS, we found significantly lower degree of methylation at 3–5 days of postnatal age as compared with that in cord levels, suggesting a delayed postnatal compensatory adaptation. Although the group difference in DNA-methylation was no longer statistically significant at 3–5 days of age – most likely due to limitations in power, the postnatal adaptation seen after CS did not reach physiological levels of epigenetic activity as that seen after VD.

The significance of higher DNA-methylation after CS is not yet understood. However, it may have important clinical implications. CS before the start of labour is one of the most rapidly increasing surgical procedures (24) and until recently, the long-term consequences on public health have not been studied. Reports on associations between CS and different diseases in later life are now emerging (5–13). The observations presented herein support that modulated or absent epigenetic modifications of specific genes may be involved. After elective CS, the 'epigenome' may acquire subtle changes in its composition which only later will have transcriptional consequences, e.g. after certain environmental triggers.

Delivery by CS is an established risk factor for later asthma and allergy (5–9). According to the prevailing 'hygiene hypothesis', altered microbial colonization of the neonatal gut and altered priming of the neonatal immune system lead to an increased risk for allergic diseases after CS (25). Our findings of higher DNA-methylation in WBC of newborn infants delivered by CS add new dimensions to how CS could be linked to immunological diseases, not only allergy and asthma.

The cause of different DNA-methylations after CS is still unclear. We speculate that maladaptive perinatal stress associated with CS plays a role. In support for such an assumption, experimental models show that adverse stress in early foetal and neonatal life affects DNA-methylation (15,26,27). However, exposures related to the surgical procedure itself may also be involved. Also although there is no relation between DNA-methylation and markers of inflammation – such as length of labour and neonatal CRP-levels, larger studies are needed to clarify these issues.

The calculation of the ratio between methylated and unmethylated DNA takes differences in total WBC number/total WBC DNA into account. But it cannot be excluded that differences in differential WBC count could have contributed to some of the observed differences in DNA-methylation between the two groups. There are larger birth-related shifts in neutrophil and monocyte counts in infants born vaginally as compared with that in those born by CS, whereas distributions of lymphocytes in cord blood does not relate to mode of delivery (3). The significance of these differences is still unclear.

The well-characterized cohort and standardized study protocol for collection and handling of blood samples are strengths of this clinical study. The conceptual framework is original, bridging gaps between molecular biology, perinatal physiology and clinical medicine. One limitation is the relatively small number of healthy infants, for which ethical approval and parental consent were given. The findings need to be confirmed and extended – methylation of specific genes remains to be studied, as well as DNA-methylation in other tissues and at later postnatal ages.

In summary, global leucocyte DNA-methylation in newborn infants delivered by CS is significantly higher as compared with levels seen in newborn infants after normal

vaginal delivery. Although currently unknown how gene expression is affected, or whether the epigenetic differences related to mode of delivery are long-lasting, our findings open a new area of clinical research.

CONFLICT OF INTEREST STATEMENT

No author has a conflict of interest to disclose.

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