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Concentrations of Pentosidine, an Advanced Glycation End-product, in Umbilical Cord Blood

HIROKAZU TSUKAHARA[a,*], NAOKO OHTA[a], SHUKO SATO[a], MASAHIRO HIRAOKA[a], KEN-ICHI SHUKUNAMI[b], MAYUMI UCHIYAMA[c], HISAKO KAWAKAMI[c], KYOUICHI SEKINE[c] and MITSUFUMI MAYUMI[a]

[a]*Department of Pediatrics, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan;* [b]*Department of Obstetrics and Gynecology, Faculty of Medical Sciences, University of Fukui, Fukui 910-1193, Japan;* [c]*Department of Research and Development, Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo 174-8555, Japan*

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Advanced glycation end-products (AGEs) are formed over several weeks to months by non-enzymatic glycation and oxidation ("glycoxidation") reactions between carbohydrate-derived carbonyl groups and protein amino groups, known as the Maillard reaction. Pentosidine is one of the best-characterized AGEs and is accepted as a satisfactory marker for glycoxidation *in vivo*. The present study was intended to measure pentosidine concentrations in umbilical cord blood from newborns with various gestational ages using our recently established high-performance liquid chromatography method [Tsukahara, H. *et al.* (2003) *Pediatr. Res.* **54**, 419–424]. Our study demonstrates, for the first time, that pentosidine is detected in most of the umbilical blood samples. This study also shows that the umbilical blood concentrations of pentosidine are considerably lower than normal adult values, but that they increase with gestation progression and fetal growth. Umbilical pentosidine concentrations were significantly elevated in newborns of mothers with preeclampsia compared to those of mothers without preeclampsia. We conclude that accumulation of AGEs and oxidative stress occurs in fetal tissues and organs *in utero* at the early stage of human life and that their accumulation is augmented in the maternal preeclampsic condition.

Keywords: Advanced glycation end-product; Newborn; Pentosidine; Preeclampsia; Umbilical blood

Abbreviations: AGE, advanced glycation end-product; HPLC, high-performance liquid chromatography; ROS, reactive oxygen species

INTRODUCTION

Normal proteins are modified over several weeks to months by the non-enzymatic Maillard reaction linking protein amino groups with carbohydrate-derived carbonyl groups, and yield the so-called advanced glycation end-products (AGEs).[1,2] AGE modified proteins increase slowly with aging and contribute to normal tissue remodeling. Pentosidine is one of several chemically characterized AGEs. Pentosidine exists in human tissues, such as skin, cartilage and aorta and in blood and urine. Its concentrations in tissues increase with age. Pentosidine is formed by sequential glycation and oxidation (thereby, it is termed "glycoxidation") reactions.[3,4] Its formation requires aerobic conditions, whereas an anti-oxidative condition inhibits such reaction. In humans, formation of pentosidine is accelerated in "oxidative stress diseases" such as diabetes, uremia, atherosclerosis, rheumatoid arthritis and Alzheimer's disease.[5–12] Increased blood concentrations of pentosidine are reported in patients with diabetes and uremia.[5,10]

However, only a paucity of information exists in reference to accumulation of AGEs, including pentosidine, in the human fetus or infants.[13] Investigation of AGE formation in these populations may be important to elucidate the molecular mechanisms of aging and tissue remodeling. The present study is intended to investigate AGE formation *in utero* and its relationship to the degree of prematurity and clinical condition. We first report pentosidine concentrations in umbilical cord blood. We describe the progressive accumulation of pentosidine with gestation advancement and body growth in the human fetus and its elevation in the maternal preeclampsic condition.

*Corresponding author. Tel.: +81-776-61-3111. Fax: +81-776-61-8129. E-mail: htsuka@fmsrsa.fukui-med.ac.jp

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SUBJECTS AND METHODS

Subjects and Blood Collection

Umbilical blood samples were obtained from 50 newborns (male/female, 21/29) with gestational age ranging from 24.4 to 41.4 weeks (mean ± SD, 35.5 ± 4.7 weeks) and birth weight ranging from 614 to 3700 g (2267 ± 810 g). Their Apgar scores (at 1 min) ranged from 2 to 10 (7 ± 2). Twenty-nine newborns were born at term and 21 were born preterm. Fourteen newborns were born by vaginal delivery and 36 were born by cesarean section. No newborns had congenital infections or major anomalies (such as brain, cardiac, pulmonary, renal or gastrointestinal anomalies), but 11 showed respiratory distress requiring supplemental oxygen and ventilator support after birth. Ten of the mothers of these newborns had been preeclampsic (showing both hypertension and proteinuria during pregnancy); the remaining 40 mothers had been non-preeclampsic.

Immediately after delivery, an umbilical cord segment was double clamped and blood was drawn gently from the umbilical vein with an 18-gauge needle and syringe. The samples were centrifuged and the supernatants were stored at −30°C until analysis. The nature and purpose of the study were explained to the parents. Their informed consent was obtained prior to enrollment.

Determination of Pentosidine

Serum concentrations of pentosidine were measured using high-performance liquid chromatography (HPLC) as described previously in detail.[12,14] The washing solution was a mixture of *n*-butanol, acetic acid, hydrochloric acid (8: 1: 1, vol/vol). CF-1 slurry was prepared by making a 5% (w/vol) suspension of CF-1 cellulose powder in the washing solvent. The pretreatment column was prepared by adding 8 ml of CF-1 slurry to a Poly-Prep chromatography column (0.8 × 40 mm^2; Bio-Rad Laboratories Inc., Hercules, CA, USA). A total of 250 µl of serum was hydrolyzed with an equal volume of concentrated hydrochloric acid at 108°C for 18 h. The cooled hydrolysate (250 µl) was mixed with 250 µl of CF-1 slurry, 250 µl of acetic acid and 2 ml of *n*-butanol and then loaded to the pretreatment column. After washing the column with 35 ml of the washing solvent, pentosidine was eluted from the column with 9 ml of 50 mM hydrochloric acid and dried under N_2 gas flow. The dry residue was then dissolved in 250 µl 1% *n*-heptafluorobutyric acid (vol/vol). An aliquot (10 µl) of each sample was applied to analytical HPLC. The HPLC eluent was 7% acetonitrile (vol/vol) containing 0.1% *n*-heptafluorobutyric acid. The HPLC system was equipped with an L-6200 intelligent pump (Hitachi Ltd., Ibaragi, Japan), an F-1050 fluorescence detector set at excitation and emission wavelengths of 335 and 385 nm, respectively (Hitachi Ltd.), and Symmetry RP18 column (3.5 µm, 4.6 × 150 mm^2; Waters Corp., Milford, MA). The flow rate was maintained at 0.8 ml/min and the column was kept at 30°C. Standard pentosidine was synthesized and purified as described previously.[14]

All analyses were performed in duplicate. The examiner was blinded to clinical and laboratory results. Intra- and inter-assay coefficients of variation were 3.6–4.7% and 5.5–9.3%, respectively. Analytical recovery tests showed that 92.8–100% of added standard pentosidine was recovered. The detection limit of our HPLC method was 17.4 pmol/ml. Values (mean ± SD) obtained from healthy adult males aged 33 ± 6 years ($n = 35$) and females aged 31 ± 5 years ($n = 31$) are 107 ± 13 and 110 ± 15 pmol/ml, respectively. Using this method, Yoshihara *et al.*[14] reported that blood and urinary concentrations increased slowly during normal aging in humans.

Statistical Analysis

Data are presented as mean ± SD and range. When pentosidine was undetectable, half the value of the detection limit (i.e. 8.7 pmol/ml) was adopted arbitrarily for data analysis. Differences between groups were examined for statistical significance using the unpaired *t*-test. Correlations between variables were assessed by linear regression. We determined a *p*-value <0.05 as a statistically significant difference.

RESULTS

Table I shows the concentrations of pentosidine in umbilical cord blood at the time of birth.

TABLE I Pentosidine concentrations in umbilical cord blood

Total ($n = 50$; M/F = 21/29)	Term newborns ($n = 29$; M/F = 12/17)	Preterm newborns ($n = 21$; M/F = 9/12)
36.6 ± 16.3 pmol/ml (<17.4–64.0 pmol/ml)	40.7 ± 15.8 pmol/ml (<17.4–64.0 pmol/ml)	31.0 ± 15.5 pmol/ml* (<17.4–56.8 pmol/ml)

Data are presented as mean ± SD and range. An arbitrary value of 8.7 pmol/ml is used for data analysis for eight newborns with undetectable results (<17.4 pmol/ml). *$p < 0.05$ vs. term newborns. These pentosidine concentrations are far below healthy adult levels (males: 107 ± 13 pmol/ml ($n = 35$); females: 110 ± 15 pmol/ml ($n = 31$)).

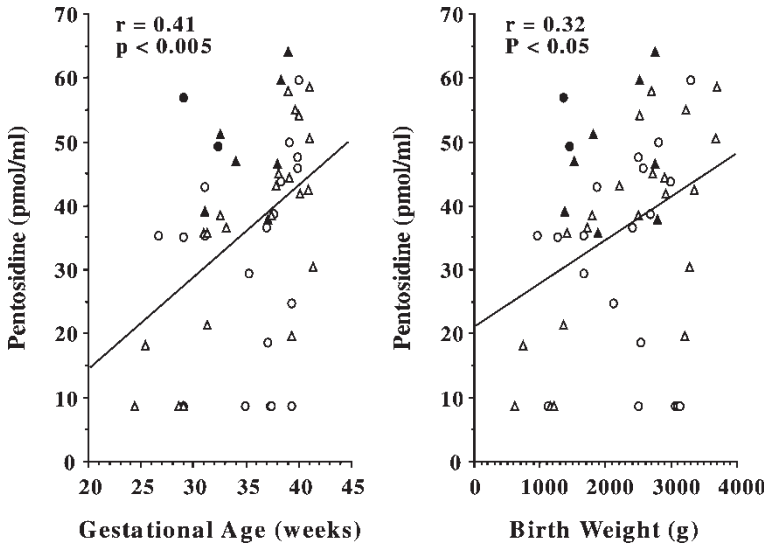

FIGURE 1 Relationships between umbilical blood concentrations of pentosidine and gestational age or birth weight. Circles and triangles are boys and girls, respectively. Open and closed symbols indicate newborns born from non-preeclampsic and preeclampsic mothers, respectively. Undetectable data are arbitrarily plotted at 8.7 pmol/ml. When all data are combined, the pentosidine concentrations are significantly correlated with gestational age ($r = 0.41$, $p < 0.005$) (left) or birth weight ($r = 0.32$, $p < 0.05$) (right).

Pentosidine was detectable (>17.4 pmol/ml) in 42 (84%) of the 50 samples. Pentosidine concentrations were considerably lower in the umbilical cord blood than normal adult values (as described in the "Subjects and methods" section). Umbilical pentosidine concentrations in the preterm newborns were significantly lower than those of term newborns. When all data of newborns were entered into the analysis, the pentosidine concentrations demonstrated significant positive correlations with gestational age at birth (Fig. 1, left) or birth weight (Fig. 1, right). No significant sex-related differences were observed (data not shown).

Next, we divided our subjects into two groups, maternal preeclampsia group ($n = 10$) and maternal non-preeclampsia group ($n = 40$). Umbilical pentosidine concentrations were elevated significantly in the preeclampsia group in comparison to the non-preeclampsia group (48.7 ± 9.5 vs. 33.6 ± 16.3 pmol/ml; $p < 0.01$) even though the gestational ages and birth weights were not significantly different (34.2 ± 3.6 vs. 35.8 ± 4.9 weeks; 2022 ± 617 vs. 2328 ± 847 g, respectively) (Fig. 1).

DISCUSSION

Pentosidine is one of the best-characterized AGEs.[1,2] It is considered to be an adequate marker for the non-enzymatic glycoxidation process. Dyer *et al.*[15] reported that skin collagen pentosidine concentrations in humans increased with strong correlation to age. Age-related increase in AGEs occurs in virtually all tissues, including skin, cartilage, aorta, lens, kidney, and brain and in blood and urine.[14–18] However, determination of AGEs in the human fetus or infants has not been performed except by Yamamoto *et al.*[13] Although accumulation of AGEs appears to be a phenomenon of aged brains, their study revealed positive immunoreactions for AGEs in fetal brains as early as 22 weeks of gestation.

The present study measured serum concentrations of pentosidine in the umbilical cord from newborns with various gestational ages using our recently established HPLC method[12,14] and sought to elucidate *in utero* AGE formation in humans. Our study demonstrates, for the first time, that detectable levels of pentosidine exist in most umbilical blood samples. Our study also shows that umbilical blood concentrations of pentosidine are considerably lower than normal adult values, but that they increase with gestation progression and fetal growth. For that reason, we infer that non-enzymatic glycoxidation occurs in fetal tissues and organs *in utero*, but its concentrations are quite low compared with normal adult levels.

Oxidative stress is involved in normal aging and many diseases.[19] Various oxidative modification products are formed from the effect of reactive oxygen species (ROS). Wolff and Dean[3] found that reducing sugars could be autoxidized by metal-catalyzed oxidative processes and generate ROS and ketoaldehydes, which contribute to AGE formation. That pentosidine is the product of "glycoxidation" is supported by *in vitro* evidence that the absence of oxygen in the incubation medium prevents pentosidine formation.[4] Pentosidine concentrations in tissues, organs and body fluids are elevated in patients with "oxidative stress diseases".[5–12]

Support for the causal role of oxidative stress in pentosidine formation is the correlation present in uremic blood between pentosidine and other oxidative stress markers.[6,7] Therefore, our results can also be taken as evidence that accumulation of oxidative stress occurs *in utero* as gestation progresses.

Preeclampsia remains a major cause of maternal morbidity and death and is a leading indication for iatrogenic premature delivery. Oxidative stress is considered to be a crucial factor in the disease process.[20–22] In pregnancies complicated by preeclampsia, ROS production in the placenta increases in connection with inadequate antioxidant defenses. Newborns born after preeclampsia had been exposed to more oxidative stress *in utero* than matched newborns.[23] Increases of ROS formation are seen by hypoxia in the fetal guinea pig brain.[24] Pentosidine concentrations in umbilical cord blood showed significant elevation in newborns born after preeclampsia. Our findings imply that oxidative stress and AGE formation are augmented *in utero* during preeclampsia. AGEs show diverse biological abilities.[1,2] They increase endothelial permeability and activate macrophages and endothelial cells with secretion of cytokines and growth factors. Those factors consequently accelerate inflammation and enhance oxidative stress. It is interesting to speculate that some of the possible mechanisms engendering preeclampsia may be related to increased formation of AGEs, including pentosidine.

In summary, our findings of the presence of pentosidine in most of the umbilical blood and of the close correlation between pentosidine and gestation progression suggest that accumulation of AGEs and oxidative stress occurs *in utero* as gestation progresses. Our findings of elevated pentosidine levels in newborns born after preeclampsia may support the view that oxidative stress and AGE formation are augmented in this disorder. Additional immunohistochemical studies using fetal or placental materials are necessary to investigate the accumulation of AGEs and oxidative stress in normal and pathological situations *in utero*.

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