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Placental Superoxide Dismutase, Genetic Polymorphism, and Neonatal Birth Weight

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Background : The roles of antioxidants in the placenta and genetic susceptibility to oxidant chemicals in relation to neonatal birth weight have not been elucidated. We determined whether the level of placental manganese superoxide dismutase (MnSOD) and its genetic polymorphism plays any role in oxidative stress and neonatal birth weight.

Methods : We measured placental MnSOD and determined MnSOD genetic polymorphism among 108 pregnant women who were hospitalized for delivery and their singleton live births in Korea. Main outcome measurements are maternal urinary malondialdehyde (MDA) and birth weight.

Results : Maternal urinary concentrations of MDA were significantly associated with neonatal birth weight (P=0.04). The enzyme level of placental MnSOD was also significantly associated with MDA concentration (P=0.04) and neonatal birth weight (P<0.01). We observed dose-response relationships between placental MnSOD and maternal urinary MDA, and neonatal birth weight after adjusting for maternal weight, height, age, and neonatal sex. After controlling for covariates, MnSOD variant genotype increased maternal urinary MDA concentrations (P<0.01) and reduced birth weight by 149 gm (P=0.08).

Conclusions : This study demonstrates that the placental level of MnSOD during pregnancy significantly affects fetal growth by reducing oxidative stress, and that genetic polymorphism of MnSOD probably modulate the effects of oxidants on fetal growth.

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Key Words: Birth weight / Genetic polymorphism / Oxidative Stress / Placental Superoxide dismutase

INTRODUCTION

Recent evidence indicates that oxidative stress on pregnant women or fetuses plays an important role in the pathophysiology of low birth weight [1-3]. Oxidative stress may result from the excessive production of reactive oxygen species or a deficiency in the protective antioxidant systems [4]. The levels of serum MDA or urinary 8-OH-dG in pregnant women with fetal growth retardation have been reported to be higher than in women with normal pregnancies [2,5], supporting the relationship between oxidative stress and low birth weight [6,7].

However, the biological mechanism of adverse pregnancy outcome by oxidative stress has not been definitively established. We suspect that such a mechanism exists, perhaps via the detrimental effect of oxidant radicals on the blood supply in the placenta, which would reduce oxygen flow to the fetus.

The placenta is a crucial organ in terms of biomolecular interactions because it provides an extensive and intimate interface between the maternal and fetal blood streams during pregnancy [3,8,9]. Therefore, the activity of antioxidants in the placenta is very important for defending against oxidative stress generated intrinsically in the placenta or loaded from the maternal circulation [3,10,11].

Superoxide, one of the oxidant radicals produced spontaneously or by hazardous chemicals either ingested or inhaled, may cause the inactivation of nitric oxide, which is a potent vasodilator of the placental vasculature [12,13]. In addition, superoxide is an important radical for lipid peroxidation [8,14]. Lipid peroxidation is a free radical chain reaction, which involves the oxidative conversion of polyunsaturated fatty acids to various oxidized products, including malondialdehyde (MDA) [4]. Manganese superoxide dismutase (MnSOD), a metalloenzyme containing manganese (Mn), catalyzes the dismutation of the superoxide radical, producing H_2O_2 and oxygen. Because MnSOD is mainly localized

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to the mitochondria of vascular endothelium in the placenta, MnSOD can prevent deleterious effects of superoxide on the vasculature [15]. The degree of lipid peroxidation in the human placenta also can be controlled by the placental activities of MnSOD, by limiting the generation of lipid peroxides [16,17].

In this report, we hypothesize that the placental level of MnSOD is associated with lipid peroxidation and birth weight. Because the placenta has been reported to be an important source of maternal lipid peroxide levels, maternal urinary MDA level may represent the oxidant-antioxidant balance of the placenta [18]. Therefore, we used the maternal urinary concentrations of MDA to assess the relationship between the placental level of MnSOD and lipid peroxidation. We previously reported that urinary 8-OH-dG and MDA are sensitive biomarkers of oxidative stress, and that some genetic polymorphisms, including MnSOD, may modify oxidative stress in pregnant women [19]. This observation suggests that certain genetic polymorphisms related to oxidative stress could cause adverse pregnancy outcomes, such as a low birth weight. A genetic polymorphism of MnSOD, T to C substitution in the mitochondrial targeting sequence, results in an amino acid codon change at the -9 position from valine to alanine [20], which would in turn decrease defense against superoxide radicals [21]. Therefore, we also hypothesize that genetic polymorphism of MnSOD mediate susceptibility to detrimental oxidant effects on birth weight.

Even though the cause of low birth weight is not known clearly, it is reasonable to believe that both environmental and genetic factors play a role [22]. In the interplay of these two factors, increased levels of oxidizing radicals and a limited response of the antioxidant system may be implicated in the pathogenesis of low birth weight. We chose to focus, in this report, on MDA and MnSOD with respect to oxidative stress and antioxidant defense, and upon the genetic polymorphism of MnSOD for the modification of the effect on birth weight.

METHODS

Study Subjects and Data Collection

We conducted a survey in 2000-2001 among 108 pregnant women hospitalized for delivery and their singleton live births. The women were otherwise healthy and had no evidence of pregnancy toxemia, hypertension, diabetes mellitus, thyroid disease, bronchial asthma, active hepatitis, chronic renal failure, or heart failure. A questionnaire administered to the women included questions on active and passive smoking, residential and employment histories, alcohol consumption and dietary patterns. Age, height, weight, date of the last menstrual period, medical history, and reproductive history were obtained from the subject's medical records. Maternal blood and urine sample, and cord blood were collected at admission and stored at -20°C until assayed. Birth weights were recorded for all infants, and gestational age was defined as the interval between the first day of the mother's last menstrual period and the date of birth. Approval to conduct this study was granted by the Institutional Review Board of Inha University Hospital in Incheon and Ewha University Hospital in Seoul. Written informed consent was obtained from all maternal participants.

Measurements of malondialdehyde (MDA)

MDA was determined by HPLC of the adduct obtained with thiobarbituric acid (TBA). The TBA reagent was purchased from Merck (Darmstadt, Germany) and was used as a 23 mmol/l solution in water. A 10 mmol/l stock standard of MDA (Aldrich, Milwaukee, WI) was prepared by dissolving 247 μℓ 1,1,3,3-tetraethoxypropane (Sigma, St. Louis, MO) in 100 ml of ethanol (40% by volume). TBA-MDA adducts were prepared in glass tubes with a polypropylene stopper. In each tube, 300μℓ of phosphoric acid, 0.5 mol/l was mixed with 50 μℓ of urine and 150 μℓ of TBA reagent. The tubes were then stoppered and heated to 95℃ and maintained at that temperature for 1 hr. Thereafter, the tubes were chilled on ice for 5 min. Methanol, 500 μℓ, was then added and the tubes were centrifuged at 5,000 g for 5 min. The samples were then transferred to glass autosampler vials and 20 μℓ was analyzed by HPLC. The concentration of the TBA-MDA adduct was determined at 532 nm on an isocratic HPLC system (DX-500, Dionex, Sunnyvale, CA). A Nova-Pak C18 column (Waters, Millipore, Milford, MA) was used and eluted with a mobile phase of potassium phosphate buffer, 50 mmol/l, pH 6.8, and methanol (58:42, v/v). The mobile phase flow was 0.8 ml/min.

Placental MnSOD Measurement

Small pieces of placental tissue (10g) were taken from the central part of each placenta immediately after delivery and stored in a freezer at -70°C. Protein lysates were prepared with solubilizing buffer (0.24M sucrose, 10 mM Tris-HCl, 150 mM NaCl, 0.5 mM EDTA, 2 mM PMSF, 2 μg/mℓ aprotinin and 2 μg/mℓ leupeptin), and the tissue was homogenized on ice using a tissue grinder. The samples were incubated on ice for 30 min and then centrifuged at 14,000 g for 10 min at 4°C. Protein quantitation was performed using a BCA protein assay kit (Sigma, St. Louis, MO).

After separation on 12.5% polyacrylamide gel, proteins were transferred to a PVDF membrane (Amersham, Piscataway, NJ). The membrane was blocked with Tris-buffered saline supplemented with 0.05% Tween-20 (TBST) containing 3% milk at 4°C overnight. The mouse monoclonal antibody, anti-MnSOD (1 μg/mℓ; UBI, Lake Placid, NY), was used to probe the Western blots, and HRP-conjugated second antibody was used for the visualization

Table 1. Characteristics of mothers and neonates

Characteristics of mothers and neonates	Mean (SD) or Number (%)
Mother (N=108)	
Age (years)	29.8 (3.7)
Height (cm)	160.9 (5.6)
Weight before pregnancy (Kg)	53.2 (6.4)
Weight at delivery (Kg)	65.8 (7.8)
MnSOD genotype	
Val/Val	78 (72.2 %)
Val/Ala	29 (26.9 %)
Ala/Ala	1 (0.9 %)
Neonates (N=108)	
Gestational age (days)	271.8 (15.2)
Birth weight (gm)	3,180.9 (472.8)
Neonatal sex	
Male	62 (57.4 %)
Female	46 (42.6 %)
MnSOD genotype	
Val/Val	75 (75.8 %)
Val/Ala	22 (22.2 %)
Ala/Ala	2 (2.0 %)

(1:2000; Amersham). The Western blots were developed with ECL Western blotting detection reagents according to the manufacturer's instructions (Amersham). Relative levels of MnSOD were calculated using the Quantity One program (BioRad, Hercules, CA).

Genotyping

Maternal and neonatal MnSOD genetic polymorphisms were analyzed by PCR in a Techne progene thermal cycler (Cambridge, England), as described previously [23]. PCR reactions were carried out in a total volume of 50 μl in the presence of 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 0.2 mM of each dNTP; 2.0 mM $MgCl_2$; 1.25 units Taq polymerase (Takara, Otsu, Japan); 20 pmol of each primer; and 100 ng of genomic DNA as a template. PCR-RFLP assays were used to distinguish a valine to alanine change at the -9 position in the signal sequence of the MnSOD gene [20].

Statistical Analysis

Associations were initially evaluated using Pearson's correlation analysis followed by multivariate analyses. Multiple regression analysis was used to investigate the relationships between placental MnSOD levels, urinary MDA concentrations, and birth weight, whilst controlling for other covariates. To explore these relationships, we used a generalized additive model with a smoothing function of the locally weighted running-line smoother. The multiple regression model to determine the effect of MnSOD polymorphism on MDA and birth weight included maternal characteristics, gestational age, dietary habits, alcohol consumption, maternal job, and neonatal sex. Statistical analyses were performed using SAS (version 6.12) and S-plus (2000). All significance testing was two-sided.

RESULTS

We analyzed 108 maternal-neonatal pairs, and the characteristics of the study participants and the distributions of MnSOD genotypes are described in Table 1. Mean gestational age was 271.8 (standard deviation [SD] 15.2) days and the mean birth weight was 3,180.9 (SD 472.8) gm. The frequencies for homozygous wild-type MnSOD (Val/Val) of mothers and neonates were 72.2 and 75.8%. The homozygous and heterozygous allelic variants were combined in the analysis because of small number of homozygous variant genotype.

Relationships between placental MnSOD and urinary MDA, and birth weight

The results of correlation analysis showed that maternal urinary concentrations of MDA were significantly associated with birth weight (r=-0.20, P=0.04). The levels of placental MnSOD were also significantly associated with the MDA concentration (r=-0.20, P=0.04) and birth weight (r=0.27, P<0.01). These statistically significant relationships were maintained in the multivariate regression models adjusted for maternal age, height, weight at delivery, and neonatal sex (Table 2).

Figure 1 shows the relationships between placental MnSOD level, maternal urinary MDA concentration, and birth weight after adjusting for maternal age, height, weight at delivery, and neonatal sex. The dose-response relationships for placental MnSOD indicate that birth weight increases with the level of placental MnSOD, which scavenges oxidant radicals, resulting in a decreased urinary MDA level.

Role of MnSOD genetic polymorphism

We controlled potential confounders in the multiple regression analysis of the influence of maternal MnSOD genetic polymorphism on oxidative stress and birth weight. Maternal urinary MDA concentrations were significantly higher in the MnSOD variant types (2.69 [95% CI, 1.32 - 4.06] (mol/mol creatinine) than in the homozygous wild type (1.83 [95% CI, 0.53 - 3.12] (mol/mol creatinine) (P<0.01). Table 3 shows adjusted mean differences of birth weight between the maternal MnSOD genotypes in the regression model. The reduction in birth weight by MnSOD genetic variant type was 149 gm (95% CI, -20 to 319 gm) (P=0.08).

When neonatal MnSOD genetic polymorphism was evaluated to figure out the relationship with maternal MnSOD genetic polymorphism or birth weight, we found that the Kappa coefficient was 0.32 (95% CI, 0.13 - 0.50) between the maternal and neonatal MnSOD polymorphisms, and that the neonatal MnSOD genetic polymorphism was not

Table 2 Multiple regression models on the association of placental MnSOD with maternal urinary MDA and neonatal birth weight

Predictors in the model	MDA		Birth Weight	
	β (SE)	p-value	β (SE)	p-value0
Placental MnSOD levels	-0.587 (0.297)	0.05	0.258 (0.096)	<0.01
Maternal Age	0.023 (0.035)	0.52	0.018 (0.011)	0.11
Maternal Height (cm)	-0.017 (0.025)	0.49	0.013 (0.008)	0.11
Maternal Weight at delivery (Kg)	-0.012 (0.017)	0.48	0.023 (0.006)	<0.01
Neonatal Sex	-0.020 (0.254)	0.94	-0.063 (0.083)	0.45

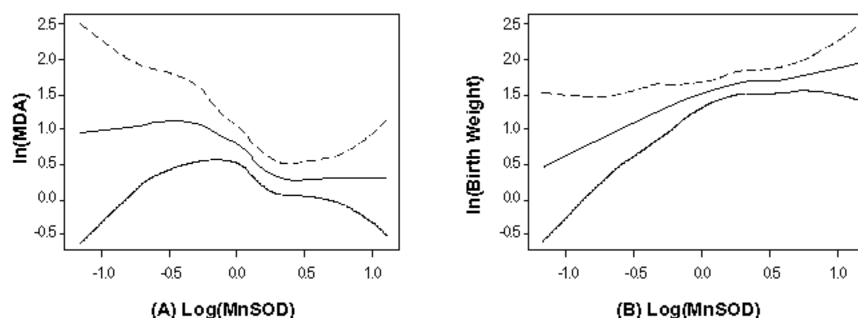

Figure1. Dose-response curves for placental MnSOD level and the concentration of maternal urinary MDA (a), and neonatal birth weight (b) after adjusting for maternal weight, height, age, and neonatal sex. Curves were smoothed using a generalized additive model with the locally weighted running-line smoother.

Table 3 Mean birth weight according to maternal MnSOD genotype after adjusting for maternal age, height, weight at delivery, gestational age, and neonatal sex.

MnSOD	Neonatal Birth Weight (gm)			
	N	Means	95% CI	p-value
Wild (Val/Val)	78	3,005	2,662 - 3,348	
Variant (Val/Ala, Ala/Ala)	30	2,856	2,493 - 3,219	0.08

MnSOD: Wild type (Val/Val); Variant type (Val/Ala, Ala/Ala)

significantly associated with birth weight.

DISCUSSION

To gain evidence as to whether antioxidant activities in the placenta are associated with lipid peroxidation and birth weight, we performed Western blot analysis for MnSOD on placental protein extractions. We found that the relative protein concentration of MnSOD was negatively associated with the maternal urinary MDA level, and positively associated with birth weight. This suggests that oxidative stress resulting from decreased placental antioxidant level, and increased lipid peroxidation could have a detrimental effect on fetal growth. We also found that the MnSOD variant types (Val/Ala, Ala/Ala) increased maternal urinary MDA concentrations ($P<0.01$) and reduced birth weight 149 gm (95% CI, -20 to 319 gm) ($P=0.08$) after controlling for potential confounders.

Antioxidant defense systems are essential to protect tissues and cells from damage caused by oxidative stress [16]. Superoxide dismutase is the primary antioxidant enzyme that inactivates the superoxide radical. Because the superoxide radical, a scavenger of endothelium-dependent nitric oxide, seems to play a major role in the pathogenesis of endothelial dysfunction, its interaction with superoxide dismutase may be important in protecting against oxidative stress [14,15,24-26]. Decreased nitric oxide availability due to increased superoxide radical levels could adversely affect placental blood flow regulation, which could, in turn, account for birth weight reduction [29]. Therefore, an imbalance between increased oxidative stress and decreased antioxidant defenses in the placenta could cause the impairment of fetal growth. MnSOD was found to be mainly localized to the vascular endothelium in the placenta, which further suggests that it has a role in preventing the deleterious effects of superoxide on the vasculature [15].

Another possible cellular injury resulting from oxidative stress is lipid peroxidation, due to the effect of reactive oxygen species on polyunsaturated fatty acids. In particular, superoxide reacts readily with nitric oxide to form peroxynitrite, a potent oxidant capable of initiating lipid peroxidation [8]. One of the lipid peroxidation products is malondialdehyde (MDA), and this is capable of inactivating many cellular proteins by forming protein cross-linkages [28]. Because a deficiency in SOD activity would lead to increased superoxide radicals, the decreased activity of SOD could be responsible for the observed increased MDA level [16]. Decreased SOD activity and increased lipid peroxides have also been described in patients with pregnancy-induced hypertension, which indicates an association between deficient antioxidant activity and increased lipid peroxidation [14-16,18,29,30]. Genetic polymorphisms have been detected in a variety of enzymes involved in the production and scavenging of reactive oxygen species [19]. One base pair transition (T to C) leads to a valine to alanine amino acid change at the -9 position of the mitochondrial targeting sequence in the MnSOD gene [20]. Consistent with our previous research, we found that MnSOD genetic polymorphism is an important genetic modifier of oxidative stress [19]. In addition, our data indicate that the adverse effects of oxidative stress on neonatal birth weight were modified by the maternal MnSOD genotype. This finding implies that pregnant women with certain genotypes related to oxidative stress regulation may be more susceptible to the adverse effect of oxidizing radicals in terms of pregnancy outcome.

These findings should be considered in light of a number of limitations and strengths. We measured urinary MDA at the time of admission for delivery and placental MnSOD after delivery, which does not necessarily reflect exposure to oxidant chemicals and antioxidant activity during the whole of the pregnancy. Therefore, we do not know which phase of pregnancy is the most critical for placental vascular insufficiency due to imbalance of oxidative stress and antioxidant capacity. However, our findings are based on objective biological markers, and the MnSOD genotypes that do not depend upon the time of study. Moreover, our results are biologically plausible and consistent with those of previous researches, which demonstrated an association

between MDA and MnSOD, and the modification of oxidative stress by MnSOD genetic polymorphism.

In conclusion, we found that an increase of MDA concentration in urine and a decrease of MnSOD level in the placenta were significantly associated with reduced birth weight, indicating that the placental level of MnSOD significantly affects fetal growth by controlling oxidative stress. The analysis also showed that the variant type (Val/Ala, Ala/Ala) of the MnSOD gene reduced birth weight via the inefficient induction of the placental MnSOD activity intrinsically or from exposure to oxidant producing chemicals.

Although conclusive evidence that the antioxidant defense system is overwhelmed by oxidative stress in low birth weight is lacking at present, our results suggest that antioxidant supplementation in pregnant women may be beneficial to fetal growth, and that this may be more beneficial in pregnant women with variant alleles of MnSOD.

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