Folic Acid Prevents Congenital Malformations in the Offspring of Diabetic Mice

KAORI OYAMA*, YOSHIHISA SUGIMURA**, TAKASHI MURASE*, AKIRA UCHIDA*, SHIZU HAYASAKA*, YUTAKA OISO**, AND YOSHIHARU MURATA*

*Department of Genetics, Research Institute of Environmental Medicine, Nagoya University, Nagoya 464-8601, Japan
**Department of Endocrinology and Diabetes, Nagoya University Graduate School of Medicine, Nagoya 466-8550, Japan

Abstract. It is well known that maternal diabetes causes various congenital malformations. Although there are many reports that folic acid (FA) administration in pregnancy reduces the risk of birth defects including neural tube defects (NTDs), a precise analysis on the preventive effect of FA against diabetic embryopathy has not been done yet. In this study, we analyzed the preventive effects of FA on congenital malformations including NTDs, cardiovascular, and skeletal malformations using a diabetic mouse model. Female mice were rendered hyperglycemic by streptozotocin and then mated. Pregnant diabetic mice were treated daily with FA (3 mg/kg body weight) or saline between gestational days (GD) 6 and 10. On GD 18, fetuses were examined for congenital malformations. FA did not affect plasma glucose levels. In the DM control group, the incidence of NTDs, cardiovascular, and skeletal malformations was 28.4%, 28.5%, and 29.7%, respectively. In the FA-treated group, the corresponding proportions reduced to 6.0%, 2.5% and 12.5%, respectively. A whole-mount TUNEL revealed an increased apoptosis in the hindbrain region of embryos from DM control group on day 9.5, and the apoptosis was decreased by FA treatment. Maternal plasma homocysteine levels on GD 9.5 were significantly lowered in DM control group compared with those in non-DM group, and FA treatment did not show a significant effect. These results indicate that FA is effective for the prevention of various diabetic embryopathy including NTDs, cardiovascular, and skeletal malformations, and suggested that this effect is independent from homocysteine metabolism and possibly mediated by decreasing the abnormal apoptosis during organogenesis.

Key words: Diabetes mellitus, Embryopathy, Folic acid, Apoptosis, Homocysteine

Received: July 7, 2008
Accepted: August 31, 2008
Correspondence to: Takashi MURASE, M.D., Ph.D., Department of Genetics, Research Institute of Environmental Medicine, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan

IT is well known that maternal diabetes during pregnancy causes a high incidence of congenital malformations in the offspring [1–4]. Even though the incidence of malformations in diabetic pregnancy has been reduced by intensive insulin treatment and glucose monitoring, it is still 2- to 6-fold higher than that in normal pregnancy [5–7]. Among the various malformations, neural tube defects (NTDs) including exencephaly, and spina bifida are the most common complications and the incidence is 2- to 3-fold higher in diabetic pregnancy [4].

Folic acid (FA) is a water-soluble B-complex vitamin. Many studies have shown that 50–70% of NTDs can be prevented by folic acid supplementation before and during pregnancy [8–11]. Additionally, it has been reported that FA may prevent other birth defects such as heart defects [12–14], cleft lip and palate [15–17], limb deficiency defects [18, 19], and urinary tract anomalies [18, 20] as well. Based on these studies the United States Department of Public Health, the Centers for Disease Control and Prevention (CDC) is recommending that women who are expected to be pregnant should take a FA supplement at least 400 μg per day.

Wentzel et al. and our group [21, 22] reported that mRNA expression of folic acid-binding protein (Folbp), a folic acid transporter, decreased in a diabetic state. It is thus suggested decreased Folbp level creates intrac-
cellular folic acid deficiency in the embryo of diabetic pregnancy. Wentzel et al. also demonstrated that FA administration prevents diabetes-induced NTDs in early rat embryos [22]. However, their study did not include the preventive effects against other congenital defects than NTDs. We therefore studied the preventive effects of FA against diabetes-induced congenital malformations in global organs using diabetic mouse model.

**Materials and Methods**

**Animal experiments**

Female ICR mice (9–10 weeks old) weighing approximately 30 g were purchased from Chubu Science Materials (Nagoya, Japan). They were housed in a standard animal facility under conditions of constant temperature (23°C), 12-h/12-h light/dark cycle, and free access to standard chow and tap water. The mice were rendered hyperglycemic by an intraperitoneal injection of streptozotocin (STZ, 240 mg/kg body weight: Sigma Chemical Co., St. Louis, MO). Seven days after the STZ injection, plasma glucose concentration in the tail vein was measured using a compact glucose analyzer (MediSafe, Terumo, Tokyo, Japan). Diabetic state was defined as a plasma glucose concentration exceeding 350 mg/dl. Diabetic female mice were mated overnight with non-diabetic male ICR mice. The morning when a vaginal plug was found was referred as gestational day (GD) 0. From GD 6 to 10, pregnant females were given a daily intraperitoneal injection of FA (Sigma Chemical Co.) to give a dose of 3 mg/kg body weight (DM + FA group). FA was dissolved in phosphate buffered saline (PBS). In the DM control group, the mice were injected with PBS in the same manner as for the DM + FA group.

All procedures were performed in accordance with institutional guidelines for animal care in Nagoya University, which conform to the NIH animal care guideline.

**Analysis of malformations**

On GD18, the pregnant mice were anesthetized with ether, and blood samples for plasma glucose determination were obtained. The uterine horns were cut open and carefully inspected for all implantations. Fetuses were extracted from the uterus, weighed, and their crown rump-lengths were measured. Using a stereomicroscope, the fetus was screened for external malformation as described previously [23, 24]. Briefly, dead fetuses were defined as “resorbed,” regardless of their size and state of development. Live fetuses with NTDs, cranial anomalies, manifest caudal regression syndrome, omphalocele or other gross malformation were categorized as “malformed”. The rate of resorptions was calculated as a percentage of the total number of implantations per litter, and the incidence of malformations as a percentage of the total number of viable fetuses per litter. After the evaluation for external malformations, fetuses were sacrificed by ether overdose. Then, approximately half the fetuses of each litter were placed in Bouin’s solution and examined for visceral malformations using a slightly modified approach to that reported previously [25–27]. Briefly, the thoracic portion of the trunk was opened ventrally with a mid-sagittal cut. After the thymus and lung were observed, the thymus, lung and atria were removed to survey the arteries. The remaining fetuses were fixed in 95% ethanol, stained with alizarin red S and/or alcian blue [28] and examined for skeletal malformations. The incidence of visceral malformations and skeletal malformations were calculated as a percentage of the total number of live fetuses per litter analyzed for visceral and skeletal malformations, respectively.

**Apoptosis assay**

Embryos were collected on GD9.5 in ice cold PBS, fixed overnight in 4% paraformaldehyde (PFA) in PBS, dehydrated through a graded methanol series and stored at –20°C until used. A whole mount TUNEL for examination of apoptosis was performed with the Takara *In Situ* Apoptosis Detection Kit (Takara Bio-medicals, Tokyo, Japan) according to the manufacturer’s instructions with minor modifications. Briefly, embryos were rehydrated, and incubated with 15 μg/ml proteinase K for 8 minutes at 37°C. After being washed with PBST, embryos were fixed again in 4% PFA for 5 minutes. After blocking the endogenous peroxidase by 3% hydrogen peroxide in methanol for 5 minutes, fragmented DNA was labeled with fluorescein isothiocyanate (FITC)-conjugated dUTP in the presence of TdT for 120 min at 37°C. Embryos were incubated with horseradish peroxidase-conjugated anti-
FITC antibody. For detecting signals, embryos were incubated with 0.3 mg/ml diaminobenzidine (DAB, Sigma) in PBSBT, in which hydrogen peroxide was added to a final concentration of 0.03%. The staining reaction was terminated by rinsing in PBST. Whole-mount DAB-stained embryos were examined using a microscope (VB-G25; Keyence Corporation, Osaka, Japan).

Maternal plasma homocysteine concentration

On GD 9.5, blood samples were collected from the pregnant dams via the femoral artery for analysis of plasma homocysteine concentration. Samples were placed into tubes containing EDTA. The tubes were immediately centrifuged and the plasma samples were kept frozen at -80°C until analysis. The plasma homocysteine levels were determined by high performance liquid chromatography.

Statistics

Unless otherwise stated, results were expressed as means ± SE. Statistical analyses were performed using one-way ANOVA followed by Fisher’s PLSD test. P values of less than 0.05 were considered to be statistically significant.

Table 1. Maternal body weights, plasma glucose levels

<table>
<thead>
<tr>
<th>Litters No.</th>
<th>Maternal BW day 18 (g)</th>
<th>Glucose day 0 (mg/dl)</th>
<th>Glucose day 18 (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-DM</td>
<td>5</td>
<td>65 ± 1</td>
<td>106 ± 8</td>
</tr>
<tr>
<td>DM control</td>
<td>8</td>
<td>57 ± 3*</td>
<td>499 ± 16*</td>
</tr>
<tr>
<td>DM + FA</td>
<td>6</td>
<td>53 ± 3*</td>
<td>489 ± 26*</td>
</tr>
</tbody>
</table>

Absolute numbers are given for litters. Other values are expressed as means ± SE. *p<0.05 vs. non-DM group.

Table 2. Reproductive outcome

<table>
<thead>
<tr>
<th>Implantations No.</th>
<th>Resorptions No.</th>
<th>Viable fetuses No.</th>
<th>Mean implantations/litter</th>
<th>Mean resorptions/litter</th>
<th>Resorptions %/litter</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-DM</td>
<td>70</td>
<td>4</td>
<td>66</td>
<td>14.0 ± 0.6</td>
<td>0.8 ± 0.3</td>
</tr>
<tr>
<td>DM control</td>
<td>111</td>
<td>38</td>
<td>73</td>
<td>13.8 ± 0.5</td>
<td>4.8 ± 0.7*</td>
</tr>
<tr>
<td>DM + FA</td>
<td>84</td>
<td>8</td>
<td>76</td>
<td>14.0 ± 0.4</td>
<td>1.3 ± 0.4*</td>
</tr>
</tbody>
</table>

Absolute numbers are given for implantations, resorptions, and viable fetuses. Other values are expressed as means ± SE. *p<0.05 vs. non-DM group. #p<0.05 vs. DM control group.
No external malformations were observed in the non-DM group. In contrast, a high rate of external malformation was observed in the DM control group (Table 4). An example of external malformations is shown in Fig. 1. The most common external malformation was NTDs including exencephaly and spina bifida. Less frequently, external malformations including cleft palate, anophthalmia, microphthalmia and limb hyperflexion were observed. FA treatment significantly decreased the incidence of total external malformations and NTDs from 34.8 ± 5.8% and 28.4 ± 5.5% to 8.9 ± 3.3% and 6.0 ± 3.0%, respectively (Fig. 2A).

**Visceral malformations**

As shown in Table 5, no visceral malformations were observed in the non-DM group. A high rate of visceral malformations was observed in the DM control group, and the most common of visceral malformation was cardiovascular malformations (CVMs), including ventricular septal defect and transposition of great arteries. Visceral malformations other than CVMs were diaphragmatic hernia, kidney agenesis, enlarged kidney, abnormal lung lobation and malpositioned ovary. In the DM control group, fetuses had CVMs including ventricular septal defect and transposition of great arteries. FA treatment significantly decreased the incidence of total visceral malformations and CVMs from 43.7 ± 10.5% and 28.5 ± 7.1% to 11.2 ± 5.3% and 2.5 ± 2.5%, respectively (Fig. 2B).

**Table 3.** Fetal size on gestational day 18

<table>
<thead>
<tr>
<th>Litters No.</th>
<th>Body weight (g)</th>
<th>Crown-rump length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-DM</td>
<td>5</td>
<td>1.56 ± 0.01</td>
</tr>
<tr>
<td>DM control</td>
<td>8</td>
<td>1.11 ± 0.03*</td>
</tr>
<tr>
<td>DM + FA</td>
<td>6</td>
<td>1.13 ± 0.01*</td>
</tr>
</tbody>
</table>

Litters are expressed as absolute numbers. Other values are expressed as means ± E. *p<0.05 vs. non-DM group.

**Fig. 1.** Diabetic embryopathy. The fetus was obtained on gestational day 18 from diabetic mothers and illustrates external malformations; exencephaly and spina bifida.

**Fig. 2.** The incidence of diabetic malformations in the fetuses. A: external malformations, EM; total external malformations, NTD; neural tube defects. B: visceral malformations, VM; total visceral malformations, CVM; cardiovascular malformations. C: skeletal malformations (SM). No malformations were observed in the non-DM group. Values are the means ± SE. *p<0.05 vs. DM control group.
Skeletal malformations were also induced by maternal DM (Table 6). FA treatment significantly decreased the incidence of skeletal malformations in fetuses of diabetic mothers from 29.7 ± 5.7% to 12.5 ± 5.5% (Fig. 2C). Anomalies included such axial skeletal malformations as caudal regression syndrome, infusion of ribs, supernumerary ribs, agnathia, micrognathia and centrum defects. Fetuses with cranial defects associated with exencephaly were not counted as skeletal malformations. Similarly, ossification delay without deformity was not included in skeletal malformations.

Apoptosis in embryos

Embryos in DM control group showed increased apoptotic cells in the hindbrain region compared with those in non-DM group, indicating the abnormal apoptosis in the neural tube cells. Apoptotic cells were apparently decreased by FA treatment (Fig. 3).

Plasma homocysteine concentration

Maternal plasma homocysteine levels on GD 9.5 were significantly reduced in DM control group compared with non-DM group (Fig. 4). There were no significant differences in plasma homocysteine levels between DM control group and DM + FA group (non-DM group; 6.28 ± 0.87 nmol/ml, DM control group; 2.52 ± 0.39 nmol/ml, DM + FA group; 3.00 ± 0.35 nmol/ml).

Discussion

In this study, we examined the preventive effects of FA against global congenital malformations in offspring from diabetic mice. We showed that FA administration decreased not only external malformations including NTDs but also visceral and skeletal malformations as well. The incidence of diabetic embryopathy was relatively high in this study compared to the incidence in previous study [2]. This is possibly due
to severe diabetic state in this study in which mean glucose level was about 700mg/dl on GD 18. Even though at this severe diabetic state, FA administration markedly reduced the incidence of diabetic embryopathy. Furthermore, FA treatment significantly reduced resorptions rate. This might reflect that the mortal malformations were prevented by FA administration.

Fetal weights and crown-rump lengths were severely reduced in DM control group compared to those in non-DM group, and FA administration did not rescue this growth retardation. There are several reports that FA prevents fetal growth retardation as well as congenital malformations [29–31]. In most of these reports, FA was administered throughout the gestation periods. During the development of embryo, nucleic acid and protein synthesis are at their peak and maternal FA requirements increase. Since FA is a cofactor for the enzyme that synthesizes nucleotides, FA insufficiency in organogenetic period may result in congenital malformations, and if FA is insufficient in the mid-latter period of pregnancy, growth retardation may occur. In this study we administered FA only during organogenesis (GD 6–10) to focus on the preventive effect of FA against congenital malformations. If we administered FA throughout all gestation period, growth retardation by diabetic pregnancy might also be prevented.

Many congenital malformations were observed in the present study, and NTDs were most common among them. Neural tube is formed in GD 8–10 by the folding and fusion of the neuroepithelium in mice [32, 33]. In the normal process of the formation of neural tube, apoptosis of neuroepithelium occurs within midline seam after fusion [34, 35]. In case the timing of the apoptosis is disturbed, it may result in NTDs. We showed here that the apoptotic cells in the neural tube were increased in DM control group compared with those in non-DM group, and that the increased apoptosis was decreased by FA treatment. These results suggest that abnormal apoptosis in the neural tube may be responsible for the NTDs in diabetic pregnancy and FA prevented NTDs by inhibiting the abnormal apoptosis.

In this study, we also showed that FA reduced the incidence of CVMs. Congenital defects in the formation of the great vessels occur as a result of inadequate migration of neural crest cells from the neural tube into the arches [36]. Hyperglycemia inhibits neural crest cell migration in fetuses [37]. In embryos of diabetic mice, it has been reported that hyperglycemia-
induced oxidative stress inhibits the expression of transcription factors such as Pax-3 [38]. We recently identified a variety of genes differentially expressed in mouse fetuses from streptozotocin-induced diabetic pregnancy by cDNA subtraction [21]. The altered expression of transcription factors in diabetic embryos may impair the migration of neural crest cells, resulting in cardiac outflow tract defects. Thus, it may be suggested that FA treatment affects the expression of transcription factors and prevents the cardiac outflow tract defects. Furthermore, Tang et al. reported that congenital heart defects in Folbp1 (–/–) mice were prevented by folic acid supplementation [39]. They demonstrated that this preventive effect is due to reduced apoptosis of cardiac neural crest cells that is associated with the expression of Pax-3 in cardiac neural crest cells. Similar mechanism might be involved in the preventive effects of FA against CVMs as observed in the present study.

Recently, Wentzel et al. and our group [21, 22] reported that mRNA expression of Folbp decreased in a diabetic state, suggesting intracellular FA deficiency in the embryo of diabetic pregnancy. FA functions as a substrate for some of the enzymes involved in the DNA synthesis, a process that is essential to the developing embryos [40]. FA is also involved in methylation reactions, which are important as the regulation mechanism of the transcriptional factors [40]. Therefore, a defective FA metabolism or FA shortage could result in a defective DNA synthesis or an impaired transcription of genes involved in the developmental process of embryos. FA treatment may ameliorate an impaired DNA synthesis or DNA methylation and prevent embryopathy caused by diabetes-associated FA deficiency.

FA plays an important role in the regulation of homocysteine metabolism and FA deficiency leads to the increase in homocysteine [40]. Homocysteine has been reported to be embryo-toxic during the process of neural tube formation [40–42] and is known to increase the reactive oxygen species (ROS) [43]. Although the mechanism of diabetes-induced congenital defects remains unclear, an excess of ROS, increased somatomedin inhibitor, myo-inositol deficiency, increased lipid peroxidation, arachidonic acid deficiency, and altered prostaglandin have been reported to be involved in the pathogenesis of diabetes-induced embryopathy [24, 38, 44–46]. Especially, the excess of ROS is thought to be particularly important. This led us to speculate that if homocysteine levels increase during diabetic pregnancy, it may be involved in the pathogenesis of congenital malformations by causing excess of ROS. Thus, we evaluated maternal plasma homocysteine levels during diabetic pregnancy. However, results showed that the plasma homocysteine levels were significantly lower in DM control mice compared with non-DM mice (Fig. 4). Therefore, it is suggested that the congenital malformations observed in the present study were not caused by the increased homocysteine. There are many reports concerning the relationship between diabetes and folate metabolism, and it is still controversial regarding to homocysteine levels in diabetes. Some reports indicated that diabetic patients who have no complications have low homocysteine levels while those who have complications tend to exhibit elevated total homocysteine levels [47, 48]. On the other hand, it has also been reported that the plasma homocysteine levels were not changed in diabetic state [49]. Our results are consistent with previous reports demonstrating that in type 1 diabetic mice induced by STZ injection, the plasma homocysteine levels diminished because the catabolism of homocysteine was enhanced by transcriptional regulation of hepatic cystathionine β synthase [47].

In conclusion, FA prevented congenital malformations including CVMs and skeletal malformations as well as NTDs in the offspring of diabetic mice. Our results also suggested that the preventive effect of FA is independent from homocysteine metabolism and possibly mediated by decreasing the abnormal apoptosis during organogenesis by FA.

Acknowledgements

This work was supported in part by a Grant-in-aid for Scientific Research from Japan Society for the Promotion Science.
References


30. Quinn PB, Cremin FM, O’Sullivan VR, Hewedi FM,


