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Modulation of Diabetes Induced Palate Defects by Maternal Immune Stimulation

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Abstract

Maternal diabetes can induce a number of developmental abnormalities in both laboratory animals and humans, including deformities of the face and palate. The incidence of birth defects in newborns of diabetic women is approximately 3 to 5 times higher than among non-diabetics. In mice, non specific activation of the maternal immune system can reduce fetal abnormalities caused by various etiologies including hyperglycemia. This study was conducted to determine whether non-specific maternal immune stimulation could reduce diabetes induced palate defects and orofacial clefts. Female ICR mice were immune stimulated prior to induction of hyperglycemia with Freund's complete adjuvant (FCA), granulocyte-macrophage colony-stimulating factor (GM-CSF) or interferon- γ (IFN γ). Streptozocin was used to induce hyperglycemia (26–35 mmol blood glucose) in females prior to breeding. Fetuses from 12–18 litters per treatment group were collected on day 17 of gestation. Palate width and length were measured and the incidence of orofacial clefts was determined. Palate length and width were both decreased by maternal hyperglycemia. Maternal immune stimulation with GM-CSF or FCA limited the degree of palate shortening from the hyperglycemia. Each of the three immune stimulants attenuated significant narrowing of the palate. Rates of orofacial clefts were not significantly different between treatment groups. Palatogenesis is a complex process driven by cellular signals which regulate cell growth and apoptosis. Dysregulation of cellular signals by maternal hyperglycemia can result in fetal malformations. Maternal immune stimulation may prevent dysregulation of these signaling pathways thus reducing fetal malformations and normalizing palate growth.

Keywords

Maternal immune stimulation; Diabetes; Palate; Development; Teratology

INTRODUCTION

Diabetes mellitus can cause fetal death and a number of developmental abnormalities in both humans and laboratory animals. The incidence of birth defects in newborns of diabetic women (both type 1 and type 2) is approximately 3 to 5 times higher than among non-diabetics (Ewart-Toland et al., 2000). Developmental abnormalities caused by maternal hyperglycemia include neural tube defects and altered formation of facial, cardiac, renal, optic and auricular structures. The teratogenic mechanism(s) have not been definitively

identified and are an area of debate in diabetes research. Current research has identified hyperglycemia, inositol deficiency, and or perturbation of arachadonic acid metabolism by excessive reactive oxygen species either independently or together as the cause of fetal malformations, reviewed by Fine et al., (1999) and Eriksson et al., (2003).

In both humans and laboratory animals, maternal hyperglycemia mainly affects fetal structures derived from neural crest tissue (Cederberg et al., 2003). The neural crest originates from the dorsolateral region of the developing neural tube. Once formed, it proliferates and migrates throughout the body forming a variety of adult structures. Cranial neural crest is found specifically around the developing brain and forms many of the craniofacial structures including parts of the brain, meninges, cranium, orbit, eye, and bones of the face and jaw, including the palate (Kanzler et al., 2000; Carstens, 2004). The maxillary and palatine bones, which make up the secondary palate, are derived from cranial neural crest developing in the midbrain region. In the mouse, midbrain neural crest proliferates and migrates to the facial region on gestational day (GD) 8 at the 11 to 16 somite stage (Carstens, 2004). Formation of the palate occurs over a number of days with palate shelf fusion occurring on GD 14. Thus there is a broad window of time when a teratogen can affect palate development resulting in abnormal formation and or incomplete closure of the palate. This broad window of sensitivity to teratogenic exposure may explain the high incidence and apparent multiple etiologies of palate malformation.

Non-specific stimulation of the maternal immune system in mice during the peri-conception period reduces a wide variety of fetal birth defects including those caused by chemical agents, hyperthermia, x-rays, and diabetes mellitus (Nomura et al., 1990; Holladay et al., 2000; Holladay et al., 2002; Hrubec et al., 2005). Maternal immune stimulation reduced or blocked digit and limb defects (Prater et al., 2004), tail malformations and cleft palates (Sharova et al., 2002), and neural tube defects (Torchinsky et al., 1997; Punareewattana and Holladay, 2004; Hrubec et al., 2006a). Diverse means of stimulating the maternal immune system, such as intraperitoneal (IP) injection of inert particles, intrauterine injection of xenogenic lymphocytes or intrauterine or IP injection of immunostimulatory cytokines, have been effective in preventing or reducing fetal defects (Hrubec et al., 2005). The specific operating mechanisms responsible for the reduction in fetal dysmorphogenesis are unknown; however, the collective literature suggests that immunoregulatory cytokines of maternal origin may normalize dysregulated apoptosis and or cell proliferation in the fetus (Sharova et al., 2000; Punareewattana and Holladay, 2004; Savion et al., 2004).

We have reported previously that maternal immune stimulation can modulate craniofacial shortening (Hrubec et al., 2006b). During this study we noticed that some fetuses from diabetic dams exhibited severe facial clefts (cheilognathoiranoschisis) in addition to other gross abnormalities. We also noticed that much of the change in craniofacial length could be attributable to diminished cranial size not necessarily alterations in the facial region. Orofacial clefts are frequently listed as a sequela of maternal hyperglycemia, yet specific studies in humans and animals demonstrating this association are limited. We conducted this study focusing on the palate to identify the effects of maternal hyperglycemia on development of palate size, shape and incidence of clefts.

MATERIALS AND METHODS

Six to 8 week old outbred ICR mice (Harlan Sprague-Dawley, Indianapolis, IN) were housed individually (males) or at 5 per cage (females) for a 2-week acclimation period. Mice were given food (NIH 31 open formula) and distilled water *ad libitum*, and were maintained at 22°C, 40–60% relative humidity with a 14/10 light/dark cycle. All procedures involving mice were reviewed by and conducted in compliance with the guidelines of the Virginia

Tech Animal Care and Use Committee at the VA-MD Regional College of Veterinary Medicine.

The five treatment groups have been described previously (Punareewattana and Holladay, 2004) and are summarized here. One group received just streptozocin (STZ, 200 mg/kg by IP injection) to induce diabetes. Three groups received one of 3 immune stimulants (Freund's complete adjuvant, 20–30 μ l, by footpad injection; GM-CSF, 8000 units by IP injection; or IFN γ 1000 units by IP injection) followed by STZ to induce diabetes. The fifth group was a non-immunestimulated non-diabetic control. We have demonstrated previously that administration of FCA, IFN γ or GM-CSF alone does not adversely alter fetal development, including development of the palate (Sharova et al., 2002; Prater et al., 2004; Hrubec et al., 2006a). Following an established procedure in our laboratory, immune stimulants were administered twice, first at 1 week and again at 1 day before STZ administration (Punareewattana et al., 2003; Punareewattana and Holladay, 2004). STZ was administered 7 days before mating. Blood glucose concentrations were determined from tail vein blood twice before mating to ensure glucose concentrations were $\geq$ 26 mmol/L (the level found previously to increase birth defects, Punareewattana and Holladay, 2004). For breeding, females were housed overnight with non-diabetic males. The presence of a vaginal plug indicated mating and was designated day 0 of gestation.

Pregnant mice were euthanized by cervical dislocation and fetuses collected on day 17 of gestation. Individual fetuses from 12 to 18 litters per treatment group were fixed in 100% ethanol. Fetuses were photographed in lateral recumbancy on a stage micrometer using an Olympus Zoom Stereo Microscope SZX7 (Olympus America Inc., Melville, NY) equipped with a CFW 1310 Scion camera (Scion Corporation, Frederick, MD) to determine facial and crown to rump lengths. Fetal heads were then removed, the lower jaw detached and the fetal palate photographed. Four fetuses with orofacial clefts were set aside for scanning electron microscopy (SEM). All remaining fetal heads were cleared in 1% KOH for 3 to 4 days until tissues were translucent. The bones were then stained with 0.01% alizarin red in 0.02% KOH for 12 hours. Heads were further cleared in 0.5% KOH for approximately one week until transparent, and were then transferred through a graded KOH:glycerine series ending in 100% glycerine. Cleared and stained palates were photographed on a stage micrometer. Palate length and width were determined from the digital images of all fetuses in each treatment group (excluding fetuses with clefts) as demonstrated in Figure 1. The fetal heads used for SEM were rehydrated and fixed/stained in 0.1 M cacodylate buffer with 2% OsO $_4$ for 2 hours. They were then dehydrated, processed for SEM, and visualized with a Phillips 505 SEM. The incidence of orofacial clefts was determined for each treatment.

One way analysis of variance (Statistix 8, Analytical Software, Tallahassee, FL) with the mother as the treatment unit was used to determine differences between the treatment groups. When a significant difference was observed ($p \leq 0.05$), a Tukey's means comparison was used to determine differences between the groups.

RESULTS

Fetal palate size and shape were altered by maternal hyperglycemia. Palate length was shortened in fetuses of diabetic dams (Fig. 2). This shortening was not observed when dams were treated prebreeding with either GM-CSF or FCA. Fetal palate width was similarly decreased by maternal hyperglycemia (Fig. 3). Administration of any one of the immune stimulants attenuated significant narrowing of the palate. Fetal palate shape was further characterized by determining the width to length ratio. Fetuses from hyperglycemic dams had lower width to length ratios than controls (Fig. 4). Thus hyperglycemia does not just reduce fetal palate size; it changes palate proportions, so that the palate is narrower for a

given length than control fetuses. Maternal treatment with either IFN γ or GM-CSF modulated significant changes in fetal palate proportion (Fig. 4).

The incidence of cleft lip and cleft palate was low (Table 1). No significant difference in the rate of clefts was observed between treatment groups, most likely due to the low overall incidence of clefts. A variety of cleft types were observed including cleft lip, cleft lip and primary palate and complete and incomplete clefts of the secondary palate (Fig. 5). A submucosal cleft (incomplete fusion of the palate bones covered with a complete layer of oral mucosa) was observed in one of the control fetuses. Hyperglycemia also altered development of the rugal folds on the fetal palate. Rugae were flattened, misshapen and occasionally missing (Fig. 5). Fetuses with clefts had wider palate dimensions and so were excluded from the measurement analysis.

DISCUSSION

Palatogenesis is a complicated multistep process involving migration and growth of neural crest tissue to form the palate shelves, followed by shelf elevation, and finally epithelial cell adhesion and mesenchymal cell differentiation (Hilliard et al., 2005). Additionally, palate formation depends on facial structure and shape, mandible development and tongue placement. (Diewert, 1981; Ricks et al., 2002). Alteration in any of these steps can result in a malformed palate. The majority of research investigating palate dysmorphogenesis has focused on the occurrence and formation of clefts. Very few studies have investigated alterations in palate shape, even though narrowing of the palate is a recognized malformation observed in a number of human syndromes including Down, Marfan, Rubinstein Taybi, and others. In one of the few studies documenting altered palate dimension, Hill et al. (2007), demonstrated reduced palate growth along the rostral caudal axis in a mouse model of Down syndrome. Reduced palate length was a result of decreased growth in all three palate bones. Palate width was also reduced, resulting in a short narrow palate similar to the morphologic findings in our hyperglycemic fetuses.

It is often cited that diabetes is associated with orofacial clefts; however, few studies have been designed to specifically investigate this association. Epidemiological studies reported odds ratios from 1.35 to 7.7 for orofacial clefts in infants of children from diabetic mothers (Jansson et al., 1996; Spilson et al., 2001). Frequently these analyses include all fetal malformations, and when stratified by disorder the sample groups are small and confidence intervals wide. Animal studies observing orofacial clefts in offspring of diabetic dams are limited (Goldman et al., 1985). We found no difference in rate of orofacial clefts between control and treatment groups. This is due in part to a cleft observed in one of the control fetuses. ICR mice (and the closely related CD-1 strain) may have an observable background rate of orofacial clefts, as cleft palates have been observed previously in control mice (Blaustein et al., 1971; Silbermann and Levitan, 1979). Additionally, stress from handling dams during pregnancy can induce palate clefts (Rosenwzeig and Blaustein, 1971) and could have played a factor in the rate of clefts observed in our study.

Cleft palates were not significantly increased in the diabetic treatment group. A possible explanation for this observation may be that these fetuses had narrower palates coupled with a higher rate of exencephaly (averaging 48%) than the other treatment groups (Punareewattana and Holladay, 2004). In mice, exencephaly appears to protect against cleft palate formation (Yasuda et al., 1991; Sato, 1994; Takagi et al., 2000). A narrow palate as observed in our diabetic group, may facilitate closure by reducing the distance palate shelves are required to elongate medially before contacting and fusing. A narrower palate may also improve tractional forces during maxillofacial development, preventing initially fused palates from separating as the face develops further (Yamada et al., 2006). The apparent lack

of response from maternal immune stimulation on cleft formation could in part be due to the low incidence of clefts observed. Alternatively, immune stimulation prevents narrowing of the palate while also reducing exencephaly rates; these two factors taken together could create greater mechanical force across the fusing palate. Some of the more compromised embryos from dams exposed to maternal immune stimulation may still exhibit clefts even under these conditions even.

The protective effects offered by maternal immune stimulation against teratogen induced malformations are not fully understood. A wide range of immune stimulants and dosing regimes appear to be effective include pre-breeding or mid-gestation injection with immune modulating cytokines, intraperitoneal (IP) injection of inert polymer particles, intrauterine injection of allogeneic or xenogeneic splenocytes, and pre-breeding injection with BCG, or FCA. In all cases, immune stimulants were given prior to teratogen exposure. When diabetic dams were immune stimulated after the onset of hyperglycemia, no protective effect was observed (Punareewattana and Holladay, 2004). This would indicate that maternal immune stimulation blocks or prevents rather than reverses the deleterious effect of hyperglycemia.

The collective literature demonstrates that stimulating the mother's immune system prevents alterations in maternal and fetal signaling molecules that regulate apoptosis and cell proliferation during development. Specifically in the diabetic pregnancy and in developing palate, maternal immune stimulation prevented alterations in regulatory factors such as tumor necrosis factor alpha (Tnf α), p53, Bcl-2, Nuclear factor κ -B (NF κ -B) transforming growth factor beta 2 (Tgf β 2), Tgf β 3, granulocyte-monocyte colony-stimulating factor (GM-CSF), interleukin 1 (IL-1), interleukin-2 (IL-2), and colony stimulating factor-1 (Fein et al., 2001, 2002; Punareewattana et al., 2003; Sharova et al., 2002). Normalization of these regulatory factors is accompanied by reduced incidence of birth defects and thus may be related to improved fetal outcome.

Formation of the palate occurs from day 8 – 14 out of a 20 gestation for a normal mouse. This long time period when the palate is sensitive to teratogenic exposure provides ample opportunity to disturb processes in palate development. Maternal hyperglycemia dysregulates cell proliferation and apoptosis in the developing embryo, likely resulting in fetal malformations (Degitz et al., 1998; Liu et al., 2005; Okano et al., 2006). Maternal immune stimulation may normalize many of these processes. This area of research holds promise for developing preventative strategies to reduce a wide range of birth defects now frequently observed in the offspring of diabetic women.

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Figure 1.

Palate dimensions were measured from cleared and stained heads as indicated by the measurement markers in the figure. Palate length was measured from the rostral condensation of premaxillary bone immediately behind the forming incisor to the caudal most portion of the darkly stained perpendicular plate of the palatine bone. Palate width was measured at the greatest distance between the lateral walls of the darkly staining perpendicular plate of the palatine bone. Variations in degree of ossification of the palatine bone with gestational stage prevented measurement in other locations or dimensions.

Palate Length

Figure 2.

Mean palate length $\pm$ SEM (measured as a ratio of palate length to crown-rump length) of gestational 17 day fetuses from diabetic dams, diabetic dams receiving prebreeding immune stimulation and non-diabetic controls. Dams were immune stimulated with either FCA (20–30 μ l), GM-CSF (8000 units), or IFN- γ (1000 units). Bars designated with different letters are significantly different ($p \leq 0.05$).

Palate Width

Figure 3.

Mean palate width $\pm$ SEM (measured as a ratio of palate width to crown-rump length) of gestational 17 day fetuses from diabetic dams, diabetic dams receiving prebreeding immune stimulation and non-diabetic controls. Dams were immune stimulated with either FCA (20–30 μ l), GM-CSF (8000 units), or IFN- γ (1000 units). Bars designated with different letters are significantly different ($p \leq 0.05$).

Palate Width to Length Ratio

Figure 4.

Mean palate width to palate length ratio for gestational 17 day fetuses from diabetic dams, diabetic dams receiving prebreeding immune stimulation and non-diabetic controls. Dams were immune stimulated with either FCA (20–30 μ l), GM-CSF (8000 units), or IFN- γ (1000 units). Bars designated with different letters are significantly different ($p \leq 0.05$).

Figure 5.

Fetal palates at gestational day 17 demonstrating some of the characteristic lesions associated with maternal hyperglycemia. Paired images are of the same individual before and after clearing to show skeletal elements or scanning electron microscopy. A-B: Normal fetus with an intact palate and a typical rugal fold pattern. C-D: Fetus with a cleft secondary palate displaying unfused maxillary and palatine bones. The cleft disrupts the rugal folds. E-F: Fetus with a cleft lip; rugal folds are normal. G-H: Fetus with a cleft lip and primary palate and an incomplete cleft of the secondary palate. Rugae are flattened and misshapen.

Table 1

Prevalence of orofacial clefts in fetuses of hyperglycemic dams and of hyperglycemic dams immune stimulated pre breeding with Interferon γ (IFN), Freund's complete adjuvant (FCA), and Granulocyte- macrophage colony stimulating factor (GM-CSF).

	Control	Treatment			
		STZ	STZ + IFN	STZ + FCA	STZ + GM-CSF
# of litters	5	18	13	18	12
Total # of fetuses	55	200	160	196	119
Cleft lips per litter (%)	0	0.4 ± 1.8	0.8 ± 2.4	0.8 ± 2.6	2.2 ± 6.3
Cleft palates per litter (%)	2.9 ± 6.4	2.6 ± 6.6	2.7 ± 4.9	1.7 ± 4.0	4.1 ± 9.6
# Litters with cleft lip	0	1	2	1	2
# Litters with cleft palate	1	4	5	2	3