Maternal Diabetes Increases the Risk of Caudal Regression Caused by Retinoic Acid

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Maternal diabetes increases the risk of congenital malformations in the offspring of affected pregnancies. This increase arises from the teratogenic effect of the maternal diabetic milieu on the developing embryo, although the mechanisms of this action is poorly understood. In the present study, we examined whether the vitamin A metabolite retinoic acid (RA), a common drug with well-known teratogenic properties, may interact with maternal diabetes to alter the incidence of congenital malformations in mice. Our results show that when treated with RA, embryos of diabetic mice are significantly more prone than embryos of nondiabetic mice to develop caudal regression, a defect that is highly associated with diabetic pregnancy in humans. By studying the vestigial tail (Wnt-3avt) mutant, we provide evidence that Wnt-3a, a gene that controls the development of the caudal region, is directly involved in the pathogenic pathway of RA-induced caudal regression. We further show that the molecular basis of the increased susceptibility of embryos of diabetic mice compared with embryos of nondiabetic mice involves enhanced downregulation of Wnt-3a expression. This positive interaction between RA and maternal diabetes may have implications for humans in suggesting increased susceptibility to environmental teratogens during diabetic pregnancy. Diabetes 51:2811–2816, 2002

Maternal diabetes is known to be associated with an increased risk of congenital malformation in the offspring of affected pregnancies (1). Indeed, congenital malformations are the leading cause of death in infants of diabetic mothers (2). Although it is clearly evident that diabetic embryopathy is the result of multifactorial interactions (3), few attempts have been made to investigate the association between diabetic embryopathy and other factors, such as food or drugs taken during pregnancy. In the present study, we aimed to test whether environmental factors can interact with the maternal diabetic milieu to alter the incidence of congenital malformations.

Although congenital anomalies that affect a number of organs, including the cardiovascular, neurological, facial, gastrointestinal, and genitourinary systems, are often found in infants of diabetic mothers (4), none of these malformations is specifically associated with maternal diabetes (5). In contrast, the rate of caudal regression is at least 250 times higher in the offspring of diabetic mothers than in nondiabetic pregnancies, and ~1% of infants born to diabetic mothers exhibit this defect (6,7). Caudal regression syndrome is characterized by premature termination of the vertebral column. It can occur as part of a complex group of malformations that include abnormalities of the anorectal, genitourinary, and nervous systems (8–10). Recent studies using mice have shown that maternal treatment with the vitamin A metabolite, all-trans retinoic acid (RA), can produce a spectrum of malformations, including vertebral truncation, terminal myelocystocele, and imperforate anus (11), which resemble caudal regression syndrome as seen in human diabetic pregnancy (8,9). To investigate in detail how an echinoderm link between RA-induced caudal regression and that associated with diabetes, we asked whether exogenous RA might interact with maternal diabetes to increase the susceptibility of the embryo to develop caudal regression and other anomalies. We found a significantly higher incidence of RA-induced caudal regression in embryos of diabetic mice compared with embryos of nondiabetic pregnancies. We also present evidence that this increased susceptibility to caudal regression is mediated via enhanced downregulation by RA of Wnt-3a expression in the embryo exposed to a diabetic milieu.

RESEARCH DESIGN AND METHODS

Induction of diabetes. Type 1 diabetes was induced in female ICR mice, aged 7–8 weeks, by intraperitoneal injection of 65 mg/kg body wt streptozotocin (ICN, Costa Mesa, CA) dissolved in 0.01 mol/l sodium citrate buffer at pH 4.5 on 3 consecutive days (12). Control mice received an equivalent volume of sodium citrate buffer. Two weeks after the first injection, mice were screened for diabetes by the measurement of glucose level in whole blood extracted from the tail vein using the Glucometer Elite (Bayer, Newbury, U.K.). Blood glucose level was closely monitored at regular intervals, and stabilization usually occurred within 3 weeks of the first day of injection. On the basis of blood glucose level, female mice were classified as nondiabetic (90–140 mg/dl), mildly diabetic (141–300 mg/dl), or severely diabetic (>300 mg/dl). Subsequent measurements, during pregnancy, confirmed that blood glucose level was maintained within these limits in all three groups (Table 1).

Females of all three blood glucose groups were mated with nondiabetic male ICR mice. At 0.5 days postcoitus (dpc), pregnant mice received an intraperitoneal injection of 25 or 50 mg/kg body wt all-trans RA (Sigma, St. Louis, MO) suspended in peanut oil; controls received peanut oil alone. Fetuses at 18.5 dpc, i.e., 1 day before birth, were removed from the uterus and examined for gross anomalies. Crown-rump length and tail length (defined as the length of the body posterior to the hindlimbs) were measured with an

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dpc, days postcoitus; RA, retinoic acid; RAR, retinoic acid receptor.
eyepiece graticule. The fetus was considered to exhibit "complete caudal regression" when it was totally tailless. For investigating the temporal changes in expression levels of Wnt-3a, embryos were harvested at different time points after RA treatment and then processed for in situ hybridization. Guidelines were followed for the use and care of laboratory animals, as set by The Chinese University of Hong Kong.

**RESULTS**

Administration of RA to pregnant ICR mice at 9.5 dpc, equivalent to the 4th week after conception of human pregnancy, caused fetuses to develop caudal regression syndrome (11). The minimum dose of RA for inducing premature vertebral truncation was 25 mg/kg, whereas 100 mg/kg caused complete caudal regression, i.e., total loss of tail. To determine whether RA may interact with maternal diabetes to affect the development of caudal regression, we induced diabetes with streptozotocin and then studied the incidence of congenital malformations in RA-treated fetuses of diabetic mice.

**Embryos of diabetic mice exhibited increased susceptibility to RA-induced caudal regression.** We found no statistically significant difference in litter size or resorption rate among pregnancies of the three diabetic states after treatment with varying doses of RA (Table 1). Hence, there did not seem to be a significant embryonic or fetal loss in any of the treatment groups. In the absence of RA, several anomalies occurred at low frequency in fetuses of severely diabetic mice but not in mildly diabetic or non-diabetic pregnancies. These anomalies include complete caudal regression, cleft palate, exencephaly, and spina bifida (Fig. 1). Upon treatment with RA at 9.5 dpc, both complete caudal regression and cleft palate showed a dose-dependent increase in frequency (Fig. 1A and B), whereas the incidence of exencephaly and spina bifida was unaffected by RA treatment at this stage of pregnancy (Fig. 1C and D).

Importantly, fetuses of mice with different diabetic states exhibited statistically significant differences in their susceptibility to RA. At 25 mg/kg RA, fetuses of both mildly diabetic and severely diabetic mice had a significantly reduced tail length/crown-rump length ratio in comparison with fetuses of nondiabetic mice (Table 1). Only a small percentage of fetuses of nondiabetic mice developed complete caudal regression, whereas there was a sixfold increase in the incidence of complete caudal regression among fetuses of mildly diabetic mice and a >16-fold increase among fetuses of severely diabetic mice (Fig. 1A). At a dose of 50 mg/kg RA, an average of 89% of fetuses within litters of severely diabetic mice developed complete caudal regression compared with only 13% in non-diabetic pregnancies (Fig. 1A).

The incidence of cleft palate induced by different doses of RA exhibited a similar relationship to the maternal diabetic state as that seen with complete caudal regression (Fig. 1B). These results demonstrate that RA treatment at 9.5 dpc synergizes with maternal diabetes to induce caudal regression and cleft palate but not exencephaly and spina bifida.

**Wnt-3a was directly involved in the genetic pathway of RA-induced caudal regression.** Caudal regression induced by RA was associated with excessive apoptosis of the tail bud (11), which contains progenitor cells for the
various tissues of the caudal embryonic region (17,18). It has been shown that Wnt-3a, a gene that encodes a secreted signaling molecule, is indispensable for tail bud development (19). Embryos with homozygous disruption of Wnt-3a exhibited many phenotypic similarities to embryos treated with RA, including premature termination of the body axis, development of excessive neural tissue, and extensive caudal cell death (11,19,20). Similar findings have been reported for the vestigial tail mouse mutant (Wnt-3avt), which results from a hypomorphic allele of Wnt-3a that causes a reduction in Wnt-3a level specifically in the tail bud. The close resemblance between embryos with absence or reduction of Wnt-3a (Wnt-3a−/− and Wnt-3avt/vt) and those treated with RA suggest that Wnt-3a may play a primary role in the pathogenic pathway of RA-induced caudal regression. We tested the prediction that Wnt-3avt/− embryos, with their subnormal Wnt-3a function, should be more susceptible to RA-induced caudal regression than Wnt-3a−/− embryos. In the absence of RA, we found no differences in the caudal length between Wnt-3avt/− and Wnt-3a−/− embryos (Fig. 2A and C). However, upon treatment with a low dose of RA (25 mg/kg), which causes a mild reduction on the caudal length of Wnt-3a−/− embryos, the extent of caudal regression in Wnt-3avt/− embryos was significantly greater than in Wnt-3a−/− embryos, whereas other parts of the embryo remained unaffected (Fig. 2B and C).
In situ hybridization analysis showed that the expression level of Wnt-3a in the tail bud of 9.5 dpc Wnt-3a<sup>vt/vt</sup> embryos was reduced compared with Wnt-3a<sup>+/+</sup> embryos, whereas Wnt-3a mRNA could not be detected in the tail bud of Wnt-3a<sup>vt/vt</sup> embryos (Fig. 3A). Five hours after RA treatment at 9.5 dpc, Wnt-3a expression level was mildly reduced in the tail bud of Wnt-3a<sup>+/+</sup> embryos, whereas in Wnt-3a<sup>vt/vt</sup> embryos, Wnt-3a mRNA exhibited dramatic reduction in the tail bud, although Wnt-3a expression in the neural tube did not seem to be affected by RA (Fig. 3B).

Hence, a correlation exists between profound inhibition of Wnt-3a expression in the tail bud and termination of axial elongation.

We previously found that the extent of caudal regression induced by RA is dose dependent (11). To determine whether the expression level of Wnt-3a mRNA is also related to the dose of RA, we compared Wnt-3a expression in ICR embryos at various time points after treatment with different dosages of RA (50 or 100 mg/kg). Figure 4A and C–H show that the higher the dose of RA, the more rapid the downregulation of Wnt-3a in the tail bud of the embryo. Taken together, these findings suggest that the molecular mechanism of RA-induced caudal regression is mediated via specific downregulation of Wnt-3a in the tail bud, with the extent of caudal regression determined by how rapidly Wnt-3a is switched off.

**Embryos of diabetic mice showed enhanced down-regulation of Wnt-3a.** Maternal diabetes can alter embryonic gene expression in association with the development of malformation (12). In view of the critical role played by Wnt-3a in the pathogenesis of RA-induced caudal regression, we examined Wnt-3a expression by in situ hybridization in embryos of nondiabetic and severely diabetic ICR mice to determine whether there is any difference between them that could account for their differences in susceptibility to development of RA-induced caudal regression. We found that there was no difference in the expression level of Wnt-3a in both the tail bud and the neural tube of embryos of nondiabetic and severely diabetic mice (compare Fig. 4A and B). However, when we studied temporal changes in the expression level of Wnt-3a after treatment with the same dose of RA (50 mg/kg), we discovered that Wnt-3a was downregulated.
The aim of this study was to determine whether maternal diabetes and exogenous RA administration might interact to increase the incidence of congenital malformations. Our results show that there is indeed a positive interaction. Fetuses of diabetic mice are more prone to develop complete caudal regression than those of nondiabetic mice when exposed to RA. Moreover, Wnt-3a expression will have no effect on expression of downstream genes, such as RARs, which act as ligand-inducible transcriptional regulators, to activate or repress transcription of downstream genes (26). The intracellular actions of RA are mediated via nuclear RA receptors (RARs), of which there are three subtypes: RAR-α, RAR-β, and RAR-γ (27). RA forms a complex with RARs, which act as ligand-inducible transcriptional regulators, to activate or repress transcription of downstream genes (28). Because RA specifically affects Wnt-3a expression in the tail bud but not in the neural tube, it seems likely that Wnt-3a is regulated by a specific RAR subtype, which is specifically expressed in the tail bud. For instance, RAR-γ is expressed in the tail bud but not in the neural tube (29). Moreover, embryos with targeted disruption of RAR-γ are completely resistant to RA-induced caudal truncation (30), thus supporting the idea that RA action on the tail bud is mediated via RAR-γ. One possibility, therefore, is that RAR-γ is upregulated in diabetes. If RA concentration is normally limiting, then this increased RAR-γ expression will have no effect on expression of downstream genes, such as Wnt-3a. However, in the presence of exogenous RA, RA-RAR-γ activity will increase, and this may enhance the downregulation of Wnt-3a in the embryos of diabetic mothers.

Another mechanism that could account for the different susceptibility of embryos of diabetic and nondiabetic mice to RA-induced malformations is that maternal diabetes may cause an elevated level of RA to be delivered to the embryo. Congenital malformations are induced in early
diabetic pregnancy, usually before the 7th gestational week in humans (5). Analysis of the preplacenta (yolk sac) or early placenta in diabetic rats shows an increased blood flow in the uterine and decidual tissues compared with normal pregnant rats (31), thus suggesting that the transfer of compounds between mother and embryo during a teratologically important period of pregnancy may be elevated. We found that the rate of downregulation of Wnt-3a is related to the dose of exogenous RA, and embryos of severely diabetic mice treated with 50 mg/kg RA downregulated Wnt-3a at a rate similar to embryos of nondiabetic mice treated with 100 mg/kg RA. It is possible, therefore, that embryos of diabetic mice may receive a higher level of RA than embryos of nondiabetic mice, even though their mothers are treated with the same dose of RA. If this is the case, then it seems very likely that the effect of maternal diabetes, in increasing the sensitivity to teratogens, will not be limited to RA. Additional studies are required to determine the type and nature of compounds that can interact with the maternal diabetic milieu.

In conclusion, this study indicates that an interaction of an environmental factor with the maternal diabetic milieu can increase the susceptibility of the offspring to congenital malformation. At a time when there is a worldwide tendency toward earlier onset of diabetes (32), more people will develop diabetes during their childbearing years; thus, there is an urgent need to understand the tendency toward earlier onset of diabetes (32), more people will develop diabetes during their childbearing years; thus, there is an urgent need to understand the cause and pathogenic mechanisms of the interaction between environmental factors and maternal diabetes on diabetic embryopathy to arrive at preventive measures. Moreover, our findings raise the more general possibility of interactions between distinct teratogenic influences potentiating the adverse effect on the embryo/fetus. In the future, it may be beneficial to consider not only the deleterious effects of individual agents but also their possible synergistic interactions with other teratogenic influences.

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