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 mechanism 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-3a*^{vt}) 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 to RA 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.

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dpc, days postcoitus; RA, retinoic acid; RAR, retinoic acid receptor.

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 a possible mechanistic 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 9.5 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

TABLE 1
Pregnancy outcome among ND, MD, and SD mice treated with various doses of RA at 9.5 dpc

	No RA			25 mg/kg RA			50 mg/kg RA		
	ND	MD	SD	ND	MD	SD	ND	MD	SD
Blood glucose (mg/dl)	125	227*	514*	127	209*	510*	115	233*	530*
±SE at 9.5 dpc	±7	±23	±49	±4	±17	±33	±11	±24	±40
Blood glucose (mg/dl)	116	218*	522*	114	240*	515*	121	227*	504*
±SE at 18.5 dpc	±5	±19	±27	±4	±12	±17	±4	±18	±23
Litters (*n*)	9	8	9	13	8	12	9	9	11
Live fetuses (*n*)	103	92	105	139	89	119	107	103	101
Litter size	11.44	11.50	11.67	10.69	11.13	9.92	11.89	11.44	9.18
±SE	±0.47	±0.57	±0.75	±0.57	±0.61	±0.73	±0.42	±0.34	±0.46
% Resorption	6.07	8.95	8.33	6.80	8.33	8.42	5.90	8.44	7.02
±SE	±2.22	±3.15	±1.97	±1.79	±3.06	±2.89	±2.15	±2.55	±2.24
TL/CRL ratio	0.43	0.43	0.42	0.28†	0.22*†	0.18*†	0.22†	0.15*†	0.04*†
±SE	±0.01	±0.01	±0.02	±0.01	±0.02	±0.01	±0.02	±0.01	±0.02

ND; nondiabetic; MD, mildly diabetic; SD, severely diabetic; TL/CRL ratio, tail length/crown-rump length ratio. *$P < 0.005$ vs. ND treated with the same dose of RA; †$P < 0.005$ vs. fetuses of the same diabetic state without RA. Fetuses analyzed at 18.5 dpc.

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.

Vestigial tail mutants. Vestigial tail (*Wnt-3a^{vt}*) mutant mice were maintained on the JF1 genetic background at the National Institute of Genetics, Mishima, Japan. Homozygotes are totally tailless, whereas heterozygotes show no overt phenotype. Matings were performed between *Wnt-3a$^{vt/+}$* and *Wnt-3a$^{+/+}$* mice or between *Wnt-3a$^{vt/+}$* and *Wnt-3a$^{vt/+}$* mice. Pregnant mice at 9.5 dpc received an intraperitoneal injection of 25 mg/kg RA or of suspension vehicle alone. Embryos were collected before or 5 h after RA treatment for in situ hybridization studies of *Wnt-3a* expression or were harvested at 14.5 dpc for measurement of caudal length, defined as the distance from the caudal limit of the hindlimb to the end of the tail. Embryonic genotype was determined by PCR amplification of DNA prepared from the yolk sac or embryo, using the polymorphic microsatellites D11Mit22 and D11Mit28, which are located proximal and distal to the *Wnt-3a^{vt}* locus, respectively (13).

In situ hybridization. The *Wnt-3a* cDNA plasmid was a gift from Roel Nusse (14). Embryos were subjected to whole-mount in situ hybridization using digoxigenin-labeled probes according to the protocol of Wilkinson (15). For facilitating comparisons, embryos of different treatment groups were marked by excision of a forelimb bud, hybridized in the same tube, and developed in the NBT/BCIP solution at 4°C overnight for the same length of time.

Nile blue sulfate staining of dead cells. The supravital dye Nile blue sulfate was used to stain dead cells in whole-mount embryos (16). Embryos of severely diabetic and nondiabetic mice were harvested at either 9.5 dpc or 24 h after treatment with 50 mg/kg RA. They were bathed in Nile blue sulfate dissolved in lactated Ringer's solution (1 in 50,000; wt/wt) for 15 min at 37°C and then thoroughly washed in lactated Ringer's solution before examination.

Statistical analysis. Statistical comparisons among nondiabetic, mildly diabetic, and severely diabetic mice that were treated with varying doses of RA were performed by ANOVA. Comparisons between *Wnt-3a$^{vt/+}$* and *Wnt-3a$^{+/+}$* embryos were performed by Student's *t* test. All statistical analyses were carried out using SPSS software (SPSS Inc., Chicago, IL).

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 nondiabetic 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. 1*A* and *B*), whereas the incidence of exencephaly and spina bifida was unaffected by RA treatment at this stage of pregnancy (Fig. 1*C* 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. 1*A*). 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 nondiabetic pregnancies (Fig. 1*A*).

The incidence of cleft palate induced by different dosages of RA exhibited a similar relationship to the maternal diabetic state as that seen with complete caudal regression (Fig. 1*B*). 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

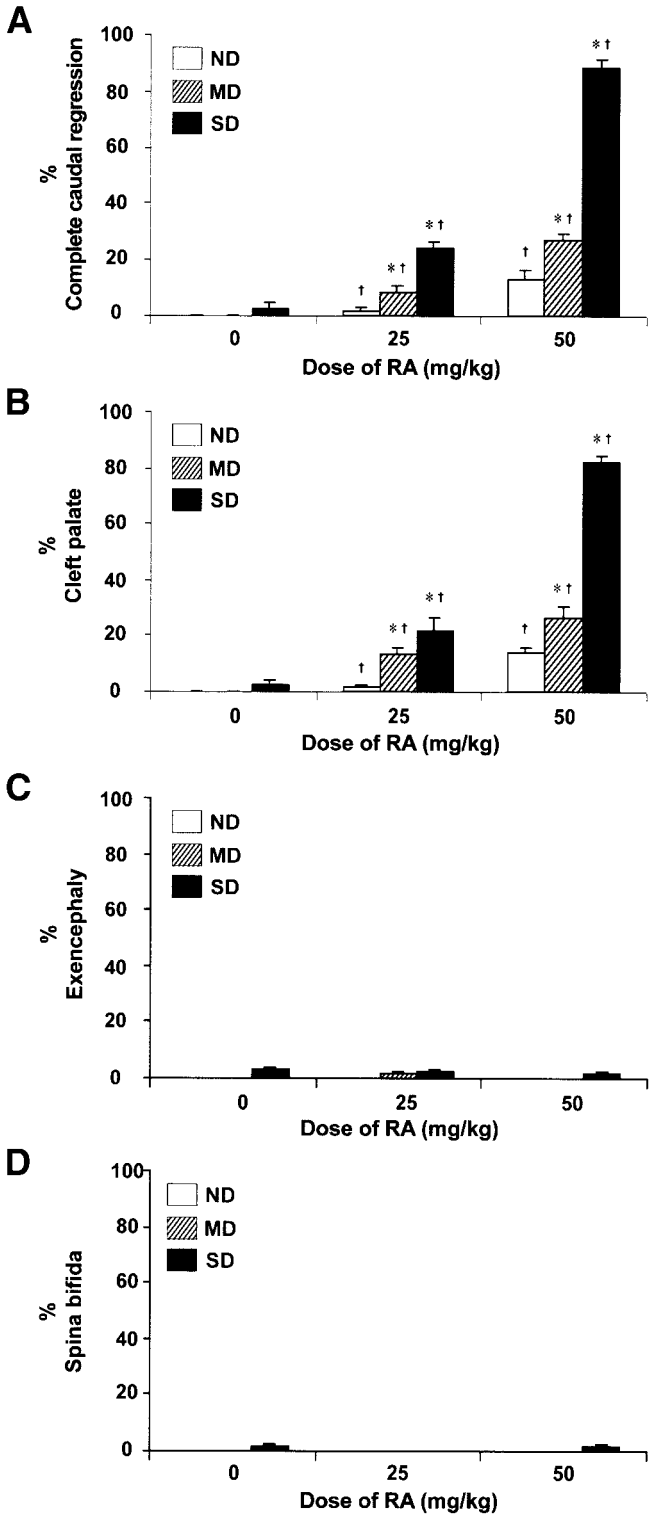

FIG. 1. Comparison of the percentage of fetuses, on a per-litter basis, of nondiabetic (ND), mildly diabetic (MD), and severely diabetic (SD) ICR mice that developed defects when exposed to RA. *A*: Fetuses of MD and SD mice exhibit a significantly increased incidence of complete caudal regression compared with fetuses of ND mice when exposed to either 25 or 50 mg/kg RA. *B*: The response profile of cleft palate is very similar to that of complete caudal regression. *C* and *D*: There is no significant increase in the incidence of exencephaly (*C*) and spina bifida (*D*) in fetuses, irrespective of the maternal diabetic state, after RA treatment. Values represent the mean ± SE of percentage of fetuses with defects in a litter. **P* < 0.005 vs. ND treated with the same dose of RA; †*P* < 0.005 vs. fetuses of the same diabetic state without RA. Sample size of each group is as shown in Table 1.

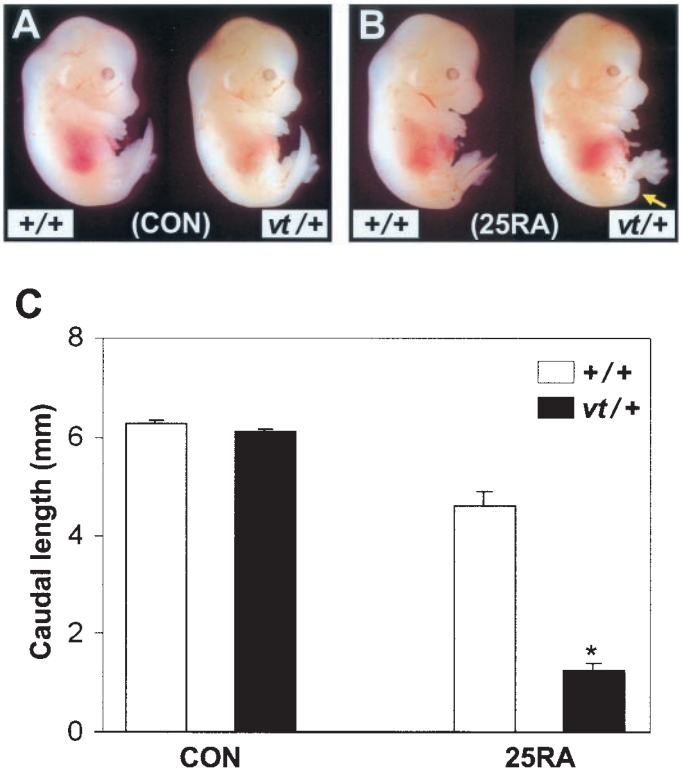

FIG. 2. Effect of RA on caudal development of *Wnt-3a*$^{+/+}$ (+/+) and *Wnt-3a*$^{vt/+}$ (*vt*/+) embryos. *A*: In the absence of RA (CON), there is no observable difference between *Wnt-3a*$^{+/+}$ and *Wnt-3a*$^{vt/+}$ embryos at 14.5 dpc. *B*: When treated with 25 mg/kg RA (25RA), the body axis of *Wnt-3a*$^{vt/+}$ embryos at 14.5 dpc is truncated at a higher axial level (arrow) in comparison with *Wnt-3a*$^{+/+}$ embryos. *C*: After RA administration, the caudal length (mean ± SE) of *Wnt-3a*$^{vt/+}$ embryos at 14.5 dpc is significantly shorter than *Wnt-3a*$^{+/+}$ embryos (**P* < 0.005), whereas the two genotypes do not differ in the absence of RA. Sample size: 22 for +/+ (CON); 26 for *vt*/+ (CON); 32 for +/+ (RA); 29 for *vt*/+ (RA).

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-3a*^{vt}), which results from a hypomorphic allele of *Wnt-3a* (11,13,21) 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-3a*$^{vt/vt}$) 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-3a*$^{vt/+}$ 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-3a*$^{vt/+}$ and *Wnt-3a*$^{+/+}$ embryos (Fig. 2*A* 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-3a*$^{vt/+}$ embryos was significantly greater than in *Wnt3a*$^{+/+}$ embryos, whereas other parts of the embryo remained unaffected (Fig. 2*B* and *C*).

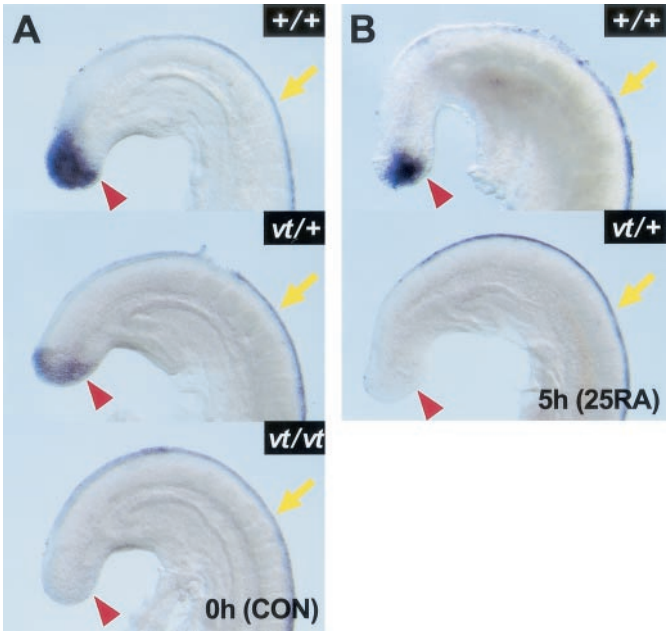

FIG. 3. In situ hybridization for *Wnt-3a* in *Wnt-3a*^{vt} embryos before (0h) or 5 h (5h) after RA administration at 9.5 dpc. *A*: Before RA treatment (CON), intensity of expression of *Wnt-3a* in the tail bud (arrowhead) of *Wnt-3a*$^{vt/+}$ (*vt/+*) embryos is intermediate between that seen in *Wnt-3a*$^{+/+}$ (+/+) and *Wnt-3a*$^{vt/vt}$ (*vt/vt*) embryos, whereas there is no difference in neural tube expression (arrow). *B*: Five hours after treatment with 25 mg/kg RA (25RA), *Wnt-3a* expression is extinguished in the tail bud of *Wnt-3a*$^{vt/+}$ embryos more rapidly than in *Wnt-3a*$^{+/+}$ embryos, whereas expression in the neural tube remains unaffected. The figure shows representative embryos from the four to seven examined in each treatment group.

In situ hybridization analysis showed that the expression level of *Wnt-3a* in the tail bud of 9.5 dpc *Wnt-3a*$^{vt/+}$ embryos was reduced compared with *Wnt-3a*$^{+/+}$ embryos, whereas *Wnt-3a* mRNA could not be detected in the tail bud of *Wnt-3a*$^{vt/vt}$ embryos (Fig. 3*A*). Five hours after RA treatment at 9.5 dpc, *Wnt-3a* expression level was mildly reduced in the tail bud of *Wnt-3a*$^{+/+}$ embryos, whereas in *Wnt-3a*$^{vt/+}$ 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. 3*B*). 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 4*A* 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 downregulation 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

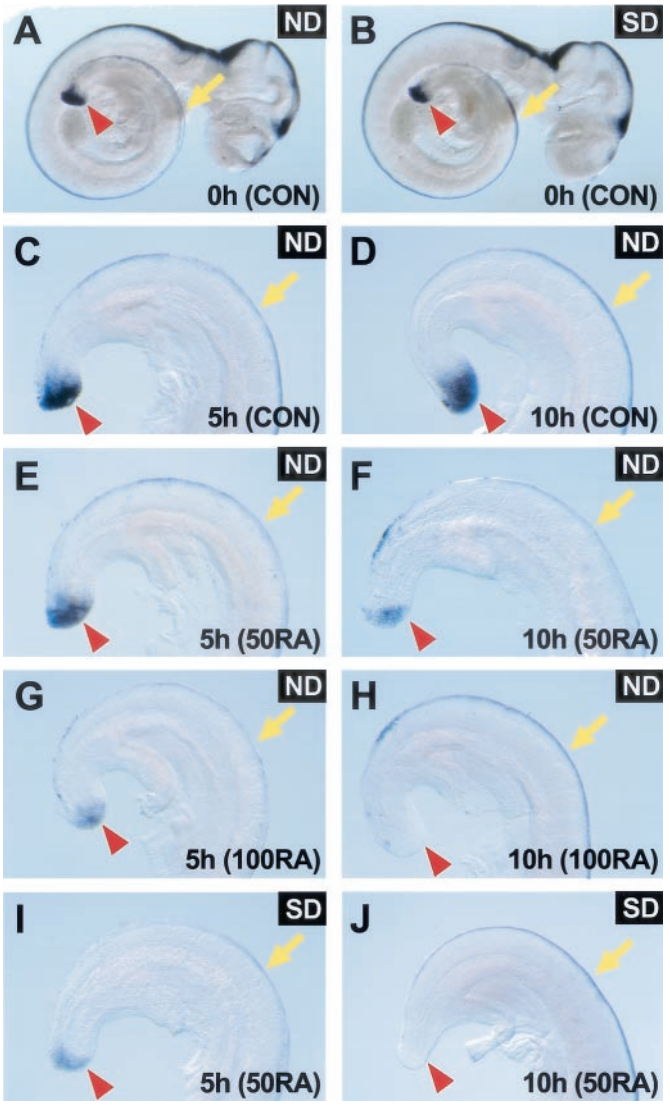

FIG. 4. Expression of *Wnt-3a* in embryos of nondiabetic (ND) and severely diabetic (SD) ICR mice before (0h) or at 5 (5h) or 10 h (10h) after treatment with different dosages of RA at 9.5 dpc. *A* and *B*: In the absence of RA (CON), there is no difference in the expression intensity of *Wnt-3a* in the tail bud (arrowhead) and in the neural tube (arrow) between embryos of ND (*A*) and SD (*B*) mice. *C* and *D*: *Wnt-3a* continues to express strongly in the tail bud of embryos of ND mice in the absence of RA at 5 (*C*) and 10 h (*D*). *E–H*: *Wnt-3a* is downregulated more rapidly in the tail bud of embryos of ND mice treated with 100 mg/kg RA (100RA; *G* and *H*) than in embryos of ND mice treated with 50 mg/kg (50RA; *E* and *F*). *I* and *J*: The speed of downregulation of *Wnt-3a* is enhanced in the tail bud of embryos of SD mice in comparison with that of ND mice treated with the same dose of RA (compare with *E* and *F*). The figure shows representative embryos from the 14–19 examined in each treatment group.

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. 4*A* 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

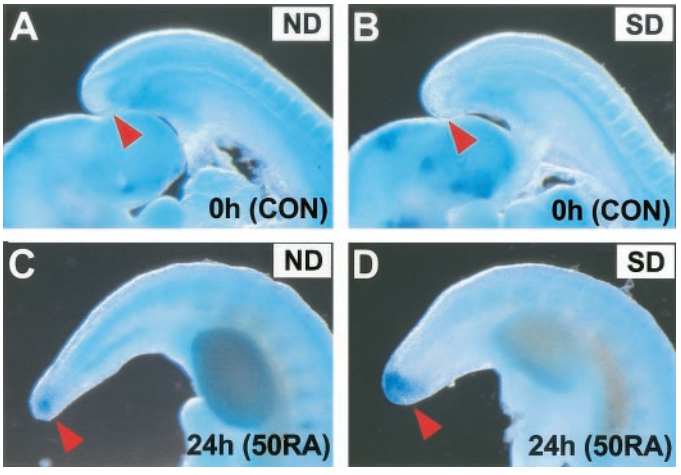

FIG. 5. Nile blue sulfate staining of dead cells in the caudal region of embryos of nondiabetic (ND) and severely diabetic (SD) ICR mice. *A* and *B*: In the absence of RA (CON), dead cells are hardly observed in the tail bud (arrowhead) of embryos of ND (*A*) and SD (*B*) mice at 9.5 dpc (0h). *C* and *D*: Twenty-four hours (24h) after treatment with 50 mg/kg RA (50RA), there is a marked increase in the number of dead cells in the caudal end of embryos of SD mice (*D*) in comparison with the amount observed in that of ND mice (*C*). The figure shows representative embryos from the 10 examined in each treatment group.

specifically in the tail bud of embryos of severely diabetic mice (Fig. 4*I* and *J*) much more rapidly than in embryos of nondiabetic mice (Fig. 4*E* and *F*), whereas no difference was found in *Wnt-3a* expression in the neural tube. Whole-mount staining of embryos with the supravital dye Nile blue sulfate provided no evidence for an increase in cell death in the tail bud of embryos of severely diabetic mice in the absence of RA (Fig. 5*A* and *B*), but considerably more dead cells were detected in the tail bud of embryos of severely diabetic mice than in embryos of nondiabetic mice after RA treatment (Fig. 5*C* and *D*). These results thus may account for the more rapid termination of axial elongation and the increased incidence of complete caudal regression in fetuses of RA-treated diabetic mice.

DISCUSSION

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*, which is critical for RA-induced caudal regression, although not affected by the maternal diabetic milieu per se, is downregulated specifically in the tail bud of embryos of diabetic mice much more rapidly than that in embryos of nondiabetic mothers after RA treatment.

In the present study, we found that after RA administration at 9.5 dpc, besides caudal regression, fetuses of diabetic mice showed increased susceptibility to develop cleft palate, but there is no elevated incidence of exencephaly and spina bifida compared with nondiabetic pregnancies. This finding probably arises from the fact that RA affects different developing organs in a stage-dependent manner (22). For instance, the developing palate is affected by RA at a similar stage to the tail bud (11,23), whereas the critical period for RA induction of exencephaly and spina bifida is earlier in gestation than 9.5 dpc (24,25). Indeed, when pregnant mice are administered RA at 8.5 dpc, when neural tube fusion of the anterior and posterior neuropores is not yet complete, we find that fetuses of diabetic mice exhibit a higher incidence of exencephaly and spina bifida than fetuses of nondiabetic mice (S.-M. Yeung and A.S.W. Shum, unpublished observations).

Why are embryos of diabetic mothers more susceptible to RA-induced malformation? We previously showed that the extent of caudal regression induced by RA is stage dependent (11). It is possible, therefore, that embryos of diabetic mothers might be developmentally retarded compared with embryos of nondiabetic mothers, thus leading to exposure to RA at different developmental stages, resulting in the increased severity of caudal regression observed in RA-treated diabetic pregnancies. To examine this point, we compared the developmental stage of embryos from diabetic and nondiabetic pregnancies, at 9.5 dpc, when RA was administered, and found no significant difference in somite number (nondiabetic 23.4 ± 0.3, severely diabetic 23.2 ± 0.3; A.S.W.S., unpublished data). This suggests that developmental retardation is unlikely to account for the increased propensity of embryos from diabetic pregnancy to RA-induced caudal regression.

An alternative possibility is that maternal diabetes may increase malformation via alteration of embryonic gene expression (12). Although embryos of diabetic mothers downregulate *Wnt-3a* in the tail bud more rapidly in response to RA than embryos of nondiabetic mothers, the two embryo types do not differ in their *Wnt-3a* expression in the absence of RA. This suggests that the diabetic milieu may act not directly on *Wnt-3a* but indirectly by altering RA signaling, which plays an important role during the embryogenesis of many developing organ systems (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 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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