Diabetic Environment and Genetic Predisposition as Causes of Congenital Malformations in NOD Mouse Embryos

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Congenital malformations such as neural tube defects and a kinky or waved vertebral column were observed at higher incidence in embryos from nonobese diabetic (NOD) female mice with overt diabetes (NOD-D; 40.3%, $P < 0.005$) or without overt diabetes (NOD-N; 8.4%, $P < 0.05$) than in control Institute of Cancer Research (ICR) mouse embryos (1%) at day 13 of gestation. In vivo and in vitro preimplantation development of NOD-N, NOD-D, and ICR embryos did not differ in rate of development, size, or morphology. Embryos cultured from one-cell to early blastocyst stage were mutually transferred to uterine horns of pseudopregnant females between NOD-D and ICR mice and examined at day 13 of gestation. There were significant decreases in ratios of implantation and of viable embryos in ICR embryos transferred to NOD-D recipients (52%, $P < 0.001$ and 14%, $P < 0.001$, respectively) compared with those in ICR embryos transferred to ICR uteri (79.2 and 56.2%) or those in NOD-D embryos transferred to ICR uteri (70.3 and 33.1%). Furthermore, 18 of 45 viable ICR embryos transferred to NOD-D dams had malformations, whereas there were no malformations in 73 viable ICR embryos transferred to ICR recipients, suggesting deleterious effects of maternal diabetic environment to embryos. On the other hand, 8 of 58 viable NOD-D embryos that were cultured in vitro and transferred to ICR uteri had malformations such as neural tube defects. This study suggests that both the presence of diabetes in the mother and a distinct genetic predisposition in embryos are involved as causes of the high incidence of congenital malformations in NOD-D embryos. Diabetes 40:1245–50, 1991

Congenital malformation occurs at a three- to fourfold higher incidence in infants of diabetic mothers than in the general population of neonates and is the single most common cause of perinatal death in infants of diabetic mothers (1–3). The presence of metabolic disorders such as hyperglycemia (4,5), hypoglycemia (6,7), and hyperketonemia (8,9) and genetic predisposition (10–12) have been suggested as causative factors. However, no single mechanism has been revealed as being the most important, and relationships among the causative factors have not been elucidated (12,13) because there has been no satisfactory method to investigate these factors together in a single system.

We found high incidence of congenital malformations such as neural tube defects in nonobese diabetic (NOD) mouse embryos from mothers both with overt diabetes (NOD-D) and without overt diabetes (NOD-N). With culture of preimplantation-period embryos and mutual intrauterine embryo transplantation between NOD and Institute of Cancer Research (ICR) mice, we analyzed diabetic environment and genetic predisposition as causes of congenital malformations in NOD embryos.

RESEARCH DESIGN AND METHODS

NOD mice used in this study were from Shionogi (Osaka, Japan) and were maintained in the Institute of Experimental Animals (Shimane Medical Univ.). Animals were bred in a room maintained at 24 ± 2°C with 60–70% relative humidity and illuminated by artificial light from 0800 to 2000. NOD-D and NOD-N mice were diagnosed weekly by urinalysis with Glucose-Pretest (Wako, Osaka, Japan) from 10 wk of age. ICR (Sic [Shizuoka Laboratory Animal Center, Shizuoka, Japan]:ICR) mice were purchased and, after at least 2 wk of breeding under experimental conditions described above, used as controls, because NOD mice were inbred from ICR mice (14). We could not use Jcl (Japan Clea, Tokyo):ICR mice, from which the NOD mice were derived, because the supply ceased during our study. Incidences of spontaneous

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CONGENITAL MALFORMATIONS IN NOD MOUSE EMBRYOS

External malformations were not significantly different between Jcl:ICR and Sic:ICR mice (15). All groups were age matched from 10 to 20 wk of age. Because NOD-D mice did not ovulate efficiently for the experimental schedule, female mice were hormonally superovulated to obtain fertilized eggs, except a group of ICR mice that was naturally mated as a control for the teratogenic effect of superovulation. Five units of pregnant mare serum gonadotropin (PMS; Serotropin, Teikoku Zoki, Tokyo) was injected intraperitoneally at 1700 3 days before embryo collection. After 48 h, 5 U human chorionic gonadotropin (HCG; Gonatropin, Teikoku Zoki) was injected similarly, and one female mouse was mated with one male. The next morning, vaginal plug was confirmed, and this day was defined as day 0 of gestation. Only 12- to 40-wk-old NOD-N male mice were used for mating with NOD-D or NOD-N female mice.

Because NOD-D females showed high incidence of absorbed embryos at term in preliminary studies, incidence of spontaneous external anomalies was examined at day 13 of gestation, when major organogenesis was almost completed. Dams were killed by cervical dislocation. After counting the number of implantations, embryos were carefully dissected out. Viability and external anomalies of embryos were observed under a dissecting microscope. The same observations were also performed with embryos that were cultured and transferred (see below).

Ratios of implantation, viable embryos, and incidences of external anomalies were analyzed by Fisher's exact test among NOD-N, NOD-D, and ICR and between in vivo-developed and culture-transferred mouse embryos.

For analysis of in vivo development, preimplantation-stage embryos were collected under a dissecting microscope by flushing the oviducts and the uterine horns with modified Whitten's (MW) medium at indicated hours after HCG injection (16). For observation of in vitro development of embryos, from 1100 to 1400 on day 0, one-cell-stage eggs were collected in MW medium by flushing the ampullae of oviducts with MW medium under a dissecting microscope. Cumulus cells were removed with MW medium supplemented with 460 U/ml bovine testis hyaluronidase (Sigma, St. Louis, MO). After washing in the same medium without hyaluronidase, embryos were cultured in MW medium in an atmosphere of 5% CO2/5% O2/90% N2 at 37°C until observation time (16). Developmental stage of each embryo was determined according to its appearance (17) with a light microscope as follows: stage 6, morula; stage 7, early blastocysts with initial, small blastocoele and persistent polar body; stage 8, blastocysts showing inner cell mass and large blastocoele; stage 9, expanding blastocysts with enlarging blastocoele filling and stretching the zona pellucida; stage 10, hatching blastocysts; stages 11-12, blastocysts that have growing trophoblasts. Means ± SD of the stage of embryos were calculated at indicated time points after injection of HCG.

Some of the cultured NOD-D or ICR embryos, which developed to morula or early blastocyst stage at day 4, were transferred to uterus of pseudopregnant NOD-D or ICR female mice. Pseudopregnant female NOD-D or ICR mice were prepared by mating with vasectomized NOD-N or ICR male mice, respectively. Vaginal plugs were confirmed at day 0, and on day 2, pseudopregnant mice were used as recipients. Hereafter, development of embryos was dated according to recipient's gestational day, because there is a lag during which an embryo adjusts to the uterus (16). Recipients were anesthetized by 70 mg/kg i.p. pentobarbital sodium (Abbott, North Chicago, IL). Embryos were introduced into uterine horns with a glass pipette (16).

Appearance of preimplantation-stage embryos in vivo or in vitro in the three groups was carefully compared with an Olympus IMT-2 light microscope equipped with phase-contrast and interference apparatuses. Furthermore, some of these morula- or blastocyst-stage embryos (>20 embryos for each condition) were observed by scanning electron microscopy (SEM) to examine surface morphology. After removal of the zona pellucida through a short incubation with 0.5% pronase (Kaken Seiyaku, Tokyo) in Hank's balanced salt solution (Gibco, Paisley, UK), embryos were washed in MW medium and transferred into a mixture of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. After >1 day of fixation, embryos were washed in buffer and attached to cover glass coated with poly-L-lysine (Sigma). These specimens were postfixed with 1% OsO4 and stained with 2% tannic acid followed by 1% OsO4. After dehydration, cover glasses with embryos were mounted on an aluminum stub, coated with gold, and observed with a Hitachi S-450 SEM at 5 kV. Several day-13 embryos with external anomalies were also prepared for SEM observation basically with the same procedures and observed at 20 kV.

RESULTS

Incidence of spontaneous external malformations in NOD-N, NOD-D, and ICR mice was examined at day 13 of gestation, when major organogenesis was almost complete (Table 1). In viable embryos, malformations such as exencephaly (Fig. 1), spina bifida, and a kinky or waved vertebral column occurred at significantly higher incidence in both NOD-D (40.3%, P < 0.005) and NOD-N (8.4%, P < 0.05) than in ICR (1%) mice. This incidence was significantly higher in NOD-D than in NOD-N (P < 0.005) mice. Also, higher incidences of intrauterine death by day 13 of gestation were noted in both NOD-D (51.3%, P < 0.005) and NOD-N (39.7%, P < 0.005) than in ICR (5%) mice. Although intrauterine death was more frequent in NOD-D than in NOD-N mice, the difference was not significant. There was no statistically significant difference in the incidences of external anomalies and intrauterine death between superovulated and naturally mated ICR mice.

Preimplantation development of NOD-N, NOD-D, and ICR embryos both in vivo (Fig. 2A) and in vitro (Fig. 2B) was observed. In vivo, embryos of the three groups developed from early blastocysts to expanded blastocystst at day 3 (88-96 h after injection of HCG). There was no delay in development or external abnormalities in NOD-D embryos compared with findings in the two other groups (Figs. 2A and 3). In vitro, >70% of embryos in the three groups cultured from one-cell stage developed to blastocyst stage at day 4 (88 and 112 h after injection of HCG, respectively). There was no significant difference in rate of development (Fig. 2B), size, or appearance, including surface morphology, among the three groups (data not shown).

Part of day 4 embryos at morula or early blastocyst stage, which developed in vitro from the one-cell stage, were mutually transplanted into the uteri of pseudopregnant female
TABLE 1
Incidence of spontaneous anomalies in mice at day 13 of gestation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Superovulation</th>
<th>Dams</th>
<th>Implant Early</th>
<th>Late</th>
<th>Viable</th>
<th>Exencephaly</th>
<th>Spina bilida</th>
<th>Kinky vertebral column</th>
<th>Other anomalies</th>
<th>Total anomalies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic NOD</td>
<td>+</td>
<td>6</td>
<td>78</td>
<td>26</td>
<td>14 (51.3)</td>
<td>38</td>
<td>11</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Non diabetic NOD</td>
<td>+</td>
<td>12</td>
<td>126</td>
<td>41</td>
<td>9 (39.7)</td>
<td>76</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>ICR</td>
<td>+</td>
<td>5</td>
<td>100</td>
<td>3</td>
<td>2 (5.0)</td>
<td>95</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ICR</td>
<td>-</td>
<td>10</td>
<td>143</td>
<td>7</td>
<td>1 (5.6)</td>
<td>135</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are given as n with percentages in parentheses. Died late, embryos with an appearance similar to viable embryos but without detectable heart beats; other, 2 cases of extreme delay in development with gross anomaly, 1 of pathological umbilical herniation in diabetic NOD, and 1 of extreme delay in development with pharyngeal arch anomaly in nondiabetic NOD; ICR, Institute of Cancer Research.

*P < 0.005 vs. nondiabetic NOD and superovulated ICR.
†P < 0.05 vs. superovulated ICR.

mice between NOD-D and ICR mice (Table 2). The incidence of external anomalies was examined on day 13 of gestation. Of 175 NOD-D morulae and blastocysts transferred to the uteri of ICR females, 58 were viable, and 8 had external anomalies, including exencephaly and a kinky or waved vertebral column. Of 321 ICR embryos transferred to NOD-D dams, 45 were viable, and 18 had external anomalies, 16 of which were neural tube defects. No external malformations were observed in 73 viable ICR embryos of 130 transferred to the uteri of different ICR female mice. There were significant decreases in ratios of implantation and viability of ICR embryos transferred to the uteri of NOD-D female mice (52 and 14%, respectively) compared with those ratios in NOD-D embryos transferred to ICR uteri (70.3%, P < 0.001 and 33.1%, P < 0.001, respectively) or in ICR embryos trans-
FIG 3. Scanning electron microscope view of diabetic NOD (A; x 593), Institute of Cancer Research (B; x 552), and nondiabetic NOD (C; x 780) mouse embryos that developed in vivo 96 h after human chorionic gonadotropin injection. Size and surface morphology of trophoblastic cells are indistinguishable among 3 groups.
which developed in vitro, showed no significant difference found in fetuses might result from deleterious effects of the complex that include MHC may be involved H-2 genes in the formed offspring of diabetic mice (19,20; R.T., H.O., O.T., of unique I-ANOD in the class II MHC (22; for review, see ref. 23). On the other hand, it has been suggested that several transition. Susceptibility to insulin-dependent diabetes in NOD is polygenetic, including the absence of I-E and the presence of I-E-8 (13,8)*; transfer experiments with NOD-N embryos and NOD-N recipients had external anomalies (Table 2). In contrast, no anomalies were found in ICR embryos transferred to ICR recipients (Table 2). These findings suggest that diabetic environment also had a deleterious effect on morphogenesis of embryos.

In embryo-transfer experiments, ratios of implantation and viability of embryos were significantly lower when NOD-D dams were recipients than with ICR recipients (P < 0.001). Thus, the diabetic environment had an untoward effect on both implantation and general postimplantation development of embryos. Incidence of external anomalies of NOD-D embryos was significantly lower when embryos were transferred to ICR uteri (Table 2) than the incidence of spontaneous anomalies in NOD-D (P < 0.005; Table 1). Furthermore, 18 of 45 viable ICR embryos transferred to NOD-D recipients had external anomalies (Table 2). In contrast, no anomalies were found in ICR embryos transferred to ICR recipients (Table 2). These findings suggest that diabetic environment also had a deleterious effect on morphogenesis of embryos.

On the other hand, 8 of 58 viable NOD-D embryos, which were cultured from one-cell to blastocyst stage and transferred to the uteri of ICR females, had neural tube defects. In contrast, there were no anomalies in viable ICR embryos that were similarly cultured and transferred to ICR uteri (Table 2). Thus, there might be a genetic predisposition for external anomalies in NOD embryos. Several studies have shown an increased frequency of chromosomal aberrations in malformed offspring of diabetic mice (19,20; R.T., H.O., O.T., H.N., S. Ando, unpublished observations). However, in this study, chromosome analysis in preimplantation embryos, which developed in vitro, showed no significant difference in incidence of chromosomal abnormalities among NOD-N, NOD-D, and ICR mice (21). Chromosomal abnormalities found in fetuses might result from deleterious effects of the diabetic environment (19,20) rather than genetic predisposition. Susceptibility to insulin-dependent diabetes in NOD is polygenetic, including the absence of I-E and the presence of unique I-A\(^{00}\) in the class II MHC (22; for review, see ref. 23). On the other hand, it has been suggested that several genes in the H-2 complex that include MHC may be involved in formation (24) or regulation of incidence (25,26) of congenital anomalies. Thus, it remains to be investigated, as a possible genetic mechanism, whether characteristics of NOD mice in the H-2 complex, which underlie the autoimmune, might also be directly or indirectly related to the high incidence of congenital malformations.

Although preimplantation development of NOD-N, NOD-D, and ICR embryos in vivo and in vitro was not significantly different, defective development or maturation of the eggs before ovulation remains to be examined further as a cause of teratogenesis in NOD embryos. Histopathological study of the reproductive organs suggests that there may be a disorder in oocyte maturation in NOD mice (27). Therefore, it cannot be denied that reproductive traits of NOD mothers, including structural and metabolic or immunological conditions of the reproductive tract, beside diabetes may affect development of embryos (27). NOD-N mice might have a different genetic composition from NOD-D in terms of incidence of congenital anomalies. Embryo-transfer experiments with NOD-N embryos and NOD-N recipients have been undertaken to examine this possibility.

In humans, neural tube defects are one of the most common abnormalities in infants of diabetic mothers, although the incidence appears to be lower than in NOD mice (22,23). Therefore, our findings in NOD mice could possibly be extrapolated to human insulin-dependent diabetic mothers. Efforts should be made to normalize the diabetic environment before and during pregnancy, as indicated in clinical reports (24) and because the incidence of anomalies in our study decreased to the minimal level when genetically predisposed NOD-D embryos were transferred to nondiabetic ICR uteri. On the other hand, possible genetic predispositions must be extensively investigated and carefully evaluated.

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