Decreased Inner Cell Mass Proportion in Blastocysts From Diabetic Rats

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Late morulae and blastocysts were recovered from streptozocin-induced diabetic pregnant rats and individually examined for numbers of inner cell mass (ICM) cells and trophectoderm (TE) cells. Compared with embryos collected from control rats, exposure to maternal diabetes significantly decreased mean ICM cell number of blastocysts recovered on day 5 of gestation, but the TE population of these embryos remained unaffected. The mean ICM proportion was therefore significantly lower than that of control embryos. These differences were not observed between the two groups of morulae collected on day 5, suggesting that the distinctive susceptibility of the ICM was expressed after blastocyst formation. On day 6, a significant inhibitory effect of diabetes was observed on the growth of both ICM and TE cells, but because the reduction was more severe in the ICM than in the TE, the mean ICM proportion of these blastocysts was again significantly lower than in control embryos. A linear quadratic relationship was obtained between the numbers of ICM cells of individual blastocysts and their respective numbers of TE cells in each of the two experimental groups. However, the slope of the curve was slower in the diabetic group than the control group. The disturbed ICM cell growth in the blastocysts from diabetic rats was found to be associated with a significantly increased incidence of cell death predominantly located in the ICM. Because it is known that excessive reduction of the ICM is incompatible with normal embryogenesis after implantation, our results suggest that the differential sensitivity of ICM and TE cells in preimplantation blastocysts may contribute to the pattern of postimplantation defects described in diabetic pregnancies. *Diabetes* 39:471–76, 1990

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Since the introduction of tight metabolic control, the successful pregnancy rate has considerably increased in diabetic women. However, early fetal losses and major malformations still occur at significantly higher rates than in the nondiabetic population (1,2). The precise mechanism leading to diabetic embryopathy remains unclear. Numerous animal studies led to the recognition that several factors were implicated in the increased incidence of embryo resorptions and malformations resulting from poor metabolic control during early organogenesis (3,4), and at least three factors (hyperglycemia, hyperketonemia, and increased levels of somatomedin inhibitors) were identified as teratogenic (5). Despite indications that the embryo is also sensitive to the induction of teratogenic effects before its implantation (6), little attention has been paid to the preimplantation period of diabetic pregnancy. It was reported that moderate concentrations of glucose, β-hydroxybutyrate, acetoacetate, and other components inhibited the development of early mouse blastocysts in vitro (7,8), and a similar effect was obtained when the culture medium was supplemented with serum from diabetic patients (9). Other studies have shown that maternal diabetes might affect the in vitro development of two-cell mouse embryos collected on day 2 (10) or the in vivo implantation rate in the rat (11). Finally, our group recently observed that the mean cell number of blastocysts recovered from diabetic rats on day 5 of gestation was significantly decreased compared with control embryos (12).

This study was designed to investigate whether the latter effect was the manifestation of a simple delay or rather reflected a more profound functional disorder induced in the embryo before implantation. For this purpose, late morulae and blastocysts were recovered from streptozocin-induced diabetic rats on days 5 and 6 of gestation and examined after differential staining (13) for their number of inner cell mass (ICM) and trophectoderm (TE) cells. The embryo differentiates into these two cell types at the time of morula-

blastocyst transition, and after implantation, the ICM cells give rise to the germ layers of the embryo and most cell types of the extraembryonic membranes (14). The underlying reasons for our study were the differential sensitivity of the ICM and TE cells previously reported for various cytotoxic agents (6) and the finding that excessive ICM cell loss is incompatible with normal organogenesis despite normal implantation (15).

RESEARCH DESIGN AND METHODS

Adult female Wistar rats (70–90 days old) were rendered diabetic by a single injection of streptozocin (Zanosar, Upjohn, Kalamazoo, MI) at a dose of 50 mg/kg body wt i.v. after an overnight fast (11), and their glycosuria was measured semiquantitatively the next day with Tes-Tape strips (M-73, range 0.1–2.0%; Lilly, Indianapolis, IN). Diabetic females (glycosuria >2%) were mated overnight with males on day 7, 14, or 21 after streptozocin treatment. The presence of a vaginal plug was designated as day 1 of gestation. Animals were killed in the morning (1000–1200) of day 5 or 6 of gestation after determination of their blood glucose level with a Glucoscot photometer (GT-4310, Daiichi Kagaku, Kyoto, Japan; range 1.1–33 mM). Females with blood glucose levels <11 mM were discarded. Embryos were gently flushed from the excised oviducts and uteri with prewarmed Ham's F-10 medium (041-01550, Gibco, Grand Island, NY) supplemented with 14.7 mg/L L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin and immediately observed on an inverted phase-contrast microscope. Morphological categories were set as follows: degenerated embryos with cytoplasmic fragmentation, morulae, early blastocysts with a nascent blastocoel, expanded blastocysts with a clear demarcated TE and ICM, hatched blastocysts, and implanting blastocysts with a collapsed blastocoel and/or a damaged TE. On day 6, ~1 ml of a 0.5% solution of Evans blue in 0.9% NaCl was injected intravenously 10 min before death, and implantation sites (small blue spots) were scored before embryo flushing (11).

Differential cell staining. The numbers of TE, ICM, and total cells of individual morulae and blastocysts collected on days 5 and 6 were counted by the differential-labeling technique previously described for mouse embryos (13) and adapted for rat embryos (16). Briefly, zonae pellucidae were removed by a 10- to 15-min incubation in 0.4% pronase in Earle's balanced saline solution (EBSS; 041-04010, Gibco) supplemented with 11 mg/L sodium pyruvate and 146 mg/L L-glutamine, and the denuded embryos were then incubated for 30 min in 10% heat-inactivated rabbit anti-rat antiserum in EBSS. After rapid washing, the embryos were incubated for 30 min in 5% guinea pig serum (used as source of complement) in EBSS containing 20 μg/ml bisbenzimide and 10 μg/ml propidium iodide and examined under a fluorescence microscope fitted for UV excitation. This treatment resulted in the selective permeabilization of the TE cells to both bisbenzimide and propidium iodide, whereas ICM cells were only stained with bisbenzimide. Under these conditions, ICM nuclei appear blue, and TE nuclei appear weakly red. For convenience, the term *ICM* was also used to designate inner cells that stained blue in morulae, although the blastocoel is not yet formed in these embryos. The ICM proportion was the ratio between the ICM cell number and the total cell number × 100 in each embryo. Cells containing scattered nuclear fragments of various sizes were considered to be dead cells and allocated to the TE or ICM according to their labeling (17). The dead-cell index was calculated separately in the TE and ICM of individual embryos. This value corresponded to the ratio between the number of dead cells and the total number of cells in the TE or ICM.

TE permeability barrier. Because our method of differential staining was based on the protection of the ICM against the antiserum-complement lysis by the TE permeability barrier (18), the possibility that maternal diabetes could have altered this barrier was tested by immunofluorescence (16). Eleven and nine ICMs of blastocysts recovered on day 5 from diabetic and control rats, respectively, were completely isolated by antiserum-complement TE lysis and exposed to fluorescein-conjugated goat anti-rabbit antiserum. They were found to be free of any labeling under blue excitation. In contrast, ICMs isolated similarly from seven blastocysts of diabetic rats and eight blastocysts of control rats and reincubated with rabbit anti-rat antibodies and fluorescein conjugates before examination were intensively labeled. These observations confirm the absence of any anti-rat antibodies on the ICM of blastocysts from diabetic and control rats and thus exclude the possibility that ICM cells of blastocysts could have been unselectively lysed by the antiserum-complement treatment.

Statistics. Developmental patterns were compared by the χ^2-test. Mean numbers of TE, ICM, or total cells were analyzed by the two-tailed unpaired Student's *t* test. Mean dead-cell indices were compared by the Mann-Whitney *U* test. Correlations were examined by least-squares regression analysis. Slopes of the regressions were compared as described elsewhere (19). Calculations were performed with a StatView 521+ program (Brain Power).

RESULTS

Embryo collection. Blood glucose level on day 5 was 23.0 ± 6.3 mM in diabetic rats and 5.10 ± 0.15 mM in control rats. At that time, the number of embryos retrieved from diabetic rats was significantly lower than control rats (7.78 ± 2.65 vs. 9.37 ± 2.09/rat, $P < 0.01$). Embryos recovered from diabetic rats presented significantly delayed morphological development ($P < 0.001$), as shown by the lower proportion of expanded blastocysts (40 vs. 74% in control rats; Table 1). Retarded embryos were either morulae and early blastocysts with a normal corresponding morphology or degenerated structures. On day 6, blood glucose level was 25.0 ± 6.0 mM in diabetic rats and 5.70 ± 0.13 mM in control rats. At that time, the mean number of embryos recovered by flushing from both control and diabetic females was lower than on day 5, suggesting that implantation had begun. The mean value found in diabetic rats (4.21 ± 2.30/rat) was not significantly different from that in control rats (3.87 ± 1.36/rat), but the morphological distribution was again significantly shifted to the less-developed stages ($P < 0.001$; Table 1). There was also a significant decrease in the mean number of implantation sites in diabetic rats on day 6 (8.27 ± 3.38 vs. 11.00 ± 1.35 for control rats, $P < 0.05$).

Cell number. No significant effect of maternal diabetes on the TE and ICM cell numbers in morulae collected on day

TABLE 1
Number and developmental stage of embryos from control and diabetic rats on days 5 and 6 of gestation

	Females	Embryos	Degenerated embryos	Morulae	Blastocysts			
					Early	Expanded	Hatched	Implanting
Day 5								
Control	24	225	3	19	36	167	0	0
Diabetic	23	179	16	33	58	72	0	0
Day 6								
Control	24	93	2	0	0	4	61	26
Diabetic	19	76	6	6	0	25	28	11

5 was found, although the proportion of morulae with a detectable ICM was slightly but not significantly lower in diabetic compared with control rats (69 vs. 79%; Table 2). The ICM proportion was similar in the two groups of morulae with ICM (Fig. 1).

In contrast, the mean ICM cell number was significantly decreased by 23% in blastocysts recovered from diabetic rats on day 5 compared with control blastocysts (Table 2). However, the mean TE cell number was not significantly different between the two blastocyst groups; the significantly decreased mean total cell number thus resulted from a preferential effect of maternal diabetes on the ICMs (Table 2). The ICM proportion was significantly lower in blastocysts from diabetic rats (27.20 ± 6.14 vs. 31.60 ± 5.16% in control rats, $P < 0.001$; Fig. 1). Approximately 10% of the blastocysts collected from diabetic rats on day 5 contained <6 ICM cells despite normal morphology, and these embryos were evenly distributed among the diabetic group. Only 1% of the control blastocysts contained <6 ICM cells. No significant relationship was found between the ICM cell number of each blastocyst and the glycemia of the corresponding diabetic female on day 5.

A significant decrease in both ICM and TE cell numbers was observed in the blastocysts recovered from diabetic rats on day 6 (Table 2). Because the reduction in cell number was greater in the ICM than the TE, the ICM proportion was significantly lower in blastocysts from diabetic rats (24.00 ± 5.43 vs. 26.30 ± 3.67% in control rats, $P < 0.05$; Fig. 1).

When all blastocysts collected on days 5 and 6 were analyzed together, the curve of best fit for the relationship between the numbers of ICM and TE cells of each embryo was linear quadratic for the embryos from both groups (Fig. 2). However, the curves were significantly different ($P < 0.01$), indicating that the differential cell growth of ICM and TE cells was significantly disturbed in the diabetic group. It was not possible to estimate the ICM proportion in implanting blastocysts because of the damaged TE, but there was a significant difference in the mean number of ICM cells in the diabetic and control groups (Table 2).

Cell death. Compared with control embryos, fragmented nuclei were observed more frequently in the ICM cells of blastocysts but not of morulae collected from diabetic rats on days 5 and 6 (Fig. 3). In contrast, fragmented nuclei were rarely observed in the TE cells of morulae and blastocysts regardless of the group or day of examination.

TABLE 2
Inner cell mass (ICM), trophectoderm (TE), and total cell number in embryos recovered from control and diabetic rats on days 5 and 6 of gestation

	Control	Diabetic
Day 5		
Morulae without ICM		
n	4	7
Total	17.00 ± 1.83	15.29 ± 3.45
Morulae with ICM		
n	15	16
ICM	5.07 ± 2.09	5.56 ± 1.83
TE	14.33 ± 2.50	15.81 ± 3.65
Total	19.40 ± 3.54	21.37 ± 3.99
Blastocysts		
n	87	74
ICM	10.01 ± 2.67	7.73 ± 2.38*
TE	21.58 ± 4.45	20.49 ± 4.14
Total	31.59 ± 6.30	28.22 ± 5.77*
Day 6		
Blastocysts		
n	35	31
ICM	14.89 ± 2.58	11.90 ± 3.19*
TE	42.11 ± 8.06	38.19 ± 7.76†
Total	57.00 ± 9.62	50.09 ± 11.21‡
Implanting blastocysts		
n	15	9
ICM	29.08 ± 5.87	20.33 ± 5.03*

Values are means ± SD where indicated.
*$P < 0.001$, †$P < 0.05$, ‡$P < 0.01$, vs. control rats.

DISCUSSION

It has recently been demonstrated that the average cell number of blastocysts recovered from streptozocin-induced di-

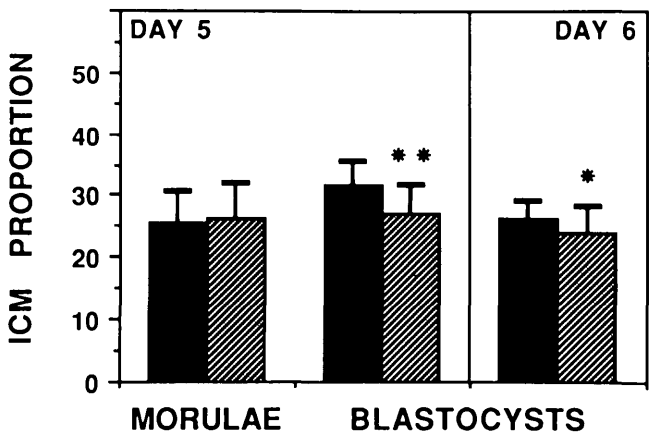

FIG. 1. Proportion of inner cell mass (ICM) cells in morulae and blastocysts (means ± SD) recovered from control (*solid bars*) and diabetic (*hatched bars*) rats on day 5 or 6 of gestation. Embryos without ICM were omitted. *$P < 0.05$; **$P < 0.001$.

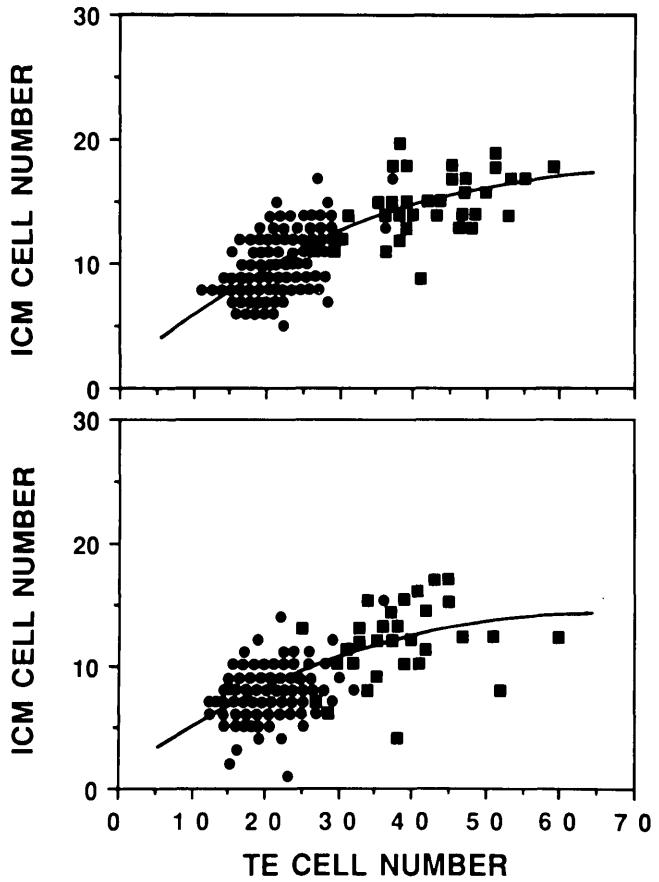

FIG. 2. Linear quadratic relationship between number of trophectoderm (TE) and inner cell mass (ICM) cells in 122 blastocysts recovered from control rats (*top*) and 105 blastocysts recovered from diabetic rats (*bottom*) on days 5 (●) and 6 (■) of gestation. For control rats, equation of best fit is $y = 1.4 + 0.48x - 0.0037x^2$; for diabetic rats, this equation is $y = 1.1 + 0.41x - 0.0033x^2$.

abetic rats on day 5 of gestation is significantly decreased compared with controls (12). Our work was designed to extend this earlier observation by means of a differential-staining technique that allows separate counting of TE and ICM cells in individual embryos (13). With this method, we observed that the deleterious effect of the maternal diabetic state was selective on the ICM cells when blastocysts were examined on day 5 of gestation. In these embryos, the average ICM contained only 77% of the normal cell number, whereas the TE cell population was normal. Examination of blastocysts on day 6 showed that growth of both the ICM and TE was inhibited by maternal diabetes, but the percentage of reduction was less for TE than ICM cells. As far as we know, this is the first study in which a disturbed maternal metabolic condition was found to induce a selective effect on a specific type of cell in preimplantation embryos.

However, evidence of a higher sensitivity of the ICM cells has been described for a large variety of physical and chemical agents tested in vitro. For instance, exposure of mouse blastocysts to [^{3}H]thymidine (20) or X rays (21) or incubation with inhibitors of proteins or RNA synthesis (22,23) or other antimetabolites (24–26) induces a significant inhibitory effect on ICM cell growth without affecting hatching, attachment, and subsequent trophoblast outgrowth of the embryo in vitro. Depletion of metabolic factors like serum (27) or amino acids (28) from the culture medium is also found to specifically suppress ICM development at the blastocyst stage. Treatment of embryos before the blastocyst stage with [^{3}H]thymidine (29), X rays (21), cyclophosphamide (30), or mitomycin C (15) also preferentially impairs ICM formation, but other antimetabolites equally inhibit the proliferation of both TE and ICM cells (26). In our study, the ICM proportion in late morulae harvested from diabetic rats on day 5 was similar to the control value. This suggests that the embryotoxic factors associated with maternal diabetes were not effective on ICM cells before blastocyst formation. This morphological event correlates with the onset of divergent gene expression of the ICM and TE (31). The statistical analysis of the relationship between ICM and TE cells clearly indicated that, in addition to a delay in morphological development and a reduction of the mean total cell number, maternal diabetes induced a functional change in the balance between ICM and TE cells. The linear quadratic equation obtained for the control blastocysts confirmed the slowing down of ICM growth compared with TE growth as the blastocyst normally expands (16). In blastocysts from diabetic rats, the same type of mathematical relationship was observed, but the ICM cell number was significantly lower than normal throughout the process of blastocyst expansion. Previous studies demonstrated that the development of the blastocyst depends on adequate interactions between the ICM and TE cells. ICM cells might migrate into the overlying polar TE when the blastocyst expands (32–35) or influence the growth of the TE without direct cellular contribution (20, 36–38).

The high proliferation rate (39,40) and the active metabolic status (25,41) needed in the ICM to support its own growth and control TE development probably render the ICM cells more sensitive. Our findings suggest that the selective inhibition of the ICM is mediated by cell death, because the increased frequency of dead cells detected in the blastocysts from diabetic rats on days 5 and 6 was restricted to the ICM. Although dead-cell indexes differ among species during blastocyst expansion (16,17,36,42,43), cell death is a regular mechanism of normal development (44), and it is therefore not excluded that the maternal diabetic state could

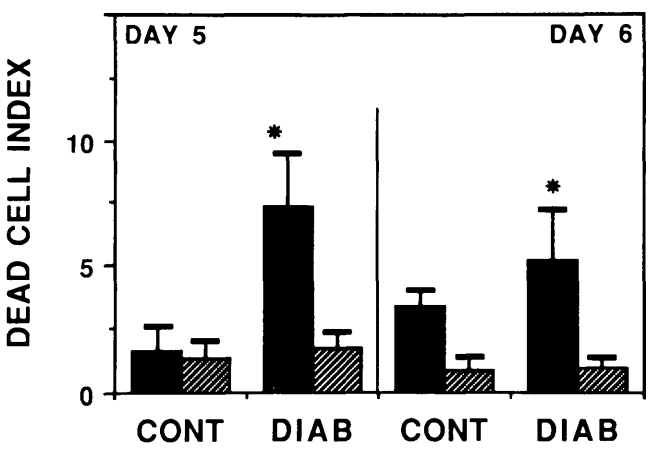

FIG. 3. Percentage of dead cells in inner cell mass (*solid bars*) and trophectoderm (*hatched bars*) of blastocysts (means ± SE) recovered from control (cont) and diabetic (diab) rats on day 5 or 6 of gestation. *$P < 0.05$.

exacerbate this normal feature and lead to a depletion of ICM cells.

Our results also suggest that blastocysts with reduced ICMs did not delay their implantation until optimum ICM cell number was obtained, because implanting blastocysts also had a significantly lower mean ICM cell number in diabetic females. This is in agreement with the belief that the role of the ICM in inducing a decidual reaction and in controlling implantation is minimal (38). However, immediate postimplantation development seems strongly dependent on a sufficient number of ICM cells (15,38,45). Therefore, we assume that the reduced implantation rate observed here and in a previous study (11) was not directly related to the diabetes-induced reduction of the ICM in the blastocysts. The decreased implantation rate could instead be explained by the presence of a lower number of developing embryos in the uterine horns, as reported in this study in agreement with other studies (46,47). The cause of this decrease is not clear because previous experiments have shown that the number of corpora lutea (our unpublished experiments) and the mean number of oocytes shed at ovulation are similar in diabetic and control rats (48,49). On the other hand, delays in oocyte maturation and reduced proportions of dividing zygotes have been reported in superovulating diabetic mice (10). The exact contribution of decreased oocyte fertilizability or viability to the decreased rate of blastocyst collection remains to be investigated.

Because derivatives of the ICM proliferate extensively after implantation and give rise to all germ layers of the embryo, it is possible that the ICM reduction observed here could be an underlying cause of the increased incidence of embryo resorptions and malformations described in diabetic rats (50–53). It is indeed believed that normal development depends on the correct balance of cell proliferation, migration, and differentiation (54). An interference affecting the relationship between ICM and TE cells could thus result in the complete disorganization of the embryo or initiate a mechanism of malformation. Alternatively, features like resorptions and malformations could be more likely to occur when the ICM of the embryo has already been damaged before its implantation. Note in this context that both elevated incidence of cell pyknosis and imbalanced cell proliferation in the neuroepithelium of early postimplantation embryos have been linked with the occurrence of neural tube defects (55,56).

A final point of interest deals with the blood glucose level at which the diabetic state would harm the preimplanted embryo development. Hence, lower streptozocin doses (35 mg/kg) were shown to produce a similar morphological development delay on day 5, as observed with 50 mg/kg of streptozocin (57). The number of nuclei per blastocyst was also reduced despite a twofold lesser increase in blood glucose levels (57). In a few such blastocysts, the ICM proportion was also reduced (unpublished observations). Non-diabetogenic doses of streptozocin (25 mg/kg) were without effect on embryo development, emphasizing the specificity of the diabetes-induced development impairment and the high sensitivity of the preimplanted embryo to the maternal diabetic state (57).

In conclusion, our study revealed the different sensitivity of rat blastocyst ICM and TE cells to maternal diabetes, because the inhibitory effect of this metabolic state was much more severe on ICM growth than TE development. Our data thus demonstrate that diabetes manifested its embryotoxic effects during the preimplantation period and that the disorder did not proceed from a simple developmental delay. These findings strongly underline the importance of early glycemic control in diabetic pregnancies and might thus be relevant to clinical practice.

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