Maternal diabetes alters extracellular matrix protein levels in rat placentas

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OBJECTIVES: The aim of this study was to determine whether maternal diabetes affects placental levels of the extracellular matrix components fibronectin, laminin, and collagen-IV.

STUDY DESIGN: Fibronectin, laminin, and collagen-IV deposition in term (day 20) rat placentas from normal and diabetic pregnancies was detected by use of Western blot, slot-blot, and immunohistochemical studies.

RESULTS: Increased placental and decreased fetal wet weight were found in offspring of manifestly diabetic rats compared with offspring of normal pregnancies. Laminin deposition was reduced whereas fibronectin levels were increased in placentas from diabetic rats. No diabetes-induced changes of collagen-IV expression and deposition were found.

CONCLUSION: The diabetes-induced alterations of laminin and fibronectin protein levels in the fetal-maternal interface may affect placental development and alter gas exchange and nutrient transfer to the offspring. This may in turn contribute to the abnormal fetal development in diabetic pregnancy. (Am J Obstet Gynecol 1998;179:772-8.)

Key words: Diabetes in pregnancy, rat, placenta, fetus, fibronectin, collagen-IV

Pregnancy in diabetic women is associated with increased fetal morbidity and mortality.[1, 2] Gestational complications are increased in diabetes despite near-normoglycemia and include macrosomia, intrauterine growth retardation, and a variety of malformations.[3] These disturbances may in part be related to changes in placental transport of maternal nutrients to the fetus.[4, 5] The notion that impaired placental function is involved in disturbed fetal development is strengthened by reports on morphologic alterations of placental ultrastructure in diabetic pregnancies.[6, 7] The precise molecular background of these findings is still unclear; however, in several other tissues of diabetic individuals changes of extracellular matrix protein expression have been found, suggesting that alterations in extracellular matrix deposition may be a general feature of diabetic complications.[8] Although these alterations in adult tissues are likely to have developed during a longer time period than the changes found in the relatively short-lived placenta, disturbances in placental extracellular matrix deposition in diabetic patients[9] and overexpression of the extracellular matrix constituents laminin-B1 and fibronectin in embryos and fetuses of streptozocin-induced diabetic rats[10] have been reported. Diabetes-induced alterations in extracellular matrix expression in embryonic and fetal tissue are thus also present during gestation. The aim of this study was therefore to investigate whether expression of the extracellular matrix components laminin, fibronectin, and collagen-IV was dysregulated in placentas exposed to maternal diabetes in the rat.

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Material and methods

Animals. Virgin female Sprague-Dawley rats 15 to 20 weeks old from a malformation-prone U substrain[11] were made diabetic by a single intravenous injection of streptozocin (The Upjohn Company, Kalamazoo, Mich) at a dose of 40 mg/kg body weight. One week after the injection, diabetic females with serum glucose concentrations exceeding 20 mmol/L were regarded manifestly diabetic. Noninjected virgin female rats of the same age and weight served as nondiabetic controls. These rats were mated with nondiabetic male U rats overnight, and in the morning a positive vaginal smear was found and that was denoted day 0 of gestation. The manifestly diabetic females that did not become pregnant during 2 weeks of mating attempts were given daily insulin treatment (5 to 10 IU subcutaneously, Novo Ultratard Insulin, Novo Nordisk Pharma AB, Malmö, Sweden); this treatment was interrupted by conception. Fetuses and placentas were harvested from nondiabetic controls or manifestly diabetic rats on gestational day 20, which represents a developmental stage repeatedly studied by our group. The fetal and placental wet weights were determined, and some placentas were selected randomly for immunohis-

tochemical study (1 to 4 per litter) and Western blot and slot-blot analysis (1 to 4 per litter). The sampling of the tissue used in the latter analyses was also performed in a randomized manner (ie, the chosen placenta was minced with scissors and homogenized, and an aliquot of the homogenate was used for the Western/slot-blot analysis).

Western blot analysis. The placentas were cut into 1 mm^3 pieces and extensively rinsed in cold saline solution to remove maternal blood. Tissue from 1 whole placenta per experiment was hand homogenized in 200 μL of 100 mmol/L sodium chloride, 40 mmol/L Tris(hydroxymethyl)-aminomethane (Tris), 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 0.2% Triton X-100, 1 mmol/L phenylmethylsulfonylfluoride, and 1 mmol/L dithiothreitol. The homogenates obtained were centrifuged at 12,000*g* for 2 minutes, and the pellet was dissolved in 200 μL of 100 mmol/L sodium chloride; 10 mmol/L Tris, pH 8.5; 5 mmol/L EDTA; and 0.2% sodium dodecyl sulfate. Aliquots were subjected to monobasic calcium phosphate precipitation followed by protein determination according to the method by Lowry et al.[12]

Ten micrograms of protein was subjected to 5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis to nitrocellulose filters by means of electroblotting (Bio-Rad Laboratories, Richmond, Calif) and subsequently incubated with a polyclonal rabbit antirat laminin (diluted 1:1000) (Chemicon Int, Temecula, Calif) followed by peroxidase-labeled donkey antirabbit immunoglobulin G (1:1000) (Amersham, Buckinghamshire, United Kingdom). The antigen-antibody complex was visualized by chemiluminescence (ECL, Western Blot Detection Reagents, Amersham, Buckinghamshire, United Kingdom) and detected by an X-Omat AR-film (Kodak, Rochester, NY). Densitometric analysis of the autoradiograms was performed with a Quick Scan Jr densitometer (Helena Laboratories, Beaumont, Tex).

Slot-blot analysis. About 35 mg of tissue from 1 placenta per experiment was homogenized in 1 mL of 137 mmol/L sodium chloride, 20 mmol/L Tris (pH 8.5), 5 mmol/L EDTA, 26 mmol/L magnesium chloride, 1 mmol/L phenylmethylsulfonylfluoride, 1 mmol/L dithiothreitol, 25 U/mL benzonase (Merck KGaA, Darmstadt, Germany), and 4 mol/L guanidine thiocyanate. After 40 minutes of incubation at 37°C, aliquots were subjected to protein determination.[12] Amounts of 2 mg, 1 mg, 0.5 mg, and 0.25 mg protein were applied in triplicate to nitrocellulose filters by means of a slot-blot device (Bio-Dot, Bio-Rad Laboratories, Richmond, Calif). Antibodies used were as follows: monoclonal mouse antimouse fibronectin (1:1000) (Sigma-Aldrich, St Louis, Mo), peroxidase-labeled sheep antimouse immunoglobulin G and biotinylated goat antirabbit-C-IV (1:10,000) (Southern Biotechnology Association, Birmingham, Ala). The immobilized polypeptide bands were revealed as described for Western blotting.

Table I. Fetal and placental weights in normal and manifestly diabetic rat pregnancies

	Normal	*Manifestly diabetic*
Fetal weight (g)	3.7 ± 0.2	2.4 ± 0.4*
Placental weight (g)	0.53 ± 0.04	0.64 ± 0.01*

Wet weights of fetuses and placentas from normal ($n = 34$) and manifestly diabetic ($n = 25$) rats are expressed as mean ± SEM.
*$P < .001$, versus corresponding controls.

Immunohistochemical studies. Eight whole rat placentas from day 20 of gestation in normal or diabetic pregnancies were fixed in formalin, sectioned (5 mm), and prepared for immunohistochemical studies by standard procedures. Antibodies used were the following: polyclonal rabbit antihuman fibronectin (1:200), (Sigma-Aldrich), goat antirabbit immunoglobulin G (1:200) (Sigma-Aldrich), monoclonal mouse antimouse laminin (1:200) (Sigma-Aldrich), biotinylated goat antimouse immunoglobulin G (1:200) (Dakopatts, Glostrup, Denmark), and biotinylated goat antirabbit collagen IV (1:100) (Southern Biotechnology Association). For detection of antirabbit collagen IV antibodies avidin and horseradish peroxidase–conjugated biotin (1:10) were used (Vector, Burlingame, Calif). Protein deposits in the tissue specimens were finally revealed by 3-amino-9-ethylcarbazol (Sigma-Aldrich) reaction with peroxidase.

Statistical analysis. Differences between means were evaluated with the aid of the 2-tailed Student *t* test.

Results

Gestational day 20 fetuses of manifestly diabetic rats were significantly growth retarded, with a mean wet weight of 2.4 ± 0.4 g compared with 3.7 ± 0.2 g in fetuses from normal pregnancies ($P < .001$) (Table I). In contrast to the diabetes-induced decrease in fetal wet weight, placentas from diabetic rats displayed augmented wet weights compared with placentas from nondiabetic rats (Table I). This increase did not compensate for the fetal weight loss, yielding an overall conceptus growth reduction of 30%. The general malformation and resorption rates of the manifestly diabetic group were 14% and 38%, respectively.

The placentas showed a marked morphologic individual variability, particularly in the diabetic rats. One morphologic alteration in some of the placentas of the diabetic rats was an increased number of putative glycogen-containing cells, primarily in the basal zone (Fig 1). Immunohistochemical analysis of placentas from normal rats revealed laminin immunoreactivity in basement membranes of the basal zone (not shown) and marked labeling in Reichert's membrane (Fig 2, *A*) and in endometrial remnants surrounding the placental

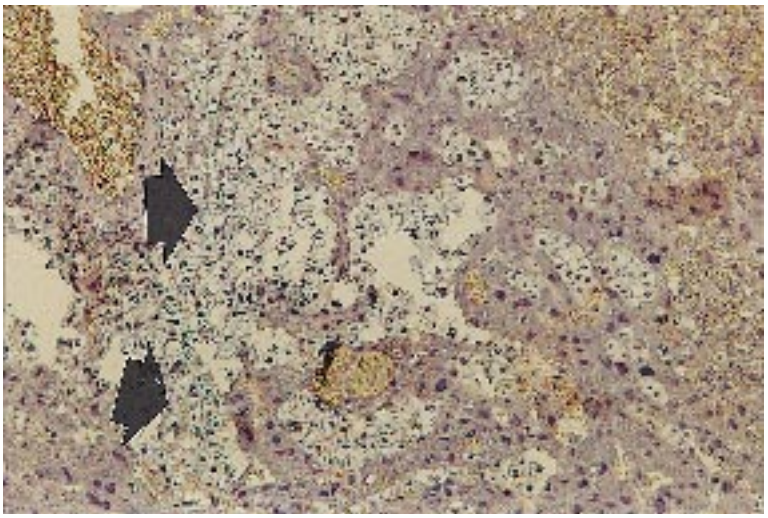

Fig 1. Placental structure. Light microscopic structure of gestational day–20 placenta from a fetus of a manifestly diabetic rat. *Arrows* point to aggregates of clear, possibly glycogen-containing cells. (Original magnification ×100.)

parenchyma (not shown). A general reduction of the intensity of laminin immunostaining was detected in the analogous placental loci from diabetic pregnancies (Fig 2, *B*), a reduction also detected by Western blot (76% ± 10% of control, $P < .05$ [see Fig 4], where the 250-kd band corresponding to the laminin B chains[13, 14] was found (Fig 3, *A*). Fibronectin showed a scattered distribution in the stroma surrounded by Reichert's membrane (not shown), in the labyrinth (Fig 2, *C*), and at the edge of the basal zone and endometrium in normal placentas (not shown). The fibronectin labeling was augmented in placentas exposed to maternal diabetes in various locations (Fig 2, *D*). Slot-blot analysis (Fig 3, *B*) confirmed the immunohistochemical observations and displayed fibronectin immunoreactivity at 204% ± 40% of control, $P < .05$ (Fig 4). The collagen-IV immunoreactivity was found in Reichert's membrane (Fig 2, *E*), labyrinth (Fig 2, *E*), basal zone (not shown), and walls of large vessels (not shown). The intensity of collagen-IV immunoreactivity (Fig 2, *E* and *F*) and slot blot (Figs 3, *C*, and 4, 98% ± 6% of control, $P > .05$) was similar in placentas from normal and diabetic pregnancies.

Comment

The major finding in this study was the changes in the concentrations of the extracellular matrix components laminin and fibronectin in the placentas of diabetic rats. These observations indicate that basement membranes may be structurally altered in placental tissues.

Basement membranes constitute extracellular matrix networks in which laminin, fibronectin, and collagen-IV are important constituents,[14] providing an epithelial-mesenchymal interface and surrounding vasculature, nerve fibers, and muscles. In addition to serving as structural proteins laminin, fibronectin, and collagen-IV are potent regulators of cellular proliferation and differentiation, providing appropriate signals that are transduced to the cell nucleus and thereby evoking changes in gene expression.[15-17]

The importance of extracellular matrix proteins for placental outgrowth and physiology is less clear. Human decidual cells synthesize and secrete extracellular matrix molecules to the pericellular environment, and cytotrophoblasts are associated with laminin, fibronectin, and collagen-IV.[18] Laminin and fibronectin were detected immunohistochemically in human chorionic villous stroma. In addition, laminin was present in basement membrane of villous trophoblast.[19] Senior et al[20] showed laminin protein associated with basement membranes of giant cells, syncytial cells, and fetal vasculature of labyrinth. Collagen-IV was shown to be deposited mainly in basement membranes and to a lesser extent in the stroma.[21] Extracellular matrix receptors are expressed in trophoblast cells,[22] and it was speculated that the extracellular matrix is facilitating trophoblast adhesion and invasion of maternal tissues. It is thus reasonable that normal placental morphogenesis depends on a specific extracellular matrix composition.

A general feature of most diabetic complications is a disturbed basement membrane composition of laminin, fibronectin, and collagen-IV, a phenomenon also demonstrated in cells cultured in high glucose concentrations in vitro.[23] Thus there may be a diabetes-induced dysregulation of extracellular matrix synthesis and deposition in various tissues with profound physiologic consequences. In developing tissues similar extracellular matrix alterations have recently been described. We have found increased messenger ribonucleic acid levels of laminin and fibronectin in rat embryos exposed to high glucose concentrations in vitro, as well as in embryos and fetuses from dia-

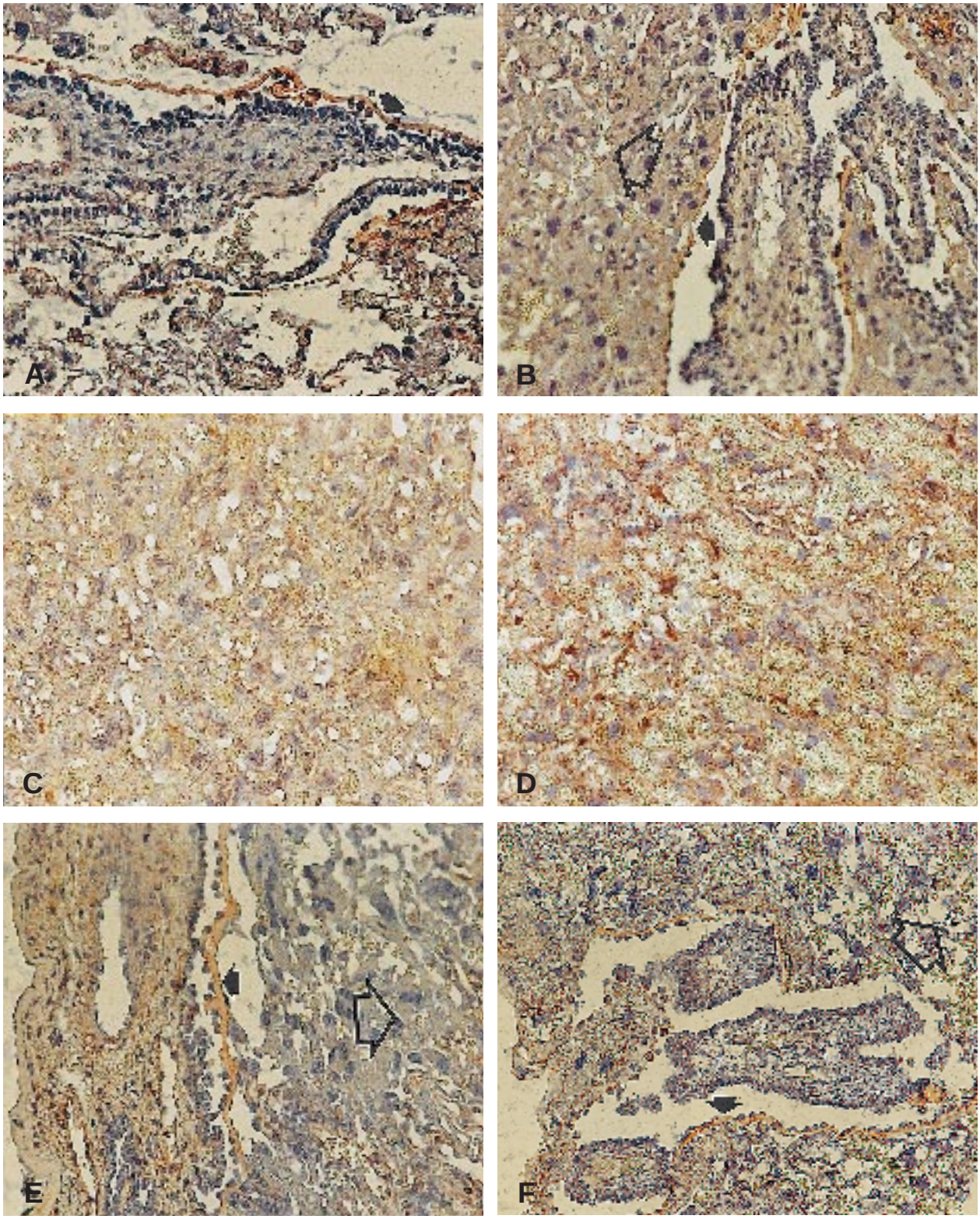

Fig 2. Immunohistochemical staining of extracellular matrix proteins. Sections of paraffin-embedded placental specimens of day 20 of gestation from normal **(A, C, E)** and manifestly diabetic **(B, D, F)** rats were stained with antibodies detecting laminin **(A, B)**, fibronectin **(C, D)**, and collagen-IV **(E, F)**. *Filled arrows* indicate Reichert's membrane and *outlined arrows* represent placental labyrinth, except in **C** and **D**, which show only labyrinth tissue. (Original magnification ×200.)

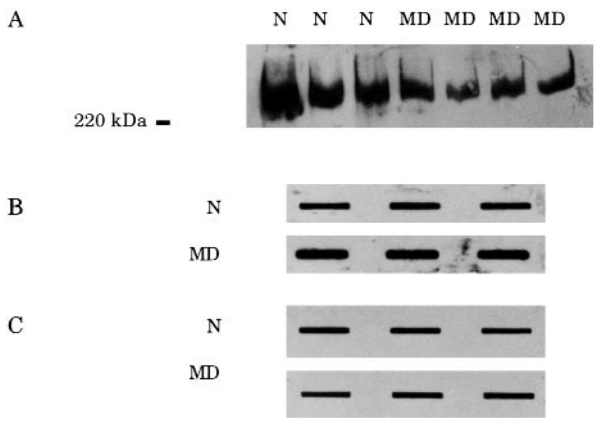

Fig 3. Western blot and slot-blot analysis of placental extracellular matrix proteins. Representative blots of extracellular matrix proteins from homogenized whole placentas of gestational day 20 from normal and diabetic rats. **A,** Laminin bands were resolved by sodium dodecylsulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Fibronectin **(B)** and collagen-IV protein **(C)** were subjected to slot-blot analysis in triplicates. *N,* Normal; *MD,* manifestly diabetic.

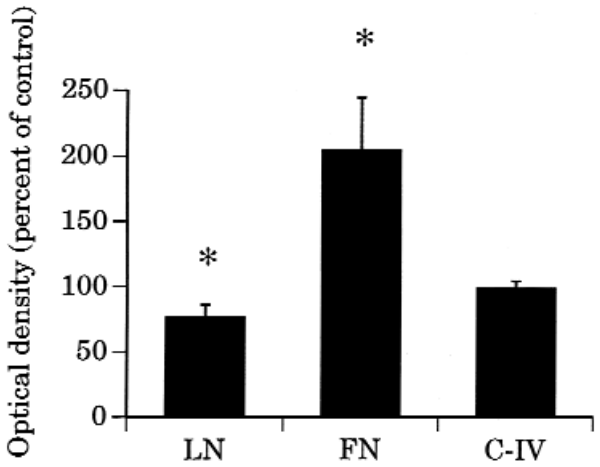

Fig 4. Levels of extracellular matrix protein immunoreactivity. Immunoreactivity of placental laminin (n = 11 in both groups), fibronectin (n = 6 in both groups), and collagen-IV (n = 8 in both groups) protein from normal and diabetic rats was quantitated densitometrically and expressed as mean percentage of corresponding normal placental tissue ± SEM. *Asterisk,* Denotes a significant difference from 100% ($P < .05$).

betic pregnancies.[10] Røge[9] reported increased fibronectin (143% of control), decreased laminin (70% of control), and decreased collagen-IV protein (75% of control) concentrations in human placentas detected by emzyme-lined immunosorbent assay, findings that were confirmed by immunohistochemical analysis of corresponding tissues. These human data are strikingly similar to our results from diabetic rats, implying similar maternal impacts on placental gene expression in human and rat diabetic pregnancies.

Katayama et al[24] found decreased laminin-B_2 messenger ribonucleic acid in cultured mesangial cells after exposure to high glucose concentrations and high osmolarity, indicating that high glucose concentrations per se repress laminin expression in these cells as in placentas from diabetic patients and rats. Thus our results on placental extracellular matrix expression fit very well with the notion of diabetes as a modulator of extracellular matrix homeostasis.

In diabetic pregnancies a wide range of reports on morphologic and ultrastructural placental abnormalities have been published. Placentomegaly appears to be the most significant effect of severely complicated diabetes in pregnant women,[25] as well as in experimental diabetic pregnancy.[27-29] The increased placental mass found in this study may be interpreted as an insufficient compensatory mechanism for the reduced uteroplacental blood flow in diabetic pregnancies.[28] Other morphologic disturbancies include thickening of the trophoblastic membranes together with higher degrees of vesiculation and vacuolization.[6] Increased content of glycogen and an increased number of glycogen cells have also been reported,[6, 30, 31] in accordance with the present finding of large aggregates of possibly glycogen-containing cells in the placentas of some diabetic rats.

Moreover, augmented cystic degeneration of rat placental spongiosa cells has been detected.[29, 32] In diabetic patients with poor blood glucose control an increased number of syncytial knots and vasculosyncytial membranes (which reduces diffusion distance over the interhemal membrane) and greater villus surface areas were found.[3] Focal thickening of human trophoblastic basement membrane,[34] larger placental capillary bed, shorter distance across fetal blood plasma, longer placental villi, but unchanged villous diameter were also found in diabetic human placentas.[7] We interpreted the latter findings as indicative of a higher degree of maturation in placentas from diabetic patients. In contrast, Gewolb et al[30] described an increased deoxyribonucleic acid content in late pregnancy placentas from diabetic rats, indicating prolonged cell division and thus a more immature state of the trophoblast tissue. This hypothesis is strengthened by the finding of a reduced number of epidermal growth factor receptors in placentas of diabetic rats[35] and women with type I diabetes.[36] Consequently, decreased epidermal growth factor signaling may be associated with delayed placental maturation. The placental immaturity may also be related to the fibronectin overexpression described in this study because higher fibronectin levels were described in trophoblastic basement membranes of immature villi compared with term villi in normal human placentas.[37]

Maternal hypertension in pregnancy may result in preeclampsia, a condition caused by structural defects of the endometrial spiral arteries leading to reduced uteropla-

cental and intervillous blood flow with oxygen desaturation of intervillous blood,[38] features shared with diabetes-complicated pregnancies.[39] Lowered laminin and unaltered collagen-IV deposition were found in placentas of patients with pre-eclampsia,[40] which agrees with the results presented here. Our finding of decreased laminin levels in placentas from diabetic rats would then allow the speculation of the existence of a common factor in pre-eclampsia and diabetic pregnancy affecting placental extracellular matrix expression. Because we do not possess any direct evidence for a common factor, this notion remains a speculation. An alternative interpretation of the data would be that our streptozocin-induced diabetic rats are to some degree experiencing pre-eclampsia and diabetes-induced hypertension. If maternal pre-eclampsia constitutes a link between maternal diabetes and placental/fetal maldevelopment, antihypertensive treatment would improve both maternal circulation and embryo-fetal development in diabetic pregnancy, as indicated by the experimental studies of Clabaut et al.[27]

In diabetic pregnancies, how could these disturbances of extracellular matrix protein composition affect the function of the placenta itself, as well as fetal growth and maturation? Experimental observations indicate placental transfer deficiency with respect to glucose,[5] amino acids,[41] and zinc,[2, 43] changes that may affect fetal development. The direct consequenses of altered placental function on fetal growth and maturation are not yet fully understood; however, evolving data strengthen the view that ultrastructural placental abnormalities have a role in the developmental and functional disturbances found in the offspring of diabetic pregnancies.

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