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Diabetes Induces Changes in Glomerular Development and Laminin-β2 (s-Laminin) Expression

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Offspring of diabetic mothers have developmental renal abnormalities; thus, we investigated the effects of the diabetic milieu on kidney development. Four groups of host rats, including insulin-deficient and insulin-treated streptozotocin-induced diabetic rats, normal controls, and insulin-treated nondiabetic rats, were prepared. After 38 days, rats received ocular implants of E14 fetal rat kidneys. Nine days later the fetal kidney grafts were harvested for analysis of glomerular development and expression of fibronectin, laminin, laminin-β2, and α-smooth muscle actin and m170, two additional markers of mesangial maturation. The rate of glomerular maturation was delayed in grafts placed in hyperglycemic, insulin-deficient diabetic rats. These glomeruli contained few mesangial cells or matrix, and laminin-β2 expression was reduced as compared with controls. Mesangial expression of α-smooth muscle actin and m170 was not detected. In contrast, grafts placed in insulin-treated diabetic animals had increased numbers of mesangial cells and expanded mesangial matrix. The content of laminin-β2 and expression of m170 and α-smooth muscle actin were also increased in these grafts. These data show that hyperglycemia and insulin status influence laminin isoform expression and play important roles in mesangial development. ***(Am J Pathol 1997, 151:1131–1140)***

The frequency of congenital malformations is increased in the offspring of diabetic mothers.[1] The numbers and severity of abnormalities in fetal development correlate with the degree of glycemic control of the mother, particularly during the first trimester.[1–3] Maternal glucose, but not insulin, readily crosses the placenta.[1] In the earliest stages of pregnancy, hyperglycemia *per se* may be detrimental to fetal development.[1] Later in the first trimester, fetal production of insulin occurs and is further stimulated by the glucose load delivered from the maternal circulation. The fetus remains persistently hyperinsulinemic as long as the mother is hyperglycemic, but fetal blood sugar levels are usually normal.[4] Experimental data suggest that metabolic abnormalities of diabetes contribute to abnormalities in kidney development in the fetus.[5–7] Yet, little is known of the mechanisms responsible for abnormalities in renal development.

Recent studies have shown that changes in extracellular matrix (ECM) composition and expression of various cell markers correlate with cellular differentiation during organ development.[8] α-Smooth muscle actin (α-SMA) expression in mesangial cells (MCs) correlates with the stages of glomerulogenesis and MC differentiation.[9] The protein identified by the monoclonal antibody m170 also has temporal-spatial changes in expression during nephrogenesis.[10] As the glomerulus matures, laminins (LMs) containing β1 chains are replaced by LMs containing β2 (s-laminin).[11] This change in LM isoform temporally corresponds to the novel expression of α3-α5 chains of collagen IV in the glomerular basement membrane (GBM). Expression of LM-β2 is functionally important in the glomerulus, as LM-β2-null mutant mice develop severe nephrotic syndrome shortly after birth.[12] Additional insights from studies of diabetic nephropathy[13] and MCs in culture[14,15] have shown that glucose and insulin regulate expression of these proteins; thus, we posited that diabetes-related changes in ECM composition would be associated with abnormalities in glomerulogenesis.

Studies of the specific effects of metabolic abnormalities of diabetes on fetal organ development are problematic because it is difficult to sample the fetus. Moreover, when diabetes is severe, fertility is reduced and fetal wastage is high, making experiments technically difficult. For these reasons we took advantage of the model of kidney development *in oculo*.[16] The progression of glomerular development was examined in normal fetal kidneys transplanted into the anterior chamber of the eye of normal and diabetic hosts. In this model, the host blood

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vessels of the eye proliferate and penetrate the graft. Vascular connections are made with the developing vasculature of the fetal kidney graft and the graft becomes perfused by the host circulation. Thus, the graft develops within the metabolic milieu of the host. In this report we describe the effects of hyperglycemia and exogenous insulin treatment of the host on glomerular maturation and ECM protein synthesis in fetal kidney grafts placed *in oculo*.

Materials and Methods

Reagents

The following reagents were obtained from commercial suppliers: streptozotocin and mouse monoclonal antibody (1A4) to α-SMA (Sigma Chemical Co., St. Louis, MO), purified pork NPH insulin (Novo Nordisk, Princeton, NJ), and fluorescein (FITC)-conjugated secondary antibodies (Jackson Laboratories, West Grove, PA). Polyclonal rabbit antibodies to rat fibronectin (FN), EHS-LM (LM-1),[17] and anti-rat GBM were prepared and characterized as described previously.[13,18,19] The anti-GBM antibody was raised by immunizing rabbits with GBM from sonicated rat glomeruli. This antibody reacts with many glomerular ECM proteins, and it is not specific for COL4A3. Mouse monoclonal antibody m170 was produced by immunization of BALB/c mice with a rat GBM preparation. At the capillary loop stage of glomerular development this antibody binds to peripheral loop GBM and mesangial matrices.[10] This antibody immunoprecipitates a 205-kd protein from MC culture supernatant and identifies a single protein by Western blot of mesangial matrix extracts. It does not cross-react with LM, FN, or collagen IV. Studies are in progress to further characterize this protein. Mouse monoclonal antibody (D5) to rat LM-β2 (s-laminin) was a gift from Dr. Joshua Sanes (Washington University, St. Louis, MO).[20,21]

Animal Model

Rats with streptozotocin-induced diabetes (DM) were prepared and characterized as described previously.[13] Male Sprague-Dawley rats weighing 200 g received 65 mg/kg streptozotocin in citrate buffer or citrate buffer alone intravenously. Five days later, the development of diabetes was confirmed by a blood sugar of greater than 350 mg/dl. One-half of the diabetic rats and an equal number of normal control animals received injections of NPH insulin (I^+) daily at 4:00 p.m. (3.5 to 5.0 U/day). The remaining half of each group received daily injections of saline (I^-). Thirty-eight days after induction of diabetes, animals in all four groups (n = 5 per group; DMI^-, DMI^+, CI^-, and CI^+) had fetal kidney grafts implanted into the anterior eye chambers.[16] Nine days later, host rats were exsanguinated under ether anesthesia and fetal grafts were harvested. Between 8:00 and 10:00 a.m. on days 0, 5, 29, and 47, blood was collected from the tip of the tail and analyzed for serum glucose using a Beckman autoanalyzer based on the glucose oxidase method. Before study and on day 29, rats were housed in metabolic cages with water and food provided *ad libitum*. Twenty-four-hour urine specimens were collected and urinary protein excretion was measured.[22]

For preparation of fetal grafts, kidneys were dissected from the fetuses of pregnant dams 14 days (E14) after the appearance of the vaginal plug. Using a small incision in the cornea, a fetal kidney was implanted into the anterior chamber of each eye of the adult host rat. The methods for fetal tissue implantation and harvest have been described in detail previously.[10,16] Grafts were harvested after 9 days as previous experience shows that glomerulogenesis occurs within this time period, and yet rejection or other inflammatory lesions, should they develop, are not observed until 25 to 30 days after implantation. Normal kidney tissue was harvested at days E14, E18, newborn, and 1 and 5 days after birth to serve as control tissue for fetal kidney grafts.

Tissue Preparation

Kidney grafts were prepared for electron microscopy or immunofluorescence microscopy by routine methods.[18,23] For electron microscopy, tissues were fixed in 2.5% glutaraldehyde, post-fixed in osmium tetroxide, embedded in Epon, stained with uranyl acetate, and processed by routine methods. For immunofluorescence microscopy, tissues were snap frozen in precooled isopentane, cryostat sectioned (4 μm), and fixed in acetone. Sections were incubated with antibodies to ECM proteins (GBM, m170, LM-1, FN, and LM-β2) and α-SMA followed by the appropriate FITC-conjugated secondary antibody. Sections were coded and examined without knowledge of sample identity using a Leitz microscope equipped for epi-illumination.

Fetal kidney LM-β2 expression was analyzed by indirect immunofluorescence microscopy using the anti-LM-β2 antibody (D5) diluted at 1:500. For analysis of LM-β2 expression in fetal kidney grafts, serial sections from each animal were stained with antibodies to LM-β2 or to rat GBM. The total number of glomeruli per section were identified and counted using frozen sections stained with anti-GBM antibodies. Four stages of expression of LM-β2 were defined in the adjacent section as shown in Figure 1. The number of glomeruli with LM-β2 staining at each stage were counted and multiplied by the score for each stage. The total score for LM-β2 expression was divided by the total number of glomeruli per section to achieve an average score for each animal. Group mean scores were calculated and compared by one-way analysis of variance (ANOVA). This scoring system for LM-β2 was devised because glomeruli easily identified by staining for other matrix proteins show variable degrees of LM-β2 expression. In contrast, LM detected by antibodies to LM-1 and FN are detected at the earliest stages of nephrogenesis, and no differences in distribution occur during glomerulogenesis. Thus, the intensity of staining for LM-1 and FN were scored on a 0 to 4+ scale. Mesangial maturation was identified by staining for α-SMA and the epitope recognized by m170.

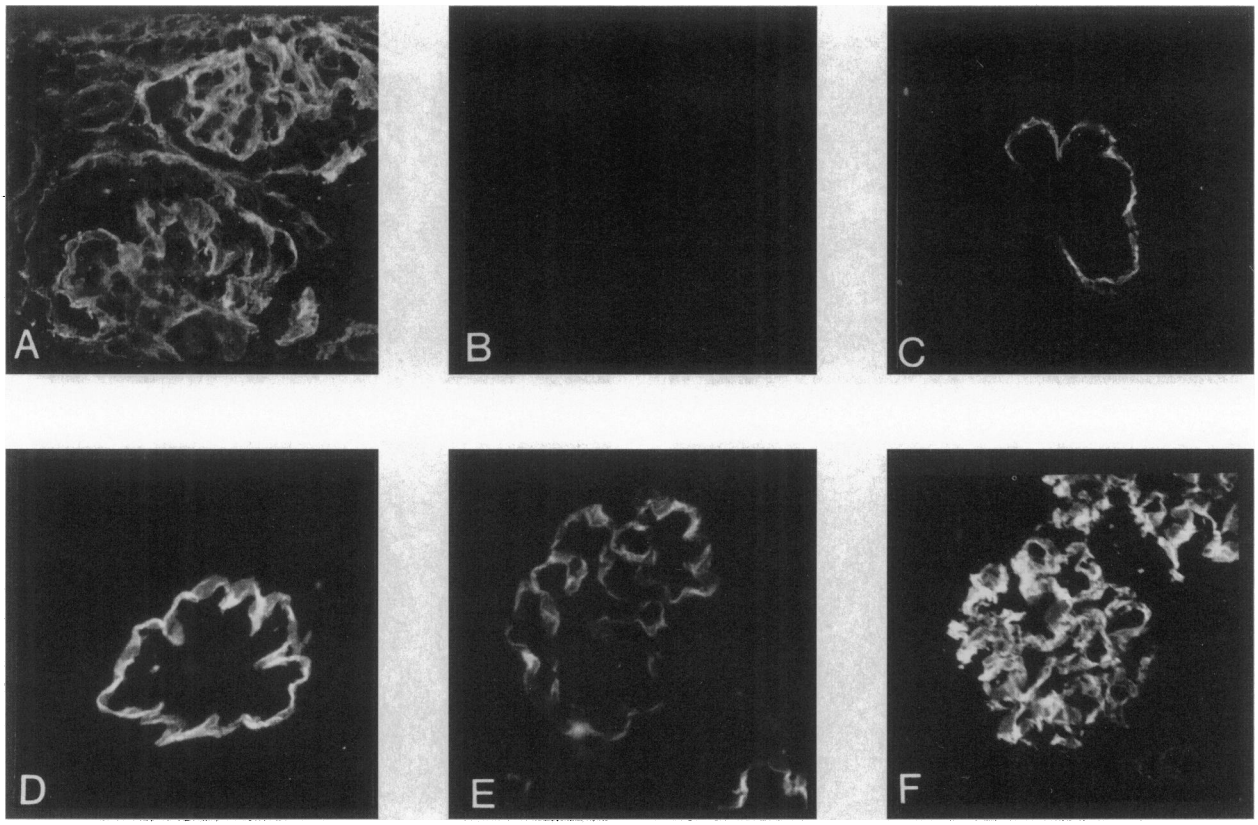

Figure 1. Stages of immunostaining for LM-β2. Serial sections were stained with antibodies to GBM and LM-β2. The number of glomeruli per section were counted in sections stained with antibody to GBM (**A**). When glomeruli were present (**A**), but none expressed LM-β2, a score of 0 was assigned (**B**). (In this example, **A** and **B** are serial sections.) Initially, LM-β2 appeared only in the periphery of glomeruli with minimal evidence of indentations (stage 1; **C**). As the glomerulus matured, invaginations of GBM stained positively for LM-β2 (stage 2; **D**). This staining pattern became more pronounced as the number of capillary loops increased and the glomerulus approached full maturity (stage 3; **E**). In the final stages of glomerular maturation, LM-β2 became more pronounced in a central distribution consistent with staining of the mesangium. Also, the intensity of staining in the capillary wall increased (stage 4; **F**). LM-β2 is not detected in tubular basement membranes. Original magnification, ×400.

Mesangial staining for these proteins was scored as present or absent. Differences in the distribution of staining for α-SMA and m170 were noted, but a method for quantifying the amount of these proteins was not performed.

Results

Animal Characteristics

The characteristics of the host rats at the time of grafting are summarized in Table 1. Insulin-deficient diabetic rats lost weight and were severely hyperglycemic. Insulin-treated diabetic rats had modest glycemic control and grew at rates 16% below normal control animals. All CI^+ animals received 3.5 U/day. Insulin dosages in the DMI^+ animals varied from 3.5 to 5.0 U/day in an attempt to maximize glycemic control. Only one DMI^- animal developed significant proteinuria and no animal died during the study period.

Fetal Grafts

Twenty-seven of the thirty-eight grafts placed into 20 rats grew and were recovered at the end of 9 days. In one CI^+ rat a single graft was placed in one eye that did not grow and in one DMI^- rat neither graft developed; therefore these animals were not included in the analysis. No statistical differences in graft success were identified between the groups (ANOVA, $P > 0.05$). Vessels from the host animal proliferated, penetrated the graft, and led to vascular perfusion of the graft as the fetal tissue developed. Glomeruli and tubules developed in grafts placed in all four groups of animals. FN and LM are normally expressed in all ECM compartments during nephrogenesis. Staining for these proteins assists in identifying the normal stages of glomerulogenesis. Because these proteins accumulate in kidneys from patients with diabetic nephropathy, they represent candidate proteins for dia-

Table 1. Animal Characteristics

	n	Weight (g)	S_{glu} (mg/dl)	U_{pro} (mg/dl)
CI^-	5	360 ± 31	140 ± 37	8.5 ± 5.4
CI^+	4	372 ± 28	263 ± 71	7.5 ± 2.5
DMI^-	4	177 ± 31	659 ± 96	22.2 ± 27.9
DMI^+	5	300 ± 27	362 ± 164	14.3 ± 5.5
ANOVA		$P < 0.001$; 1,2 > 3,4*	$P < 0.001$; 1,2 < 3,4*	$P > 0.05$

The results represent the mean ± 1 SD.
*1 = CI; 2 = CI^+; 3 = DMI^-; 4 = DMI^+.

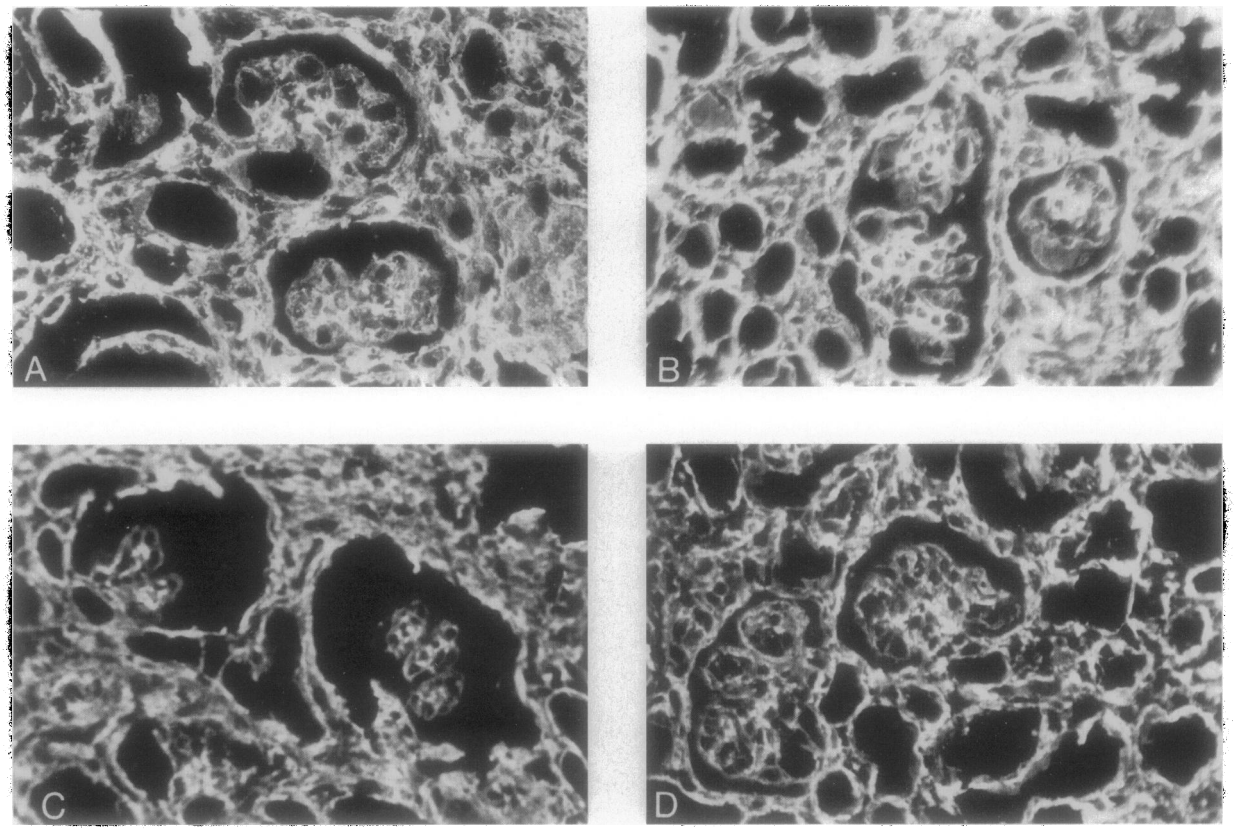

Figure 2. Immunostaining of fetal grafts for FN. Samples of fetal kidneys grafts from CI^- (**A**), CI^+ (**B**), DMI^- (**C**), and DMI^+ (**D**) hosts. Note the generalized staining of tubulointerstitium and GBM for FN with comparable intensity (4+) and distribution in all groups of fetal grafts. Original magnification, ×250.

betes-related abnormalities in glomerulogenesis. All ECM compartments of the developing kidney grafts stained positively for FN (Figure 2) and LM-1 (antibody raised to EHS-LM; Figure 3). The temporal-spatial pattern of staining paralleled the stage of glomerular development and the sites of localization of these proteins were consistent with their localization in mature kidneys. Although diabetes-associated abnormalities can influence synthesis of these proteins,[13,15,24,25] no significant differences in the intensity or pattern of staining for these proteins were identified between the experimental groups (Mann Whitney U, $P > 0.05$[18]).

Electron microscopy confirmed that glomeruli developed in all four groups of animals. No differences in the thickness of the GBM or the distribution of the double basement membrane or its fusion were observed between the groups. Glomerular epithelial cells appeared to differentiate in a normal fashion, and foot processes were well formed in all experimental groups. Endothelial cells were fenestrated and appeared normal in all samples. The major change in glomerular development observed between the experimental groups was in the mesangium. This was best evidenced in the electron micrographs (Figure 4) and in specific stains for markers of MC maturation. All stages of glomerulogenesis were noted in grafts from CI^- and CI^+ animals, including development of MCs and matrix. Although some glomeruli in grafts from CI^+ animals had a modest degree of MC hyperplasia, mesangial matrix expansion was not observed in grafts from CI^+ rats. In grafts from DMI^- rats, the mesangium did not develop. Few mesangial cells were identified and mesangial matrix was not apparent. Striking changes in the mesangium were observed in the grafts from DMI^+ rats. There was a marked increase in the number of mesangial cells in grafts from rats that were hyperglycemic and received exogenous insulin therapy. Also, large accumulations of mesangial matrix were apparent. These changes gave rise to a marked expansion of the mesangial area with an apparent reduction in the capillary space.

Markers of mesangial maturation were analyzed to confirm the changes observed by electron microscopy. Analysis of normal fetal kidney tissue from E14 to postnatal day 5 confirmed previously described changes in expression of α-SMA.[9] MC progenitors and vascular smooth muscle cells express α-SMA. In the normal fetal kidney, α-SMA appears in MC progenitors before their migration into the vascular cleft. Expression increases until the mesangium is fully mature and then the intensity of expression of α-SMA diminishes. Expression of α-SMA in arterioles steadily increases during development and remains high in arterioles of mature kidneys. α-SMA expression was evident in blood vessels in all groups, but it was not detected in glomeruli in fetal grafts placed in DMI^- rats. α-SMA was detected in mesangial areas of maturing glomeruli in grafts placed in CI^-, CI^+, and DMI^+ rats. In grafts from DMI^+ rats, the intensity of mesangial staining for α-SMA was sometimes increased; however,

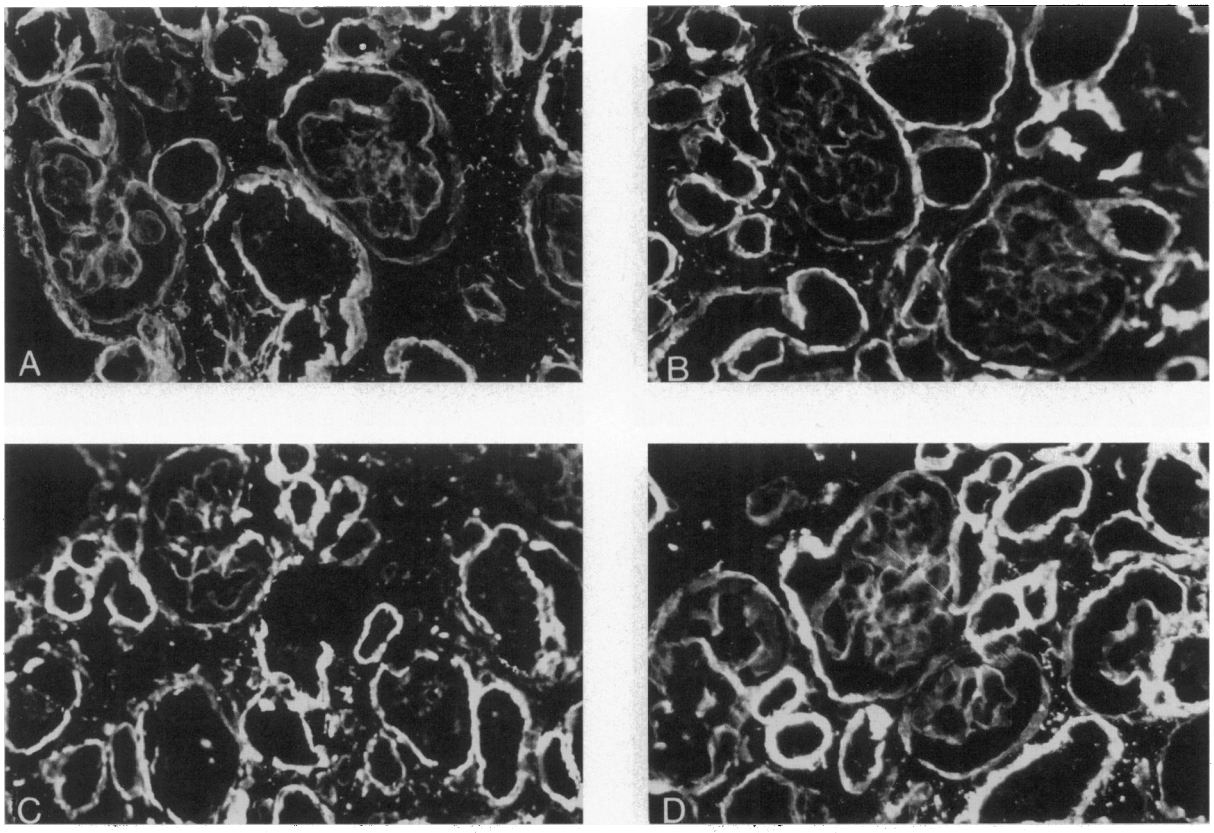

Figure 3. Immunostaining of fetal grafts for LM. No distinguishable differences in the pattern or intensity of staining for EHS-LM (laminin-1) in tubular basement membrane, GBM, or Bowman's capsule were noted between the groups (all groups 4+; CI^- (**A**), CI^+ (**B**), DMI^- (**C**), and DMI^+ (**D**)). Original magnification, ×250.

specific quantitation was not performed. Patterns of staining for α-SMA are shown in Figure 5.

During normal fetal kidney development, all basement membranes initially stain positively with the m170 antibody. As the mesangium matures, it condenses and a pinched waist appearance develops at the vascular pole. During this time, the epitope recognized by m170 appears in the mesangium and increases in intensity until the time of birth. As this matrix protein becomes concentrated in the mesangium, it diminishes and often disappears from other renal ECM compartments. Heterogeneity of staining for m170 at the time of birth reflects variable degrees of maturity of individual glomeruli. A similar pattern of expression was observed in fetal kidney grafts placed in control rats, yet at the termination of the study the majority of staining was confined to the glomerulus, which corresponds to other characteristics of glomerular maturity. In grafts placed in DMI^- rats, staining with m170 was evident in all basement membranes at the time of termination of the study, but no condensates of m170 were detected in mesangial areas. In grafts placed in DMI^+ rats, similar to control animals, minimal staining of ECM regions other than mesangia was identified. The intensity and area of staining with m170 was increased in most glomeruli in DMI^+ samples. These changes are shown in Figure 6.

Because changes in LM isoform correlate with the stages of glomerulogenesis,[11] insulin regulates LM isoform expression by MCs in culture,[15] and the major changes in nephrogenesis that were seen in this study occurred in the glomerular mesangium, we examined the expression of LM-β2 in the fetal kidney grafts. First, normal fetal and newborn rat kidneys were examined by indirect immunofluorescence to establish a scoring system for the normal pattern of expression of LM-β2. LM-β2 is not detectable in normal E14 fetal kidneys. Minimal staining is observed at E18. On the day of birth many glomeruli stain positively for LM-β2 and the number increases through postnatal day 5 until all glomeruli stain positively for LM-β2. LM-β2 first appears in a continuous linear pattern in the GBM at the early capillary loop stage. As development proceeds, the loops begin to invaginate and GBM staining corresponds to the rich capillary loop structure. At the time corrugation of the capillary loops is apparent, LM-β2 is first detected in the afferent and efferent arterioles. Condensation and positive staining for LM-β2 in the mesangium is the last to occur and corresponds to the fully mature pattern of staining. These stages and the stages of LM-β2 expression used for scoring experimental tissues are shown in Figure 1. The experimental results are shown in Figure 7. Significant differences in the number of glomeruli expressing LM-β2 were observed. The amount of LM-β2 and its extension from the peripheral capillary loop to the condensed mesangium differed between the four animal groups. DMI^- rats had very little expression of LM-β2 despite significant

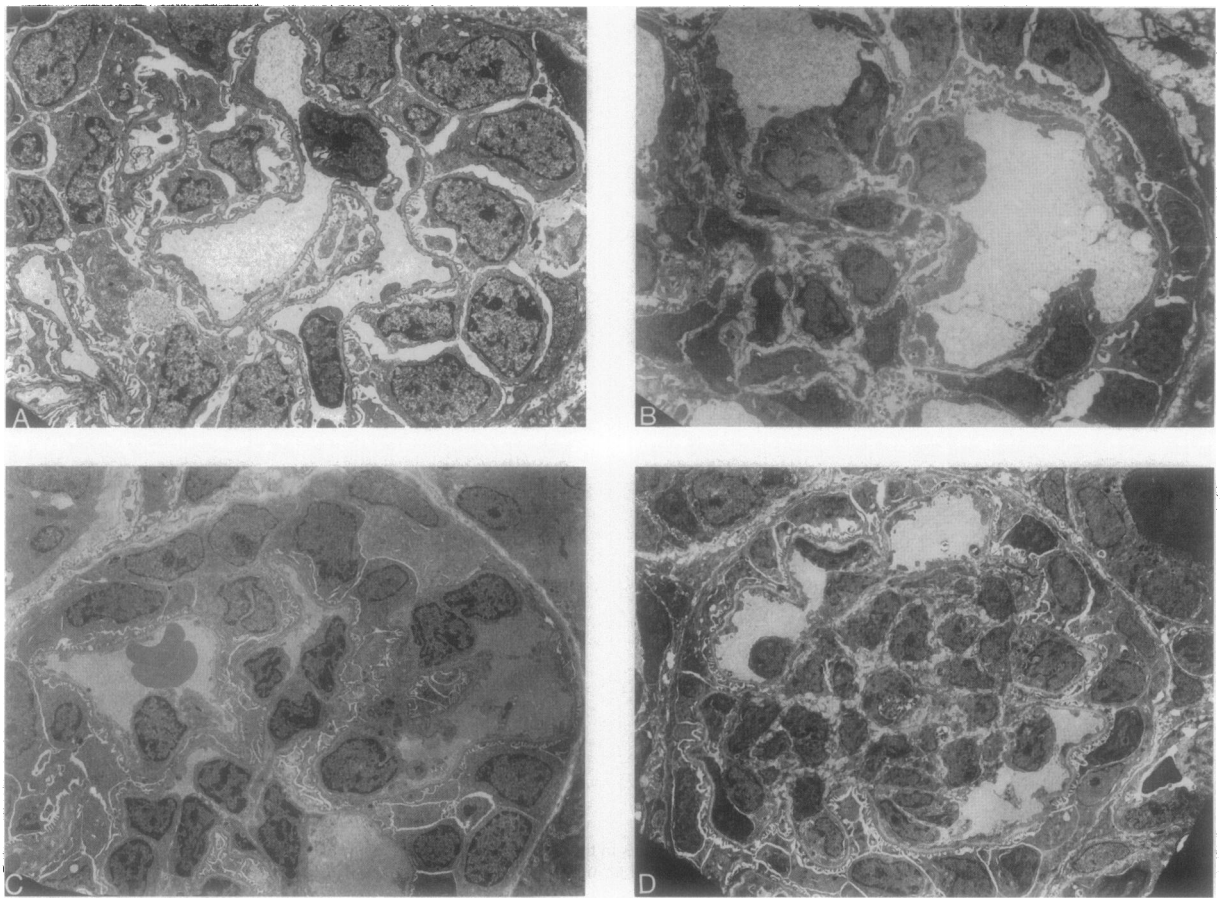

Figure 4. Electron micrographs. Representative micrographs of fetal kidney grafts from CI^- (**A**), CI^+ (**B**), DMI^- (**C**), and DMI^+ (**D**) are shown. In each case the glomerulus has formed. In most areas, the GBM has fused and foot processes are apparent on podocytes. In both **A** and **C**, the mesangium is not apparent. In CI^+ samples (**B**) a few MCs are noted in the central part of the glomerulus and small amounts of loose mesangial matrix are seen surrounding these cells. A graft from a DMI^+ rat is shown in **D**. A significant increase in mesangial cellularity and a striking expansion of the mesangial matrix are observed. Capillary lumens are patent, but their dimensions are reduced as a result of the marked expansion of the mesangium. Original magnification, ×2500.

numbers of glomeruli detected in serial sections as determined by staining with anti-GBM antibodies. Animals treated with insulin had significantly more LM-β2 expression than either normal controls (CI^-) or insulin-deficient diabetic rats (DMI^-). Grafts from DMI^+ animals that were hyperglycemic and received higher doses of insulin than CI^+ rats had a significant increase in LM-β2 score. The mean scores for expression of LM-β2 were as follows: DMI^-, 0.45 ± 0.03; CI^-, 1.05 ± 0.15; CI^+, 1.53 ± 0.18; and DMI^+, 2.05 ± 0.26 ($P < 0.001$, ANOVA).

Discussion

The results of this study show that alterations in circulating levels of glucose and insulin have significant effects on glomerulogenesis, particularly in the mesangium. Severe hyperglycemia and insulin deficiency (DMI^-) were associated with delayed maturation of the glomerulus. Glomeruli formed and no structural abnormalities were detected in glomerular epithelial or endothelial cells; yet the mesangium did not develop. MCs, mesangial matrix, and other markers of mesangial maturation were not detected. This was in marked contrast to the findings in insulin-treated, hyperglycemic rats in which the mesangium was severely expanded with encroachment on the capillary lumens. As the most striking differences between grafts placed in DMI^- and DMI^+ rats were the number of MCs and the amount of mesangial matrix, we analyzed two other markers of MC differentiation, α-SMA[9] and m170.[10]

In normal fetal kidneys and grafts placed in control animals in this study, α-SMA expression begins in undifferentiated mesenchymal cells that appear destined to become MCs. The intensity of staining for α-SMA increases as the MC progenitors migrate into the vascular cleft of the developing glomerulus. As glomeruli reach full maturity, α-SMA expression in MCs diminishes. Few α-SMA-positive cells were identified in the mesangium of DMI^- grafts even though α-SMA expression was abundant in developing arterioles. This argues that the environment in the DMI^- rats did not directly interfere with α-SMA expression but supports the conclusion that there was delayed/impaired maturation of the mesangium. In grafts placed in DMI^+ rats, the intensity of staining for α-SMA remained high even in mature glomeruli. This suggests that some characteristics of the fetal phenotype

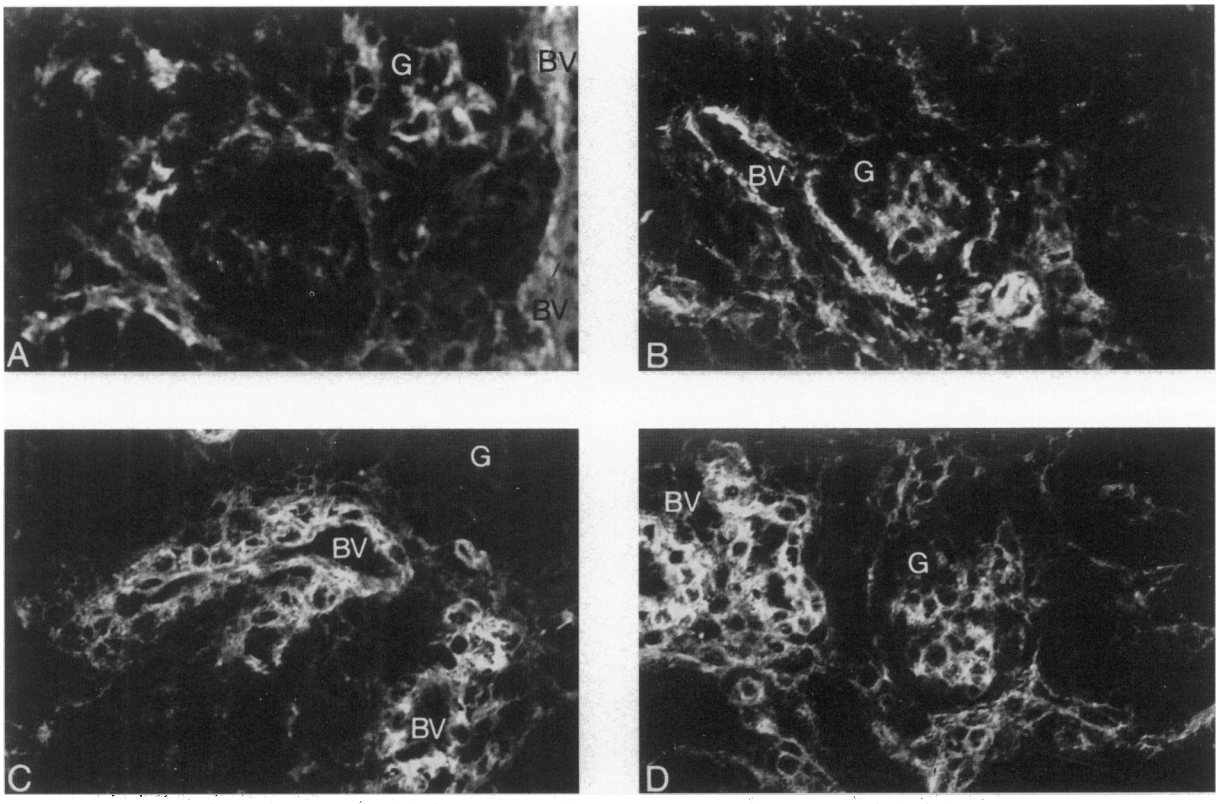

Figure 5. Staining for α-SMA. α-SMA-positive cells are observed in the undifferentiated mesenchyme, arterioles (BV), and the mesangium of the developing glomeruli (G). The patterns and intensities of staining are similar in grafts from CI^- (**A**) and CI^+ (**B**) rats. Although significant staining for α-SMA is identified in arterioles in grafts from DMI^- animals, no staining for α-SMA is seen in the mesangial areas of developing glomeruli (**C**). Significant staining for α-SMA is seen in developing arterioles and glomeruli in grafts from DMI^+ rats (**D**). Original magnification, ×400.

persisted in these grafts. We have previously shown that insulin directly alters MC cytoskeletal arrangement and increases α-SMA expression,[26] which is consistent with observations reported here. These changes have been postulated to represent an activated phenotype.[27,28] The present observations suggest that the metabolic environment of DMI^+ rats prevented the MCs from entering a quiescent state as usually occurs when glomeruli become fully mature.

The monoclonal antibody m170 identifies a 205-kd protein in matrix extracts from normal rat glomeruli and cultured MCs (unpublished observations). In the present study we show that the distribution of this epitope varies temporally and spatially during nephrogenesis. During early stages of nephrogenesis, the epitope recognized by m170 is present in all basement membranes but becomes restricted to the mesangium when the glomerulus reaches maturity. In grafts placed in DMI^- rats, this protein is expressed normally in ECM compartments other than the mesangium. Failure to identify the m170 epitope in the mesangium corresponds to other evidence that indicates that MC development is retarded in DMI^- rats. In grafts placed in DMI^+ rats, expression of the m170 epitope was limited to the mesangium, was increased in area and intensity, and in some cases extended into the extra-glomerular mesangium. These observations suggest that the expression of this protein is influenced by hyperglycemia and circulating insulin levels as well as other factors that determine MC differentiation. Studies are in progress to complete the characterization of this protein.

In part, the findings we expected were based on studies of glomerular structure and ECM composition in diabetic nephropathy, and in part on *in vitro* studies of the effects of glucose and insulin on MC proliferation and matrix synthesis. In the absence of insulin, hyperglycemia suppresses MC proliferation *in vitro*[29]; thus, it is possible that hyperglycemia directly suppressed MC proliferation *in vivo*. Delayed glomerular maturation in DMI^- rats in this study is consistent with *in vivo* data showing that generalized fetal and kidney development are retarded in infants of diabetic mothers with severe hyperglycemia in the first trimester.[1,3] The direct role of glucose has been demonstrated in studies of metanephric organ cultures in which high concentrations of glucose are associated with suppression of cellular proliferation, increased apoptosis, impaired mesenchymal induction by the ureteric buds, and alterations in the extracellular matrix.[5,6] Insulin is also a potent MC mitogen[30–32]; thus, it is likely that abnormalities in glucose and insulin that typify the DMI^- rats account for the delayed maturation of the mesangium. This is further supported by changes seen in grafts placed in DMI^+ rats. Striking changes in glomerulogenesis occurred in grafts from DMI^+ animals in which the number of MCs and the amount of mesangial matrix was markedly increased. As insulin is known to stimulate

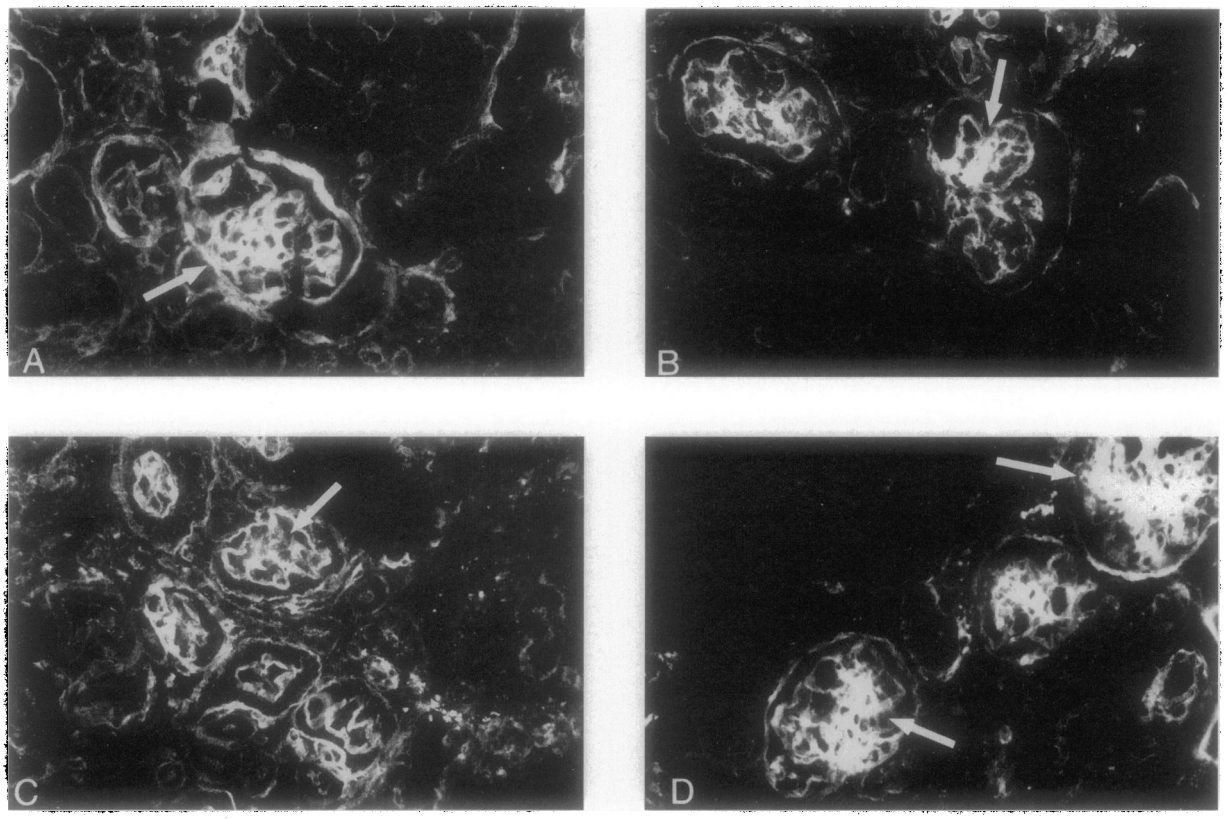

Figure 6. Staining of fetal kidney grafts with m170. In grafts from both CI^- (**A**) and CI^+ (**B**) rats, the m170 epitope is detected in tubular basement membranes, Bowman's capsule, GBM, and mesangial matrix (**arrows**). In grafts from DMI^- rats distinct mesangial staining is not apparent (**arrow**) even though this protein is expressed in other ECM compartments (**C**). In grafts from DMI^+ rats (**D**), minimal staining of the tubular basement membranes is seen, yet the intensity and area of positive staining of mesangial matrix is greater than in the other three groups. Original magnification, ×250.

MC proliferation *in vitro*, the increase in MC number in grafts from insulin-treated animals suggests this may occur *in vivo*. These observations suggest that hyperglycemia and hyperinsulinemia are important to the development of mesangial hyperplasia and matrix accumulation. These changes might occur in pregnancies in which the mother is persistently hyperglycemic and in which high glucose loads stimulate persistent increases in fetal insulin synthesis.

Considerable data have shown that organogenesis requires coordinated, temporal-spatial expression of various genes, including those that encode growth factors.[33,34] Growth factors stimulate rapid cellular proliferation to facilitate population of a growing organ, followed by differentiation signals that direct cells to express a mature phenotype. In part, coordinated, region-specific expression of unique ECM components directs cellular differentiation.[35,36] The temporal-spatial relationships between growth factors, their receptors, and ECM proteins during nephrogenesis are just being defined.[37] In the present study perturbations associated with diabetes have provided new insights into this process as well as the potential adverse impact of diabetes on glomerular development. Abnormalities of glucose and insulin influenced the evolution of change in LM isoform expression in the glomerulus.

LM is a large heterotrimer that is a critical component of all ECM compartments.[38] A large number of LM chains[17] variably assemble to create individual isoforms with limited distribution throughout the body.[20,39] LM-β2 is present in the glomerulus[18] and in the neuromuscular system where it serves as a stop signal for neurite outgrowth as neurites contact the motor end plate of muscle cells.[20,21,40] In the kidney, LM-β2 is present in GBM, mesangial matrix, and afferent and efferent arterioles,[20] but it is not identified in tubular basement membranes or Bowman's capsule. Its abundance in the glomerulus and limited distribution in other renal ECM compartments suggests that this isoform serves a unique function, yet currently its function in the glomerulus in unknown.

Changes in isoforms of LM occur with kidney development[36,41] and are thought to play an important role in cellular differentiation. Miner and Sanes[11] have shown that the appearance of LM-β2 in GBM temporally correlates with a switch in the chain composition of collagen IV to include α3-α5, which persists in the mature glomerulus. In the present study, similar patterns of LM-β2 expression were seen in the GBM and arterioles in grafts from control animals, and we show that LM-β2 expression appears last in the mesangium. In DMI^- rats with marked reduction in LM-β2 expression, GBM and glomerular epithelial and endothelial cells developed normally. This was not unexpected as the glomerulus develops normally, including podocyte foot processes, in LM-β2 null-mutant mice.[12] Detailed descriptions of the mesangium in LM-β2 null-mutant mice were not reported,[42] but failure

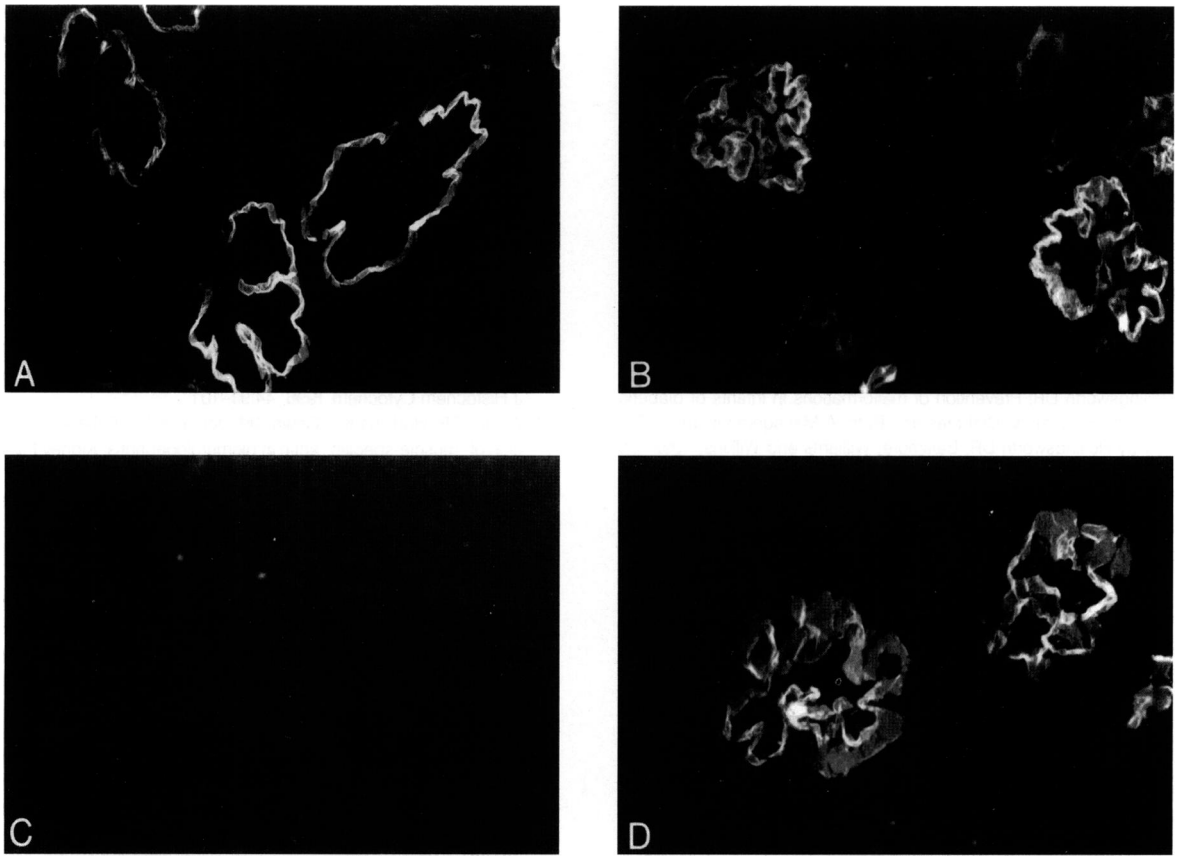

Figure 7. Immunostaining for LM-β2 in fetal grafts from the four experimental groups. LM-β2 is apparent in developing glomeruli in grafts from CI^- (**A**) and CI^+ (**B**) rats. The average stage of glomerular maturation was slightly higher in CI^+ rats as evidenced in **B** by the greater degree of capillary loop development. Despite the presence of glomeruli, there is no staining for LM-β2 in this sample from a DMI^- rat (**C**). The most intense staining for LM-β2 was observed in grafts from DMI^+ rats (**D**). Central collections of LM-β2 staining the mesangium were noted in some glomeruli (**arrow**). Original magnification, ×400.

of the mesangium to develop in DMI^- rats with reduced expression of LM-β2 suggests that the change in LM isoform expression may be required for induction of MC migration into the glomerulus and subsequent MC differentiation. Alternatively, MC differentiation might be required to signal a shift in the isoform of LM expressed by glomerular epithelial and endothelial cells. MC-capillary wall contacts maintain the structural integrity of the glomerulus[43]; thus, inadequate development of the mesangium might account for the nephrotic syndrome that developed shortly after birth in LM-β2 null-mutant mice.[12]

Despite apparent normal maturation of the capillary loops in DMI^- grafts, LM-β2 expression was markedly reduced. This suggests that hyperglycemia might be an important regulator of LM-β2 expression. Insulin also appears to be important. Insulin increased the number of MCs and the amount of mesangial matrix but also increased the intensity and distribution of LM-β2. These changes were particularly marked in DMI^+ rats that received higher doses of insulin than CI^+ rats. These findings suggest that insulin had a significant positive effect on induction of LM-β2 synthesis and thereby may play an important role in glomerular development. We have shown previously that insulin modulates the amount and chain composition of LM heterotrimers secreted by mesangial cells in culture.[15] Studies to examine the specific effects of glucose and insulin on LM-β2 synthesis by MCs in culture are in progress.

This study shows that the metabolic environment of the diabetic host has direct effects on kidney development. The most striking effects are on MC differentiation and glomerular matrix synthesis. There was diminished expression of LM-β2, retarded MC differentiation, and reduced expression of mesangial α-SMA and the m170 epitope in the hyperglycemic, insulin-deficient animals. The presence of insulin in the host allowed glomeruli to fully mature in a fashion similar to normal fetal kidney. Of note, grafts from animals that were both hyperglycemic and received exogenous insulin developed marked MC hyperplasia and mesangial matrix expansion. In this environment, the switch in LM isoform to include LM-β2 and expression of α-SMA and the m170 epitope in the mesangium occurred in the normal sequence consistent with maturation of the mesangium; however, the MC hyperplasia and expanded mesangial matrix suggests that the completely mature, quiescent state of the MCs was not achieved. It is of particular note that the most marked abnormalities occurred in the mesangium, as this is also the area of the kidney most affected by diabetic nephropathy. Diabetic nephropathy is characterized by nodular expansion of the mesangial matrix with increased accumulation of collagen IV and LM[13] and increased expression of α-SMA.[27] As the structural changes seen in the mesangium of grafts placed in DMI^+ animals were similar to those seen in diabetic nephropathy, this model may be useful for future studies to elucidate the contribution of

various factors in the pathogenesis of diabetic nephropathy.

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