Autocrine Activation of the IGF-I Signaling Pathway in Mesangial Cells Isolated From Diabetic NOD Mice

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Mesangial cells isolated from NOD mice after the onset of diabetes have undergone a stable phenotypic change. This phenotype is characterized by increased expression of IGF-I and downregulation of collagen degradation, which is associated with decreased MMP-2 activity. Here, we investigated the IGF-I signaling pathway in mesangial cells isolated from NOD mice before (nondiabetic NOD mice [ND-NOD]) and after (diabetic NOD mice [D-NOD]) the onset of diabetes. We found that the IGF-I signaling pathway in D-NOD cells was activated by autocrine IGF-I. They had phosphorylation of the IGF-I receptor β-subunit, phosphorylation of insulin receptor substrate (IRS)-1, and association of the p85 subunit (phosphatidylinositol 3-kinase [PI3K]) with the IGF-I receptor and IRS-1 in D-NOD cells in the basal state. This was also associated with increased phosphorylation of ERK2 in D-NOD mesangial cells. Inhibiting autocrine IGF-I from binding to its receptor using an IGF-I–neutralizing antibody or inhibiting IGF-I signaling pathways using a specific PI3K inhibitor or a specific mitogen-activated protein kinase/extracellular response kinase inhibitor decreased phosphorylated ERKs in D-NOD cells. Importantly, this was associated with increased MMP-2 activity. The addition of exogenous IGF-I to ND-NOD activated signal transduction. Therefore, we conclude that the IGF-I signaling pathway is intact in both D-NOD and ND-NOD cells. However, the phenotypic change in D-NOD cells is associated with constitutive activation of the IGF-I signaling pathways, which may participate in the development and progression of diabetic glomerulosclerosis. Diabetes 51: 182–188, 2002

Iterations in the availability of IGF-I or an altered response to IGF-I may play a role in diabetic nephropathy. Mesangial cells are critical determinants in the accumulation of extracellular matrix (ECM) in the glomeruli of patients with diabetic nephropathy (1). These cells express IGF-I receptors and synthesize IGF-I (2–6). We have shown that glomerular mesangial cells isolated from mice with autoimmune type 1 diabetes (NOD) exhibited a stable phenotypic change after the onset of diabetes (7). This stable change was characterized by increased IGF-I synthesis and increased cell proliferation. After blocking autocrine IGF-I with a neutralizing antibody, the number of detectable IGF-I surface receptors was found to be increased approximately threefold in diabetic NOD mice (D-NOD) compared with that in cells isolated from nondiabetic NOD mice (ND-NOD). This stable phenotypic change may be present in other experimental models of diabetic nephropathy because comparable phenotype changes were found in mesangial cells isolated from a model of spontaneously occurring type 2 diabetes with nephropathy (db/db) after the onset of diabetes (8). We showed that excess IGF-I secretion by mesangial cells could contribute to extracellular matrix deposition in diabetic nephropathy through a decrease in MMP-2 synthesis (9). A preliminary report in which mesangial cells from patients with type 2 diabetes and nephropathy had an altered phenotype (10) suggested that this observation may apply to patients. We compared intracellular IGF-I signaling pathways in mesangial cells isolated from NOD mice before and after the spontaneous onset of diabetes to determine whether the phenotypic change in mesangial cells isolated from diabetic mice resulted from changes in these pathways. The IGF-I signaling pathway was intact in mesangial cells isolated from both D-NOD and ND-NOD. The constitutive activation of IGF-I signaling pathways, apparently through an autocrine IGF-I loop due to increased synthesis and release of IGF-I, may contribute to decreased degradation and increased collagen accumulation.

RESEARCH DESIGN AND METHODS

Reagents. Tissue culture plates were obtained from Nunclon (Nalge Nunc). Reagents for SDS-PAGE and immunoblotting were obtained from Novex (San Diego, CA). HEPES, phenylmethylsulfonyl fluoride (PMSF), aprotinin, leupeptin, benzamidine, EDTA, sodium pyrophosphate, sodium fluoride and sodium orthovanadate, insulin, Triton X-100, Tween 20, bovine serum albumin (BSA; Fraction V), glycerol, and NaCl were from Sigma Chemical (St. Louis, MO). PD98059 and LY294002 were purchased from Calbiochem (La Jolla, CA). Nitrocellulose membranes were from Amersham Pharmacia Biotech (Piscataway, NJ). Recombinant human IGF-I-1 and a specific neutralizing monoclonal antibody for IGF-I were purchased from Upstate Biotechnology (Lake Placid, NY). All antibodies and the protein A agarose were obtained from Santa Cruz (Santa Cruz, CA).

Isolation and propagation of mesangial cell lines. Mesangial cells were isolated from NOD mice before and after the spontaneous onset of diabetes as previously described (7). Briefly, glomeruli were isolated from kidneys of 4- to 6-month-old D-NOD and ND-NOD mice. Diabetic mice had glycoculia for 4–8 weeks before sacrifice and were receiving two insulin injections each day. Nondiabetic mice had normal glucose tolerance, as determined by a glucose
tolerance test before sacrifice. Several lines of mesangial cells were derived from each of several D-NOD and ND-NOD mice (7). In each experiment, two separate mesangial cell lines derived from different D-NOD mice and two separate mesangial cell lines from two different ND-NOD mice were used between passages 15 and 28.

**Cell culture and experimental design.** Three days before the collection of protein, cells were plated in either 6-well plates or 75 cm² flasks in B medium containing 20% fetal bovine serum and 6 mmol/l glucose as previously described (7). Twenty-four hours before collection, the medium was replaced with B medium containing 0.1% BSA. Cell number was determined in duplicate wells at each experimental time point. Phosphorylation of IGF-I receptor and insulin receptor substrate (IRS)-1 and IRS-2 were examined after exposure to either IGF-I (50 ng/ml) or insulin (50 ng/ml) for 10 min. To block activation of the signaling pathway, a neutralizing IGF-I antibody (34 µg/ml, PD98059 or a specific inhibitor of protein-activated protein kinase (MAPK)/extracellular response kinase (ERK) kinase (MEK), an upstream kinase activator of ERK (20–40 µmol/l final concentration), or a specific phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002 (4 µmol/l final concentration), was added to the culture medium for 12 and 36 h.

**Immunoprecipitation and Western blotting.** Each well of a 6-well plate was washed twice with cold PBS and 100 µl cold lysis buffer (20 mmol/l Tris, 140 mmol/l NaCl, 3 mmol/l EDTA, 10 mmol/l NaF, 10 mmol/l Na pyrophosphate, 2 mmol/l NaVO₄, 10% glycerol, pH 7.4, 1% Triton X-100, aprotinin, leupeptin, and PMSF) was added for 2 min before harvesting the cell layer by scraping. Cell lysates were incubated for 45 min at 4°C, and the insoluble material was removed by centrifugation at 20,000g for 30 min at 4°C. Samples were analyzed by electrophoresis through 6% (IRS-1, IRS-2, and IGF-I-RIP), 8% (PI3K-p85, ERK-1/2, phospho-ERKs) polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes. After overnight incubation at 4°C in Tris-buffered saline, 5% milk, or 1% milk plus 1% BSA and 0.05% Tween-20, the blots were exposed to antibodies recognizing either IRS-1, IRS-2, IGF-I-RIP, PI3K-p85, ERK-1/2, phospho-ERKs, and antiphosphotyrosine PY20 or PY99 for 1 h at room temperature. The primary antibodies were revealed using the corresponding goat or donkey peroxidase–conjugated secondary antibodies (1/2,000–1/4,000) for 1 h. Peroxidase activity was detected using the Santa Cruz chemiluminescence kit.

For immunoprecipitation experiments, 250 µg protein extract was incubated with either IGF-I-Rβ or IRS-1 antibody for 1 h at 4°C, followed by the addition of protein A agarose overnight. The resulting protein A antibody conjugate was centrifuged at 4°C and washed four times with PBS (pH 7.4). The final pellet was resuspended in PBS, sample buffer was added, and the mixture was boiled for 3 min before analysis as described above.

**Zymography for matrix metalloproteinases.** To determine whether blocking IGF-I activation increased MMP-2 in diabetic mesangial cells, medium was collected from cells exposed to PD98059 for 24 h or LY294002 for 36 h. Cell supernatants were electrophoresed and incubated for 24h in 2.5% Triton X-100 and incubated overnight in collagenase as previously described (11).

**Statistical analysis.** Comparison between groups was performed using one-way analysis of variance and Tukey’s multiple comparison test. Changes in MMP-2 activity after exposure to PD98059 or LY294002 were analyzed by ‘t’ test.

**RESULTS**

**IGF-I receptor and IRS-1 and -2 protein expression.** IGF-I receptor proteins in mesangial cells were assessed by Western immunoblot. Lysates from at least six separate experiments on each of two mesangial cell lines from two separate ND-NOD mice and two other mesangial cell lines from two D-NOD mice were examined. Serial dilutions of a large pool of mesangial cell lysates were run with each gel to provide a comparison between gels. The amount of protein contained in each sample was expressed as a fraction of the standard. Results for each cell line are expressed as the mean ± SD of 6–7 cell lysates from individual collections (D-NOD vs. ND-NOD mesangial cell lines, ***P < 0.001). A representative gel is shown.

**IGF-I receptor and IRS-1 and -2 phosphorylation.** IGF-I receptor activation leads to tyrosine phosphorylation of its β-chain and binding of the regulatory (p85) subunit of PI3K (13). We examined these events by immunoprecipitating cell lysates with IGF-I receptor antibody followed by Western immunoblotting with antibodies against phosphorylated tyrosine residues (PY99) and the p85 subunit of PI3K. Two bands were identified (Fig. 3). Based on its molecular weight and the effect of added IGF-I, the lower band corresponded to the β-chain of the IGF-I receptor (Fig. 3, middle panel). We found that the IGF-I receptor was phosphorylated in the basal state in mesangial cells isolated from D-NOD mice but not in those from ND-NOD mice (Fig. 3, middle panel). In all cell lines, incubation for 5 min in the presence of 50 ng/ml IGF-I resulted in a prominent phosphorylation of the IGF-I receptor β-chain. Interestingly, whereas incubation with a similar dose of insulin had no effect on phosphorylation of the β-chain of the IGF-I receptor, it induced phosphorylation of a band migrating above the IGF-I receptor (Fig. 3, middle panel). We postulated that the protein phosphorylated by insulin may be the β-chain of insulin receptor, based on its homology with the β-chain of IGF-I receptor and its molecular weight (14).

The p85 subunit of PI3K was shown to spontaneously associate with the β-chain of the IGF-I receptor. The amount of p85 associated with IGF-I receptor was greater in mesangial cells from diabetic mice (Fig. 3, lower panel). The addition of IGF-I/A to media increased the association of the p85 subunit to the IGF-I receptor in both the ND-NOD and the D-NOD cell lines.
The effect of IGF-I on IRS-1 and -2 phosphorylation was studied by immunoprecipitating cell lysates using a mixture of IRS-1 and -2 antibodies. The precipitates were examined by Western immunoblots. Because the amount of IRS in mesangial cells from D-NOD mice was twofold greater, the quantity of extract loaded for the Western blot after immunoprecipitation was halved (Fig. 4, top panel).

Under these conditions, the phosphorylated forms of both IRS-1 and -2 were detected. Examination of mesangial cells in the basal state revealed that phospho-IRS-1 was increased in those cells isolated from diabetic mice compared with those isolated from ND-NOD mice. A 10-min exposure to 50 ng/ml IGF-I resulted in a twofold increase in IRS-1 phosphorylation, whereas IRS-2 phosphorylation was unchanged. Stimulation with 50 ng/ml insulin had no
Effect of a neutralizing anti-IGF-I antibody, a PD3K inhibitor, and a MEK inhibitor. Mesangial cells synthesize and secrete IGF-I (2–5). Therefore, an IGF-I-neutralizing antibody was added to cell culture medium to determine whether autocrine secretion was responsible for the activation of IGF-I pathway in mesangial cells isolated from D-NOD mice. IGF-I receptor β-chain and IRS-1 phosphorylation was assessed by immunoprecipitation with the corresponding antibodies, followed by Western immunoblotting with an anti-phosphorylated tyrosine antibody. The addition of the IGF-I-neutralizing antibody (Fig. 6A) blocked basal phosphorylation of the IGF-I receptor in mesangial cells from D-NOD mice. We confirmed by zymography that it also increased MMP-2 activity as previously reported (data not shown) (9). Furthermore, the addition of the neutralizing antibody reduced the amount of phospho-IRS-1 in D-NOD mesangial cells to a level that was similar to that of resting mesangial cells isolated from ND-NOD mice (Fig. 6B, lower panel). In contrast, the neutralizing antibody did not affect the amount of phospho-IRS-1 in mesangial cells from ND-NOD mice (Fig. 6B, lower panel).

After exposure to an IGF-I-neutralizing antibody, the amounts of phosphorylated ERK1 and -2 were only decreased in D-NOD mesangial cells, whereas they were unchanged in similarly treated ND-NOD mesangial cells (2–5). PD3K activation is an important downstream event after IGF-I receptor activation (17). The increased amount of p85 subunit associated with the IGF-I receptor and IRS (Figs. 3 and 4) suggested that PD3K might be implicated in the downstream events of the intracellular IGF-I pathway, such as phosphorylation of MAPKs. This was investigated using LY294002, a specific PD3K inhibitor (18). LY294002 did not alter the growth curve of mesangial cells isolated from either ND-NOD or D-NOD mice. After the addition of LY294002, the growth of mesangial cells was decreased to levels similar to those of ND-NOD cells (18A). Thus, the increase of MAPK phosphorylation was due to autocrine secretion of IGF-I, not to PD3K activation.
LY294002 for 12 h, the basal levels of phosphorylated ERK1 and -2 decreased in D-NOD mesangial cells (57.9 ± 5.93% and 63.3 ± 4.34%, respectively) but not in ND-NOD mesangial cells (Fig. 7B). This decrease was similar to that obtained with the IGF-I-neutralizing antibody (51.6 ± 7.77 and 56.9 ± 2.26%, respectively). No further inhibition was observed with the addition of both the PI3K inhibitor and the IGF-I antibody (data not shown). When D-NOD cells were exposed to the specific MEK inhibitor PD98059 for 12 h, phosphorylated ERK1 and -2 decreased by 54 and 80% of baseline, respectively (data not shown).

**Zymography.** To determine whether the inhibition of IGF-I signaling resulted in increased MMP-2 activity, D-NOD and ND-NOD mesangial cells were treated with LY294002 for 36 h or PD98059 for 16 h. Medium was collected, and zymography was performed to assess MMP-2 activity. MMP-2 activity was increased only in D-NOD cells after treatment with LY294002 (134 ± 0.94%, \( P < 0.0001 \)) (Fig. 8A) or PD98059 (136 ± 9.3%, \( P < 0.05 \)) (Fig. 8B).

**DISCUSSION**

The purpose of the current study was to determine whether the phenotypic change in IGF-I expression in mesangial cells isolated from D-NOD mice resulted from specific alterations in its signaling pathways. We found that the IGF-I signaling pathway was activated in mesangial cells isolated from D-NOD mice due to the synthesis of excess IGF-I. For instance, the -subunit of the IGF-I receptor was significantly increased in mesangial cells isolated from D-NOD mice when compared with those from nondiabetic littermates. These data are consistent with our previous report that diabetic cells had more surface IGF-I receptors when autocrine IGF-I was blocked with a neutralizing antibody to IGF-I (7). In addition, the -subunit of the IGF-I receptor was significantly increased in mesangial cells isolated from D-NOD mice when compared with those from nondiabetic littermates. These data are consistent with our previous report that diabetic cells had more surface IGF-I receptors when autocrine IGF-I was blocked with a neutralizing antibody to IGF-I (7).
mesangial cells isolated from diabetic mice. In addition, whereas ERK1 and -2 levels were found to be similar in all cell lines from diabetic and nondiabetic mice, activated (phosphorylated) ERK2 was increased only in the basal state of cells isolated from diabetic mice. Although the addition of exogenous IGF-I further increased levels of phosphorylated ERKs in D-NOD cells, the increase was less prominent than in ND-NOD cells.

The IGF-I signaling cascade has been investigated in many cell types, including vascular smooth muscle cells (19) and fibroblasts (20). Oemar et al. (8) reported the induction of tyrosyl phosphorylation of nuclear proteins by IGF-I in mesangial cells isolated from a model of type 2 diabetes (db/db). However, to our knowledge, there are no reports on the activation of this pathway in mesangial cells isolated from a model of type 1 diabetes, such as the NOD mouse.

Because cells isolated from D-NOD mice synthesized more IGF-I at baseline (7), we added a neutralizing antibody to IGF-I to determine whether there were also abnormalities in the IGF-I signaling pathway. We found that the addition of this antibody prevented phosphorylation of the IGF receptor β-subunit, reduced the amount of phospho-IRS-1, and decreased the amounts of phosphorylated ERK1 and -2 in the cells isolated from diabetic mice.

Finally, because the amount of p85 subunit associated with the IGF receptor was increased in cells isolated from diabetic mice, we used an inhibitor (LY294002) to determine whether downstream signaling events were affected. Importantly, after the addition of LY294002, we found decreased levels of phosphorylated ERK1 and -2 in D-NOD cells, suggesting that the IGF-I–dependent signal transduction from PI3K to ERKs was inhibited. Although it was previously reported in MCF-7 breast cancer cells (21), this pathway has not been explored in glomerular mesangial cells.

Thus, the IGF-I signaling pathway is intact in mesangial cells isolated from both D-NOD and ND-NOD mice. However, the levels of activated ERK were relatively increased in the D-NOD cells. Stimulation with IGF-I, either autocrine or exogenous, activated signal transduction in both D-NOD and ND-NOD mesangial cells, although the magnitude of the response differed markedly. Removal of autocrine IGF-I with a neutralizing antibody decreased the activation in the D-NOD mesangial cells but had little effect on mesangial cells isolated from ND-NOD mice, even though they synthesized and secreted small amounts of IGF-I under basal conditions.

We have previously shown that excess IGF-I synthesized by mesangial cells isolated from D-NOD mice resulted in decreased MMP-2 activity. Conversely, removal of the autocrine IGF-I caused an increased in MMP-2 activity (9). These data suggest that overexpression of IGF-I may result in decreased degradation of ECM and lead to an accumulation of ECM, a characteristic feature of diabetic nephropathy. A recent report suggests that constitutive activation of MEK (MEK1, upstream from ERK) leads to activation of MMP-2 in a rat fibroblast cell line (22). In contrast, we found that activation of ERK1 and -2 is associated with decreased MMP-2 activity in diabetic mesangial cells, since blocking IGF-I activation through either the PI3K or MAPK pathway increased MMP-2 activity. This suggests that MAPK regulation of MMP-2 activity may be cell- or tissue-specific.

In conclusion, the constitutive overexpression of IGF-I in mesangial cells isolated from D-NOD mice leads to autoactivation of the IGF-I signaling pathways, including increased basal expression and phosphorylation of signaling components (ERK1 and -2). This may lead to decreased ECM degradation and appears to be part of the phenotypic changes induced after the onset of diabetes.

REFERENCES

