The Diabetic Milieu Modulates the Advanced Glycation End Product–Receptor Complex in the Mesangium by Inducing or Upregulating Galectin-3 Expression

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Nonenzymatic glycation has been implicated in the pathogenesis of the dysregulated tissue remodeling that characterizes diabetic glomerulopathy, via the formation of advanced glycation end products (AGEs) and their binding to cell surface receptors. Several AGE-binding proteins have been identified so far, including *p60*, *p90*, and the adhesive and growth-regulating lectin galectin-3 (Gal-3), the components of the so-called AGE-receptor complex. This study aimed to evaluate the mesangial expression of the AGE-receptor complex and its modulation by the diabetic milieu, both in vivo, in nondiabetic versus streptozotocin-induced diabetic rats, and in vitro, in mesangial cells exposed to either normal glucose (NG) levels (5.5 mmol/l), as compared with high glucose (HG) levels (30 mmol/l) and iso-osmolar mannitol (M), or to native bovine serum albumin (BSA), as compared with glycated BSA with AGE formation (BSA-AGE) and glycated BSA in which AGE formation was prevented by aminoguanidine (BSA-AM). In vivo, Gal-3 protein and mRNA were not detectable in glomeruli from nondiabetic rats until 12 months after initiating the study. On the contrary, in diabetic rats, Gal-3 expression was observed at 2 months of disease duration, and it increased thereafter. Both *p60* and *p90* immunoreactivities were observed at the glomerular level with slightly increased expression of *p90*, but not *p60*, in diabetic versus nondiabetic animals. In vitro, Gal-3 was not detectable in mesangial cells cultured in NG (although it became evident after a certain number of passages in culture), whereas Gal-3 was detectable in cells grown on BSA. Prolonged exposure (2–4 weeks) of mesangial cells to HG but not to M, as well as growing cells on BSA-AGE and, to a lesser extent, BSA-AM, induced or significantly increased the expression of Gal-3, both protein (up to 2.65-fold) and mRNA (up to 3.10-fold) and its secretion in the medium (by ~50%). Both *p60* and *p90* were demonstrated in mesangial cells under NG conditions, and the expression of *p90*, but not *p60*, was upregulated by ~20% by HG or BSA-AGE. These results indicate that *1*) under basal conditions, Gal-3, unlike *p90* and *p60*, is not detectable in the mesangium but becomes expressed with aging and *2*) the diabetic milieu induces or upregulates Gal-3 production, whereas it increases only slightly the expression of *p90*, but not *p60*. Gal-3 expression or overexpression may modulate the AGE-receptor–mediated events by modifying the function of the AGE-receptor complex. Additionally, it may exert direct effects on tissue remodeling by virtue of its adhesive and growth-regulating properties. *Diabetes* 49:1249–1257, 2000

Nonenzymatic glycation has been implicated in the pathogenesis of diabetic glomerulopathy through the formation of advanced glycation end products (AGEs) and their binding to cell surface receptors (1,2). The AGE-receptor–mediated pathway is involved in both the removal of AGEs and the modulation of dysregulated tissue remodeling that leads to mesangial expansion (3,4).

Several AGE-binding proteins have been identified, including *p60*, a 50-kD protein homologous to a component of the oligosaccharyltransferase complex OST-48 (5); *p90*, an 80-kD protein homologous to the protein kinase C substrate 80K-H; galectin-3 (Gal-3), a 32-kD protein previously known as Mac-2 or carbohydrate-binding protein-35 (6); RAGE, a 35-kD member of the immunoglobulin superfamily of receptors (7); and the macrophage scavenger receptor (type II) (8). At present, *p60*, *p90*, and Gal-3 are believed to act as a molecular complex (the so-called AGE-receptor complex), with the first implicated mainly in AGE degradation and the latter two in cell activation (2). However, the molecular mechanisms by which AGE-binding proteins associate and cooperate with each other to specifically recognize the highly heterogeneous AGE structures and to mediate their effects remain unknown.

Gal-3, the most recently identified AGE-binding protein, belongs to the lectin family (9,10). It interacts with the β-galac-

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AGE, advanced glycation end product; AM, aminoguanidine; BSA, bovine serum albumin; BSA-AGE, glycated BSA with AGE formation; BSA-AM, glycated BSA in which AGE formation was prevented by AM; CRD, carbohydrate recognition domain; ECL, enhanced chemiluminescence; ELISA, enzyme-linked immunosorbent assay; Gal-3, galectin-3; HG, high glucose; HMC, human mesangial cell; M, mannitol; NFDM, nonfat dry milk; NG, normal glucose; PBS, phosphate-buffered saline; RMC, rat mesangial cell; RT, room temperature; SSC, sodium chloride and sodium citrate; TBS, Tris-buffered saline; UV, ultraviolet.

toside residues of several cell surface and matrix glycoproteins via the carbohydrate recognition domain (CRD), which it shares with the other galectins. It is also capable of peptide-peptide homo- and heterodimeric associations mediated mainly by its unique short NH_2-terminus after the proline-glycine-alanine-thyrosine–rich repeating domain. These structural properties enable Gal-3 to exert multiple functions (10). As an adhesion molecule, Gal-3 mediates homotypic cell adhesion (11) and downregulates cell adhesion to laminin (12), thus producing an antiadhesive effect that has been involved in tumor invasion and metastasis (13); however, under certain circumstances, Gal-3 may favor cell adhesion (14). Moreover, it mediates IgE-dependent cell activation (15), thus suggesting a role for this molecule in allergy (10). Intracellularly, it acts as a pre-mRNA–splicing factor (16) and participates in the regulation of cell proliferation and death, the latter due to its sequence homology and association with *bcl-2* (17). By virtue of its proproliferative (17,18) and antiapoptotic (17) actions, it is considered as an immediate early gene (19) possibly implicated in tumor growth.

The AGE-receptor function of Gal-3 was suspected on the basis of the isolation of a sequence corresponding to Gal-3 by screening an expression library from activated macrophages with an anti-*p90* antibody. Moreover, it was demonstrated by evidence of a high-affinity binding of Gal-3 for AGEs (6).

At present, scant information is available regarding the glomerular/mesangial expression of the components of the AGE-receptor complex, particularly that of Gal-3. Furthermore, it is still unclear whether the diabetes-induced enhanced formation and accumulation of AGEs within the kidney and mesangium are associated with changes in the levels of the AGE-binding proteins. These changes might in fact modify the AGE/AGE-receptor interactions and, in the case of Gal-3, also exert AGE-independent effects on glomerular remodeling.

To address these issues, we have conducted an in vivo and in vitro study aimed at evaluating both the glomerular/mesangial expression of the components of the AGE-receptor complex and their modulation by the diabetic milieu.

RESEARCH DESIGN AND METHODS

Experimental design

In vivo studies. Adult (aged 8–10 weeks) male Sprague-Dawley rats (Harlan Nossan, Milan, Italy) were divided into nondiabetic (n = 22) and diabetic (n = 22) groups. Rats were made diabetic by a single injection (via the caudal vein) of streptozotocin at a dose of 60 mg/kg body wt in citrate buffer (pH 4.5) (20). The animals were housed and cared in keeping with European Community regulations, and they received water and food ad libitum. Metabolic control was monitored by measuring body weight and blood glucose (by the glucose oxidase method) at regular intervals. When needed, diabetic animals were given supportive insulin treatment (Ultratard [Novo Nordisk, Copenhagen, Denmark] 4 IU/kg body wt twice a week) to prevent ketosis without significantly affecting blood glucose levels. The animals were studied 1 (n = 4), 2 (n = 6), 4 (n = 6), and 12 (n = 6) months after initiating the study. Under anesthesia with ketamine (Ketalar [Parke-Davis, Morris Plains, NJ] 60 mg/kg body wt i.m.) and xylazine (Rompum [Bayer, Leverkusen, Germany] 7.5 mg/kg body wt i.m.), the kidneys were removed, stripped of surrounding fat, and washed in sterile saline solution. A transverse slab was cut from the right kidney, fixed in 4% formaldehyde, and embedded in paraffin for subsequent immunofluorescence analysis of Gal-3, *p60*, and *p90* expression. The remaining kidney tissue was used for isolation of glomeruli (by sequential sievings) and total RNA extraction for the measurement of glomerular Gal-3 gene expression.

In vitro studies. Glomerular mesangial cells were isolated from human and rat kidneys and characterized as previously reported (3,21). Because the Gal-3 expression pattern was shown to vary with cell aging (i.e., increased basal and reduced serum-stimulated levels) (22), cells between the 3rd and the 15th passages (for human mesangial cells [HMCs]) and between the 5th and the 27th passages (for rat mesangial cells [RMCs]) were used in these experiments. Preliminary data showing unchanged rates of proliferation and apoptosis, unchanged patterns of matrix protein and cytokine production, and unchanged expression of mesangial cell-specific markers (Thy1.1, vimentin, and smooth muscle actin) indicated that these cells do not undergo detectable phenotypic changes at advanced passages in culture. Cells were seeded in 35- or 100-mm culture dishes (Falcon; Becton Dickinson, Lincoln Park, NJ) and grown to confluence in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 17% fetal bovine serum and 2 mmol/l L-glutamine and antibiotics (all obtained from Flow Laboratories, Irvine, Scotland, U.K.) at 37°C in a 95% air and a 5% CO_2 humidified atmosphere (3,21). Cells were exposed to one of the following 2 sets of conditions: *1*) media containing high glucose (HG) levels (30 mmol/l) as compared with those containing iso-osmolar mannitol (M) (24.5 mmol/l + 5.5 mmol/l glucose) or media containing normal glucose (NG) levels (5.5 mmol/l) for 1–4 weeks, or *2*) glycated bovine serum albumin with AGE formation (BSA-AGE) as compared with glycated BSA in which AGE formation was prevented by aminoguanidine (AM) (BSA-AM) or with nonglycated or native BSA, all previously prepared and either coated onto the culture dish or added to the culture media, for 4 days (3). Based on previous observations in fibroblasts showing that Gal-3 expression is cell cycle–dependent (i.e., it increases in proliferating cells and is markedly stimulated [up to 100-fold] by serum [23]), cells were studied at *1*) the lag phase (nonconfluent and slowly proliferating cells), *2*) the log phase (subconfluent and actively replicating cells), and *3*) the plateau phase (confluent cells growth arrested by serum deprivation for 48 h). Cells under these conditions were then detached and processed for immunofluorescence, flow cytometry, or Western blot or Northern blot analyses. Serum-free conditioned media were collected and stored for the assessment of Gal-3 content by enzyme-linked immunosorbent assay (ELISA).

Methods

BSA preparations. The BSA preparations used in these studies were obtained by incubating 1 mg/ml BSA (fraction V; Sigma) for 8 weeks at 37°C in phosphate-buffered saline (PBS) (containing antibiotics and protease inhibitors) in the presence or absence of 0.5 mol/l glucose ± 0.1 mol/l AM emisulphate (all obtained from Sigma) under sterile conditions (3,4,24). At the end of the incubation period, BSA preparations were dialyzed against PBS for 48 h under stirring to remove contaminating substances and were then purified through heparin-sepharose CL-6B columns (Pharmacia, Uppsala, Sweden). The endotoxin content of each preparation was assessed by the Limulus amoebocyte lysate assay (E-Toxate; Sigma) and was found to be negligible (<0.2 ng/ml). The assessment of AGE levels was performed by both fluorometric (25) and immunoenzymatic (26) methods. A polyclonal antibody raised against RNase-AGE, which does not recognize specific AGE structures, or a monoclonal antibody raised against BSA-AGE, which also recognizes N^{ε}-carboxymethillysine, was used in the competitive ELISA measurements. Results showed significantly increased AGE levels in BSA exposed to 0.5 mol/l glucose, as compared with native BSA, with increments ranging from 5- to 10-fold. Values were 88 ± 16 vs. 16 ± 2 relative fluorescence U/mg protein, as assessed fluorometrically, 43.7 ± 2.3 vs. 4.6 ± 0.5 AGE U/mg protein, as assessed by the ELISA using the polyclonal antibody, and 51.9 ± 3.2 vs. 4.8 ± 1.0 AGE U/mg protein, as assessed by the ELISA using the monoclonal antibody (P < 0.001 in all cases). These values are similar to (or lower than) those detected in the AGE preparations used in previous in vitro and in vivo studies (3,4,27,28), and they are also comparable to AGE levels observed in sera of diabetic patients (26,29) and in kidneys of experimental diabetic animals (30). Coincubation of BSA with 0.1 mol/l AM almost completely prevented AGE formation; AGE levels were only 50% higher than those in native BSA. The assessment of Amadori product in our BSA preparations was performed by boronate affinity gel chromatography (31), which revealed a slightly increased Amadori content in BSA-AGE versus BSA (7.6 ± 2.8 vs. 4.1 ± 0.5%, P < 0.01). To rule out the possibility that changes in the expression of the components of the AGE-receptor complex could be induced by Amadori products, we conducted additional experiments using BSA preparations subjected to reduction with a 200 molar excess of sodium borohydride (Sigma) at room temperature (RT) for 10 min initially and then on ice for 50 min. This procedure was followed by extensive dialysis (24). BSA preparations were coated onto the culture dish at the concentration of 0.05 nmol/cm^2 surface area by allowing it to dry under laminar flow. Dried coated dishes were then sterilized by overnight exposure to ultraviolet (UV) light in a tissue culture hood and rinsed with sterile deionized water. Based on earlier observations showing that soluble AGEs produce different effects on mesangial cell proliferation and matrix production, as compared with immobilized AGEs (3,32), we also conducted experiments in which glucose-modified or unmodified BSA preparations were added to the culture media at the concentration of 0.5–2.0 nmol/ml, as in previous publications (3,4,27).

Immunohistochemistry/cytochemistry. Cells were detached by trypsinization, seeded on sterile multiwell coverslips (Falcon), and maintained for another 24–48 h under the previous experimental conditions. At the end of this period, cells were fixed with 2% paraformaldehyde, washed with PBS, and

stored for subsequent immunofluorescence analysis (33). Fixed cells and deparaffinized kidney sections were incubated with PBS plus 1% BSA for 30 min at RT to block nonspecific reactivity. Slides were then washed and incubated for 1 h at RT with the primary antibody against Gal-3 (rabbit anti–rat affinity-purified polyclonal [34] or mouse anti–human B210 and A3A12 monoclonal [35] antibodies diluted 1:100 to 1:200), *p60* and *p90* (antisera produced in rabbit using purified recombinant OST-48 and 80K-H proteins [5] diluted 1:100 and 1:400, respectively), or Thy 1.1 (mouse monoclonal antibody from Serotec [Blackhorn Bicester, U.K.] diluted 1:100 as a positive control for mesangial cells). Specimens were washed and incubated for 1 h at RT with a swine anti–rabbit IgG or a rabbit anti–mouse IgG antibody conjugated with fluorescein (Dako, Glostrup, Denmark) and diluted 1:50–1:100. In some experiments, fixed cells were washed and counterstained with propidium iodide (1 µg/ml) for 20 min at RT. After washing, coverslips were mounted and preparations were observed and photographed using a Zeiss 310 Laser Confocal Microscope (Zeiss, Jena, Germany). Experiments in which the primary antibody was omitted or substituted with the appropriate nonimmune serum served as negative controls. Immunohistochemistry on kidney sections was performed in parallel with the immunoperoxidase method. Briefly, deparaffinized samples were treated with 0.3% H_2O_2 in methanol to block endogenous peroxidases and were then incubated with the primary antibody for 30 min at RT, followed by the biotinylated peroxidase-conjugated secondary antibody for 30 min at RT and the peroxidase substrate for 5 min to reveal the reaction. Subsequently, the sections were counterstained with hematoxylin for 3 min, dehydrated, mounted, and observed. Glomerular expression of Gal-3 was evaluated semiquantitatively by assigning it a grade (0 indicated no staining, and 1–4 indicated 1–10, 11–25, 26–50, and >50% of positive glomerular area, respectively). At least 50 glomeruli per animal were scored by an investigator blinded to the group assignment, and results were expressed as percent of glomeruli for each scoring grade.

Flow cytometry. For flow cytometry analysis (33), cells were detached using the cell dissociation solution (Sigma), fixed in 4% formaldehyde for 30 min, and then washed. After centrifugation, the pellet was incubated with the anti–rat or anti–human Gal-3 antibody (diluted 1:50 to 1:100) for 1 h at 37°C and was washed and incubated again with the corresponding secondary antibody conjugated with fluorescein (diluted 1:50 to 1:100) for 45 min at 37°C. One aliquot was used for evaluating autofluorescence (omission of both antibodies), and another aliquot was used to assess the nonspecific binding of the secondary antibody (omission of the primary antibody). After washing, the pellet was resuspended in PBS and analyzed in an Epics Profile flow cytometer (Coulter, Mialeah, FL).

Western blot analysis. To obtain cell lysates for Western blot analysis (36), cells were scraped, pelleted by centrifugation, and disrupted by incubation for 15 min at 4°C in lysing buffer (25 mmol/l Tris-HCl, pH 7.4, 0.2% SDS, 50 mmol/l NaCl, 0.5% Na-deoxycholate, 2% Nonidet P-40, 1 mmol/l phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, and 2 µg/ml leupeptin) under continuous agitation. After centrifugation, the supernatant was collected and assayed for protein content by using the Bradford method with a protein microassay kit (Pierce, Rockford, IL). Protein samples (10–15 µg) were added with an equal volume of sample buffer 2× (100 mmol/l Tris-HCl, pH 7.4, 5% SDS, 10% saccharose, 1 mmol/l Na_2 EDTA, 0.025% bromophenol blue, and 0.1 mol/l dithiotreitol) separated by SDS-PAGE (10% acrylamide) and transferred by electroblotting using a Mini Protean II (Bio-Rad Laboratories, Hercules, CA) onto polyvinylidene fluoride membranes (Amersham, Amersham, U.K.). The membranes were incubated overnight at 4°C under agitation with Tris-buffered saline (TBS)-Tween (TBS plus 0.5% Tween 20) plus 5% nonfat dry milk (NFDM) to block the nonspecific reactivity, and they were then probed for 1 h at RT under agitation with the primary antibody diluted 1:100 (anti–Gal-3), 1:750 (anti-*p60*), or 1:3,000 (anti-*p90*) in TBS-Tween plus 3% NFDM. Subsequently, the membranes were incubated for 45 min at RT with a goat anti–rabbit or a rabbit anti–mouse IgG antibody conjugated with peroxidase (Dako) diluted 1:1,500 in TBS-Tween plus 3% NFDM, and were washed and developed with an enhanced chemiluminescence (ECL) reagent (Amersham). To detect Gal-3 dimers, which are stable in SDS under reducing conditions, exposure to the ECL reagent was prolonged to 15 min. Immunocomplexes were revealed by autoradiography and quantitated by scanning densitometry with a Gel Doc 2000 Densitometer equipped with the Quantity One software (Bio-Rad Laboratories). Results were normalized to the signal of heat shock protein 70, as revealed by the use of a mouse monoclonal antibody (Sigma).

ELISA. ELISA measurements of Gal-3 content were performed on conditioned media from mesangial cells. In this assay, the anti-Gal 3 antibody was coated onto a 96-well plate (Nunc, Roskilde, Denmark) at 37°C overnight, and residual binding sites were saturated with PBS containing 1% BSA, 0.01% Thimerosal, and 0.05% Tween 20 for 1 h at 37°C. The medium samples or the standard (rat or human) Gal-3 were then added for 3 h at 4°C, after which the same anti–Gal 3 antibody (diluted 1:100 to 1:200) and the secondary antibody conjugated with peroxidase were added, both for 1 h at RT. Finally, the reaction was revealed by addition of the substrate for 15 min at RT, and absorbances at 450 nm were measured using an automated microtiter plate reader (Titertek Multiscan; Flow Laboratories). Results were normalized to the DNA content of the correspondent monolayer, as measured fluorometrically as previously reported (3).

Northern blot. Total RNA was extracted from isolated glomeruli and mesangial cells by the guanidine thiocyanate-phenol-chloroform method using the RNAfast kit (Molecular Systems, San Diego, CA) and quantified by measuring the absorbance at 260 nm in a Beckman DU-65 spectrophotometer (Beckman Instruments, Fullerton, CA). Transcripts were measured by Northern blot analysis (3) using the 1,058-bp rat Gal-3 cDNA (37), the 2,200-bp human Gal-3 cDNA (14), and the 2,200-bp human β-actin cDNA (3) as probes. Total RNA samples (30 µg per lane) were electrophoresed under denaturing conditions in 1.2% agarose gel (Life Technologies, Gaithersburg, MD), transferred onto Hybond-N nylon blotting membranes (Amersham), and UV-fixed in Spectrolinker XL-1000 UV Crosslinker (Spectronics, Westbury, NY). Filters were prehybridized in Rapid-Hyb Buffer (Amersham) for 15 min at 65°C in the Mini Oven MK-II (Hybaid, Teddington, Middlesex, U.K.) and were then hybridized for 2 h in the same solution containing 25 ng of cDNA labeled using the Prime-IT II Random Primer Labeling Kit (Stratagene, La Jolla, CA) and 50 µCi of [α-^{32}P]dCTP (3,000 Ci/mmol) (Amersham) as a precursor. Filters were sequentially washed for 15 min at 65°C with sodium chloride and sodium citrate (SSC) 2×, SSC 1×, and SSC 0.1× plus 0.1% SDS. The filters were then exposed to Hyperfilm-MP autoradiography films (Amersham) with the aid of intensifying screens at –70°C. Quantification of Gal-3 mRNA levels was performed by scanning densitometry, and results were normalized to the signal of the "housekeeping" gene β-actin to account for differences in sample loading.

Statistical analysis. Values are expressed as means ± SD. We also calculated the percentage of change in values compared with controls. Statistical significance was evaluated by one-way analysis of variance followed by the Student-Newman-Keuls test for multiple comparisons. All statistical tests were performed on raw data.

RESULTS

In vivo studies. In diabetic rats, metabolic derangement was evidenced by impaired body weight and increased blood glucose levels versus nondiabetic control rats (Table 1).

Gal-3 protein was not demonstrable in glomeruli from nondiabetic rats at 1,2 (Fig. 1*A*), and 4 months, but it became detectable at 12 months (Fig. 1*B*). At this time, 18.5% of all glomeruli were still negative, but 81.5% were positive, although most of them (48.3% of total) were positive in ≤25% of glomerular area (Table 2). Expression of Gal-3 was already observed in glomeruli from diabetic rats at 2 months of disease duration (Fig. 1*D*), and it increased thereafter (Fig. 1*E*). At 12 months, Gal-3 protein was detected in 100% of glomeruli; staining covered >50% of glomerular area in 47.7% of all glomeruli, indicating a more pronounced expression compared with age-matched nondiabetic controls (Table 2). Glomerular mRNA expression for Gal-3 followed the same pattern as Gal-3 protein, with undetectable levels in nondiabetic rats (Fig. 2*A*) and a visible 1.1-kb band in diabetic rats (Fig. 2*B*) at 2 months. Conversely, Gal-3 was expressed in tubuli from both nondiabetic and diabetic rats, with an increased staining in diabetic versus nondiabetic animals at all time points examined (Fig. 1*C* and *F*).

Both *p60* and *p90* were demonstrable in glomeruli of nondiabetic rats, whereas they were only barely detectable at the tubular level (Fig. 3*A* and *B*). The glomerular expression of *p90*, but not *p60*, was slightly increased by diabetes (Fig. 3*C* and *D*).

In vitro studies. No Gal-3 was demonstrable in mesangial cells cultured in NG before a certain number of passages in culture (Figs. 4*A* and 5*A*); thereafter, it became progressively detectable (Fig. 6*A* and *B*): Gal-3 levels in 20th-passage RMCs were 2.15-fold higher than those detected in 12th-passage RMCs, as assessed by Western blot analysis (Fig. 6*A* and *B*). The mRNA expression showed the same pattern, with no visible signal in early passage cells (Fig. 2*C*) and a progres-

TABLE 1
Metabolic parameters in nondiabetic and diabetic rats at 0, 1, 2, 4, and 12 months

	n	Time (months)	Age (weeks)	Body weight (g)	Blood glucose (mmol/l)
Nondiabetic rats	22	0	12	248 ± 12	5.57 ± 0.3
	4	1	16	368 ± 10	5.49 ± 0.4
	6	2	20	438 ± 11	5.79 ± 1.0
	6	4	28	552 ± 17	5.84 ± 0.8
	6	12	60	612 ± 16	5.79 ± 1.3
Diabetic rats	22	0	12	251 ± 16	24.87 ± 2.8*
	4	1	16	242 ± 44*	24.98 ± 1.1
	6	2	20	311 ± 29	25.08 ± 1.3*
	6	4	28	308 ± 41*	25.17 ± 1.8*
	6	12	60	349 ± 62*	25.09 ± 2.1*

Data are presented as mean ± SD. *P < 0.001 vs. age-matched nondiabetic rats.

sively more evident band in the more advanced passages (data not shown). Cells grown on BSA always showed a diffuse (cytoplasmic) staining at immunofluorescence (Fig. 4*D*), corresponding to a peak at flow cytometry (Fig. 5*D*) and a band at Western blot (Fig. 6*C* and *D*) and Northern blot (Fig. 2*D*) analyses. Gal-3 expression showed slight changes according to the replicative state of the cell, showing an ~30% increase during the log phase compared with the lag and plateau phases (Fig. 6*A*–*D*).

Prolonged exposure (2–4 weeks) of RMCs or HMCs to HG, but not to M, induced Gal-3 protein (Figs. 4*A*–*C* and 5*A*–*C*) and mRNA expression (Fig. 2*C*), or it significantly increased their levels (up to 2.65-fold) when Gal-3 was expressed under basal conditions (Fig. 6*A* and *B*). Increases were ~75% more pronounced in early-passage than in advanced-passage cells and ~50% higher in the log phases than in the lag or plateau phases of cell growth (Fig. 6*A* and *B*). Likewise, coated BSA-AGE (and BSA-AM to a lesser extent) significantly increased protein (Figs. 4*D*–*F*, 5*D*–*F*, and 6*C* and *D*) and mRNA expression (Fig. 2*D*); this effect was more pronounced in early-passage (up to 2.38-fold) than in late-passage (up to 1.67-fold) cells and, to a lesser extent (by ~45%),

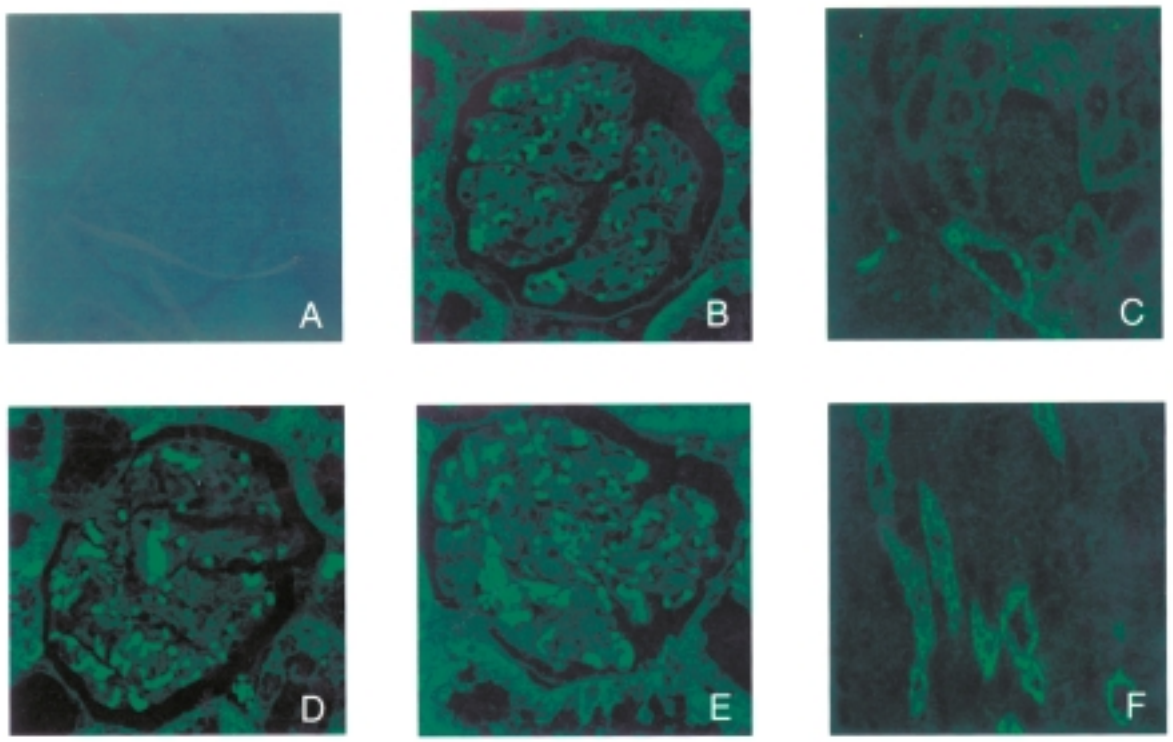

FIG. 1. Immunofluorescence analysis of Gal-3 expression in kidney sections from nondiabetic and diabetic Sprague-Dawley rats at 2 and 12 months. *A*: Glomerular expression of Gal-3 in a nondiabetic rat at 2 months. *B*: Glomerular expression of Gal-3 in a nondiabetic rat at 12 months. *C*: Tubular expression of Gal-3 in a nondiabetic rat at 2 months. *D*: Glomerular expression of Gal-3 in a diabetic rat at 2 months. *E*: Glomerular expression of Gal-3 in a diabetic rat at 12 months. *F*: Tubular expression of Gal-3 in a diabetic rat at 2 months.

TABLE 2
Semiquantitative analysis of Gal-3 immunoreactivity in glomeruli from nondiabetic and diabetic rats at 0, 1, 2, 4, and 12 months

Grade	*n*	Time (months)	Age (weeks)	Percent of glomeruli	
				Nondiabetic	Diabetic
0 (0%)	4	1	16	100.0 ± 0.0	100.0 ± 0.0
	6	2	20	100.0 ± 0.0	17.3 ± 3.3*
	6	4	28	100.0 ± 0.0	9.2 ± 3.1*
	6	12	60	18.5 ± 3.0)	0.0 ± 0.0*
1 (1–10%)	4	1	16	0.0 ± 0.0	0.0 ± 0.0
	6	2	20	0.0 ± 0.0	21.5 ± 3.3*
	6	4	28	0.0 ± 0.0	12.8 ± 2.3*
	6	12	60	17.3 ± 3.5	7.3 ± 4.2*
2 (11–25%)	4	1	16	0.0 ± 0.0	0.0 ± 0.0
	6	2	20	0.0 ± 0.0	27.0 ± 2.6*
	6	4	28	0.0 ± 0.0	21.5 ± 3.3*
	6	12	60	31.0 ± 2.4	15.7 ± 3.0*
3 (26–50%)	4	1	16	0.0 ± 0.0	0.0 ± 0.0
	6	2	20	0.0 ± 0.0	19.7 ± 3.5*
	6	4	28	0.0 ± 0.0	34.2 ± 2.9*
	6	12	60	17.0 ± 2.7	29.3 ± 5.1*
4 (>50%)	4	1	16	0.0 ± 0.0	0.0 ± 0.0
	6	2	20	0.0 ± 0.0	14.5 ± 2.4*
	6	4	28	0.0 ± 0.0	22.3 ± 3.4*
	6	12	60	16.2 ± 4.2	47.7 ± 6.2*

Data are presented as mean ± SD. *P < 0.001 vs. age-matched nondiabetic rats.

in actively replicating cells versus slowly proliferating or starved cells (Fig. 6*C* and *D*). Soluble BSA-AGE also enhanced Gal-3 expression, as compared with native BSA, although these increases were modest (up to 1.53-fold) (data not shown). Previous borohydride reduction of BSA preparations to remove Amadori products did not affect Gal-3 expression in cells grown on these proteins compared with cells grown on unreduced preparations (Fig. 6*G*). Gal-3 secretion in the medium was also increased (P < 0.001) in response to both HG (by 53%) and BSA-AGE (by 50%) versus corresponding controls (Fig. 7). By immunofluorescent confocal microscopy, cells cultured under HG and BSA-AGE showed a distinct patchy distribution of Gal-3 fluorescence, in addition to the diffuse pattern, with scanning analysis indicating both a cytoplasmic and cell surface localization of granules (Fig. 4*G*). Western blot analysis also showed increased levels (up to 2.8-fold) of SDS- and dithiotreitol-resistant Gal-3 dimers under these conditions (Fig. 6*E* and *F*).

Both *p60* and *p90* were demonstrable in mesangial cells cultured in the presence of NG (Figs. 8*A* and *C* and 9*A* and *C*) and BSA (Fig. 9*B* and *D*). The expression of *p90* was increased by

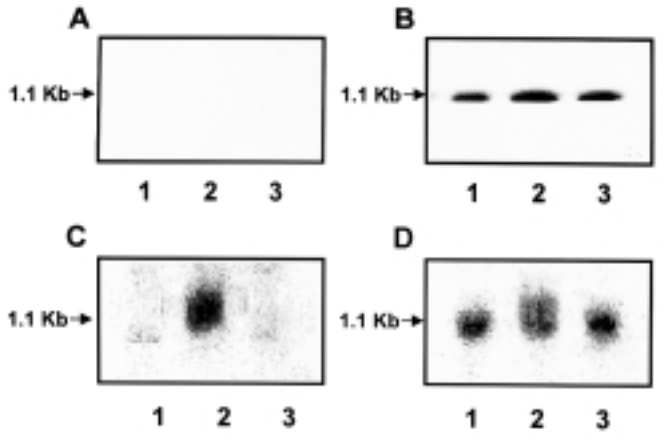

FIG. 2. Northern blot analysis of Gal-3 mRNA expression in glomeruli from selected nondiabetic (*A*) and diabetic (*B*) Sprague-Dawley rats at 2 months and in eighth-passage RMCs grown for 4 weeks in NG (lane 1), HG (lane 2), and M (lane 3) (*C*) and for 4 days on BSA (lane 1), BSA-AGE (lane 2), and BSA-AM (lane 3) (*D*).

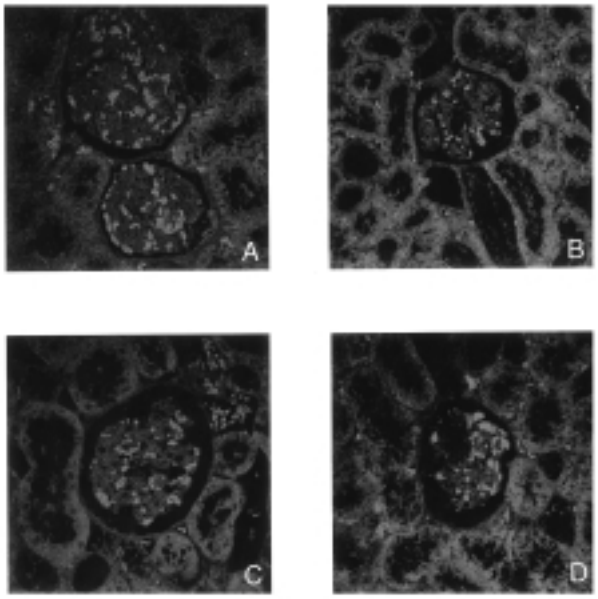

FIG. 3. Immunofluorescence analysis of Gal-3, *p60*, and *p90* protein expression in kidney sections from nondiabetic and diabetic Sprague-Dawley rats at 2 months. *A*: Renal expression of *p60* in a nondiabetic rat. *B*: Renal expression of *p90* in a nondiabetic rat. *C*: Renal expression of *p60* in a diabetic rat. *D*: Renal expression of *p90* in a diabetic rat.

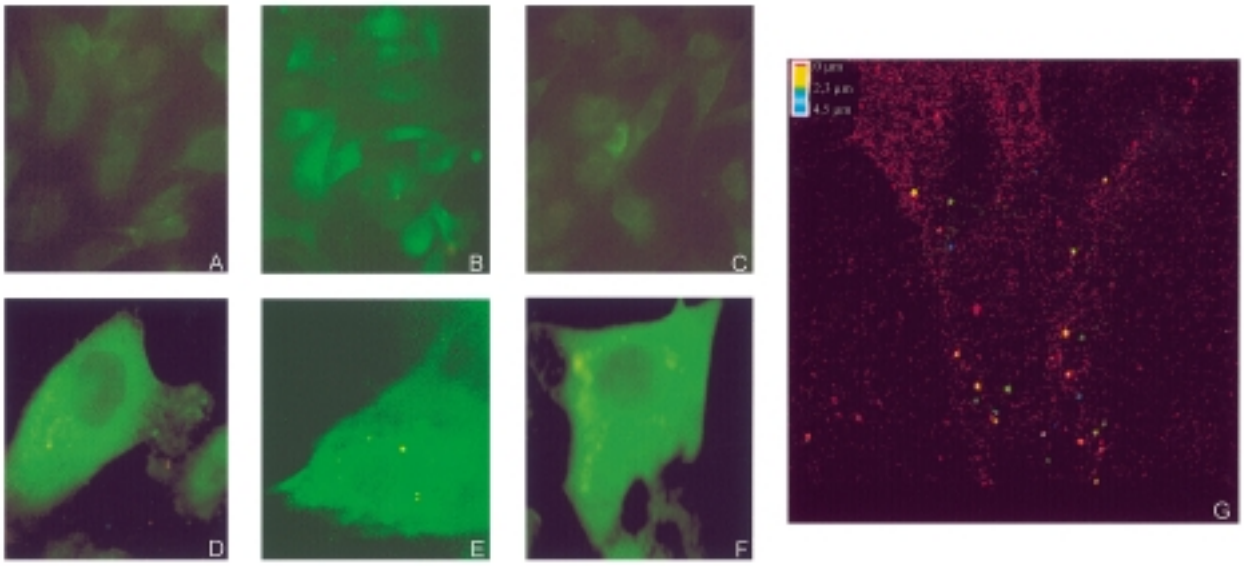

FIG. 4. Immunofluorescence analysis of Gal-3 protein expression in eighth-passage RMCs grown for 4 weeks in NG (*A*), HG (*B*), and M (*C*) and for 4 days on BSA (*D*), BSA-AGE (*E*), and BSA-AM (*F*) and scanning confocal microscopy image of RMCs grown on BSA-AGE (*G*).

24% by HG (Figs. 8*D* and 9*C*) and by 22% by BSA-AGE (Fig. 9*D*), whereas that of *p60* was not changed by either HG (Figs. 8*B* and9*A*) or BSA-AGE (Fig. 9*B*).

DISCUSSION

This study describes both the glomerular/mesangial expression of the components of the AGE-receptor complex, which include *p60*, *p90*, and the novel AGE-binding protein Gal-3, and their modulation by the diabetic milieu. The results indicate that *1*) under basal conditions, Gal-3, unlike *p90* and *p60*, is not detectable in the mesangium but becomes expressed with aging, and *2*) the diabetic milieu induces or upregulates Gal-3 production, whereas it only slightly increases the expression of *p90*, but not *p60*.

The finding that Gal-3 is not detectable under normal conditions at the glomerular/mesangial level, both in vivo and in vitro, is consistent with previous reports in early human embryos and postnatal kidney samples (38) as well as in the experimental animals and mesangial cells derived from them (39). Our results also show that cell cycle variations do not play a major role in modulating Gal-3 expression within the mesangium, whereas aging markedly influences it. The fact that this molecule becomes detectable in glomeruli from aging nondiabetic animals supports the hypothesis that its expression in advanced passage mesangial cells cultured in NG is an age-dependent phenomenon and not the result of a phenotypic change (dedifferentiation) induced by the in vitro environment. The expression of Gal-3 in cells grown on native BSA suggests that this age-dependent process may be accelerated by cell-protein interactions, possibly due to the adhesive properties of Gal-3 (12). However, the expression of Gal-3 under this condition might also be induced by the low but measurable amount of AGEs contained in these preparations (3); likewise, the positive Gal-3 staining observed in glomeruli from older rats might be related to the progressive AGE accumulation occurring in aging animals (2).

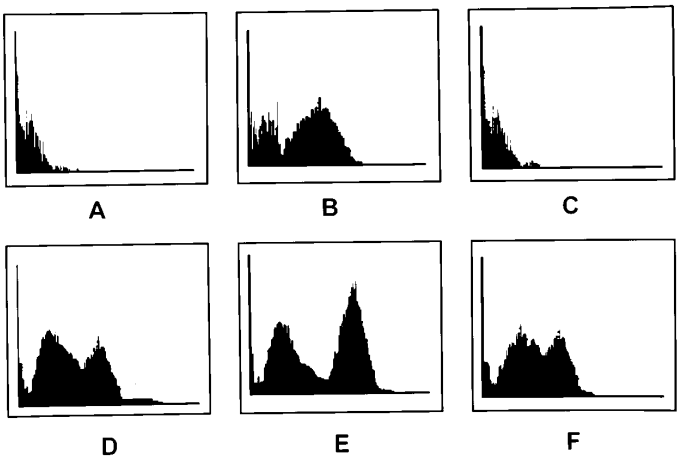

FIG. 5. Flow cytometry analysis of Gal-3 protein expression in eighth-passage RMCs grown for 4 weeks in NG (*A*), HG (*B*), and M (*C*) and for 4 days on BSA (*D*), BSA-AGE (*E*), and BSA-AM (*F*). The first peak indicates autofluorescence.

The finding that both *p60* and *p90* are normally expressed in the glomerulus indicates that these proteins participate in mediating cell binding of AGEs at this level, as previously shown (2,4). The fact that these components of the AGE-receptor complex are weakly expressed in tubuli also indicates that they may not be operating in transducing the effects of AGEs at the tubular level. The report that BSA-AGE binding into the renal cortex of experimental animals was localized primarily to proximal tubules (and was significantly enhanced by diabetes) (40) suggests that the interaction of AGEs with tubular epithelial cells is mediated by other AGE-binding proteins, possibly RAGE (41).

Additionally, the finding that the expression of Gal-3 in the diabetic milieu is induced or upregulated, both in vivo and in vitro, whereas that of *p60* and *p90* is not or is only modestly affected, is in keeping with a recent report on human umbilical vein endothelial cells exposed to AGEs (42) and also with preliminary data on NOD mice; however, a significant reduction in *p60* levels was reported in this study (43). The findings that the effects of HG and AGEs on Gal-3 content in mesangial cells were modestly affected by the proliferative

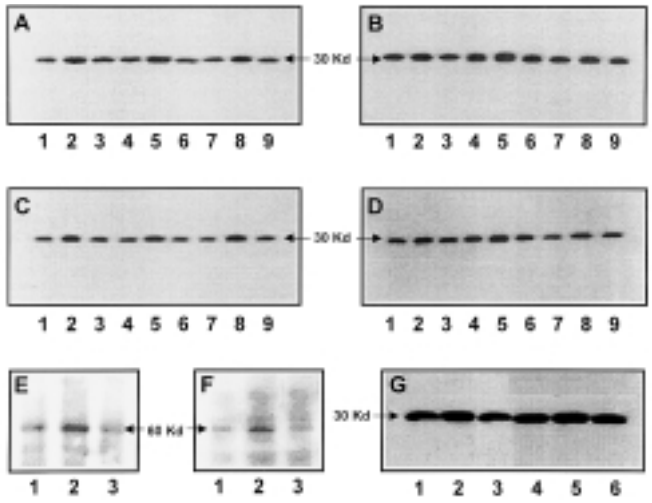

FIG. 6. Western blot analysis of Gal-3 protein expression in 12th- and 20th-passage RMCs grown in NG, HG, and M for 4 weeks and on BSA, BSA-AGE, and BSA-AM for 4 days. Monomeric form in the 12th-passage (*A*) and the 20th-passage (*B*) RMCs (lanes 1, 2, and 3 represent NG, HG, and M cells, respectively, in the lag phase; lanes 4, 5, and 6 represent NG, HG, and M cells, respectively, in the log phase; and lanes 7, 8, and 9 represent NG, HG, and M cells, respectively, in the plateau phase). Monomeric form in the 12th-passage (*C*) and the 20th-passage (*D*) RMCs (lanes 1, 2, and 3 represent BSA, BSA-AGE, and BSA-AM cells, respectively, in the lag phase; lanes 4, 5, and 6 represent BSA, BSA-AGE, and BSA-AM cells, respectively, in the log phase; and lanes 7, 8, and 9 represent BSA, BSA-AGE, and BSA-AM cells, respectively, in the plateau phase). *E*: Multimeric forms in the 12th-passage RMCs (lane 1 indicates NG, lane 2 indicates HG, and lane 3 indicates M). *F*: Multimeric forms in 12th-passage RMCs (lanes 1, 2, and 3 indicate BSA, BSA-AGE, and BSA-AM). *G*: Monomeric forms in 12th-passage RMCs (lanes 1, 2, and 3 indicate unreduced BSA, BSA-AGE, and BSA-AM, respectively, and lanes 4, 5, and 6 indicate sodium borohydride reduced BSA, BSA-AGE, and BSA-AM, respectively).

state, but markedly influenced by the passage in culture, further support the concept that the cell cycle dependence of Gal-3 production is less evident in the mesangium than in other tissues, whereas cell aging plays an important role in modulating Gal-3 expression.

Both an increased synthesis and a reduced breakdown of Gal-3, the latter being due to the reported reduction of the activity of metalloproteinases (known to enzymatically cleave Gal-3 [12]) might be implicated in mediating the upregulation of this lectin induced by the diabetic milieu. The induction of Gal-3 in glomeruli of diabetic and aging animals prompts the speculation that the increase in levels of circulating and tissue AGEs, which occurs in both conditions (though to different extents), alters the expression pattern of the AGE-receptor complex. The concept that the effect of chronic hyperglycemia or prolonged incubation in HG are in fact mediated, at least partly, by AGEs is further supported by the time lag between induction of diabetes or exposure to HG and occurrence of Gal-3 expression or overexpression as well as by the similarity between HG- and AGE-induced changes. On the other hand, the fact that removal of Amadori products by borohydride reduction did not affect Gal-3 levels, as compared with the corresponding unreduced preparations, suggests that these early sugar adducts do not play a major role in regulating Gal-3 expression.

The distinct patchy distribution of Gal-3 fluorescence and the increased levels of Gal-3 dimers observed in cells exposed

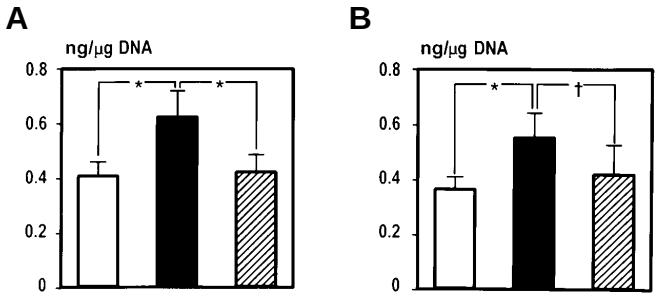

FIG. 7. ELISA assessment of Gal-3 protein content (means ± SD) in media from 12th-passage RMCs grown for 4 weeks in NG (□), HG (■), and M (▨) (*A*) and for 4 days on BSA (□), BSA-AGE (■), and BSA-AM (▨) (*B*). **P* < 0.001; †*P* < 0.01.

to the diabetic milieu suggest that Gal-3 undergoes dimerization/multimerization. This phenomenon has been previously reported to be a concentration-dependent process mediated by the NH_2-terminal domain, occurring when Gal-3 is bound to its ligands via the CRD (9), whereas the COOH-terminal domain appears to be implicated in multimerization only in the absence of saccharides (44). Because the interaction between AGEs and Gal-3 also involves the CRD, Gal-3 multimerization in response to HG or AGEs could be a consequence of the increased Gal-3 expression and binding induced by AGEs. Moreover, it is compatible with the reported Gal-3 AGE-receptor function, as shown in monocytes-macrophages (6).

Taken together, these data indicate that diabetes-induced glomerular injury could be mediated also by a modification of the expression pattern of the components of the AGE-receptor complex, possibly altering the function of this receptor. These changes might be compensatory for the increased AGE levels by producing an increment in the glomerular/mesangial AGE-binding capacity. On the other hand, the prevailing qual-

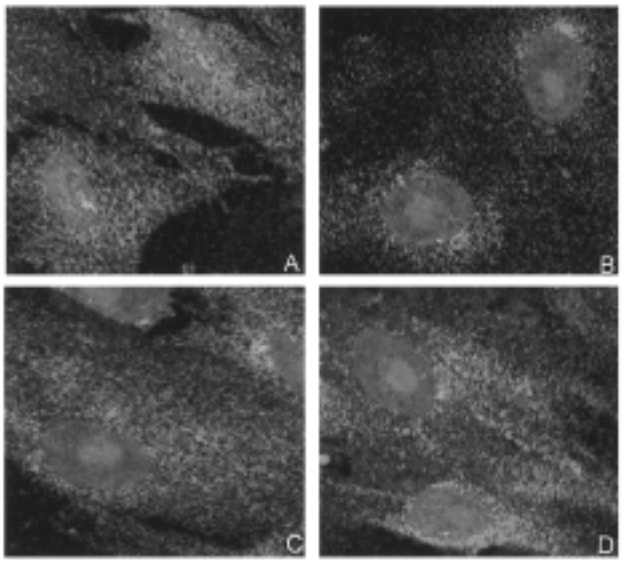

FIG. 8. Immunofluorescence analysis of *p60* (*A*) and *p90* (*C*) protein expression in eighth-passage RMCs grown for 4 weeks in NG versus *p60* (*B*) and *p90* (*D*) protein expression in eighth-passage RMCs grown for 4 weeks in HG.

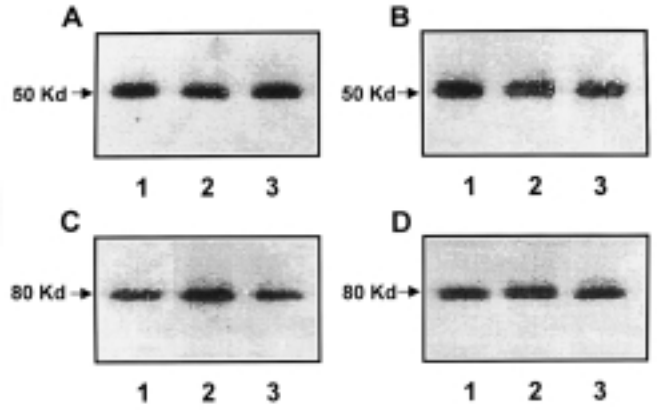

FIG. 9. Western blot analysis of p60 (*A*) and p90 (*C*) protein expression in eighth-passage RMCs grown for 4 weeks in NG (lane 1), HG (lane 2), and M (lane 3). Western blot analysis of *p60* (*B*) and *p90* (*D*) protein expression in eighth-passage RMCs grown for 4 days on BSA (lane 1), BSA-AGE (lane 2), and BSA-AM (lane 3).

itative nature of the alterations in the components of this receptor, particularly the induction of Gal-3, could imply that pathways not operating under normal conditions (or not required for binding the small amounts of AGEs formed under these circumstances) are activated as a consequence of changes in the AGE-receptor complex induced by the diabetic milieu. Based on the possible association of Gal-3 with *p90* (6), it may be speculated that the induction of Gal-3 expression by hyperglycemia enables *p90* to transduce the AGE-mediated effects on cell activation. The slight increase in *p90* expression associated with Gal-3 induction could be part of this scenario. However, the impact of the altered expression of the AGE-receptor complex on the development of diabetic glomerulopathy is difficult to establish. This complication is a result of the dual function of AGE receptors mediating both AGE degradation and AGE-induced cell activation as well as of not having complete knowledge of the molecular mechanisms underlying Gal-3 receptor function and its interaction with other AGE-binding proteins.

The increased expression of Gal-3 occurring in diabetes may have further implications in the pathogenesis of glomerulopathy due to the multifunctional nature of this lectin. Enhanced Gal-3 levels may in fact participate in the pathogenesis of mesangial expansion by modifying cell-matrix interactions. Altered cell contacts might favor the increased synthesis and reduced degradation of matrix components, which lead to their accumulation within the mesangium. Gal-3 overexpression may also contribute to the expansion of the cell compartment by stimulating cell growth and proliferation and macrophage activation (39). Finally, the antiapoptotic effect of Gal-3 might also be involved, even though this effect would be inhibited by the interaction of the CRD with ligands, such as saccharides and, possibly, AGEs (17).

In conclusion, these results suggest that hyperglycemia, in addition to increasing AGE formation and accumulation within the mesangium, is capable of modulating the AGE-receptor–mediated effects and possibly influencing directly the process of glomerular remodeling by modifying the expression pattern of the AGE-binding protein and adhesive lectin Gal-3. In this regard, hyperglycemia appears to accelerate and/or exacerbate the effect of aging, with both conditions possibly acting through an increased deposition of AGEs.

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