

Early Glomerular Macrophage Recruitment in Streptozotocin-Induced Diabetic Rats

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Diabetic glomerulosclerosis is defined by increased glomerular extracellular matrix (ECM) that is mainly synthesized by mesangial cells that underwent an activation mediated by cytokines and growth factors from various cellular origins. In this study, we tested whether macrophages could infiltrate the glomeruli and influence ECM synthesis in experimental diabetes. To test our hypothesis, we initially studied the dynamics of glomerular macrophage recruitment in streptozotocin-induced diabetic rats at days 1, 2, 4, 8, 15, and 30 by using quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) on isolated glomeruli and immunohistochemistry and morphometry. We then assessed the role of macrophages on the basis of the pharmacological modulation of their recruitment by insulin or ACE inhibitor treatments and by X-irradiation-induced macrophage depletion at days 8 and 30. Macrophages were recruited within the glomeruli at the very early phase of hyperglycemia by using RT-PCR CD14 detection from day 2 and by using ED1 immunohistochemistry from day 8. This glomerular macrophage infiltration was associated with an increase in α_1 -chain type IV collagen mRNA. In parallel, the diabetic glomeruli became hypertrophic with an increase in the mesangial area. Macrophage recruitment was preceded by or associated with an increased glomerular expression of vascular cell adhesion molecule 1, intracellular adhesion molecule 1, and monocyte chemoattractant protein 1, which contributes to monocyte diapedesis. Glomerular interleukin-1 β mRNA synthesis was also enhanced as early as day 1 and could be involved in the increase in ECM and adhesion molecule gene expressions. Insulin treatment and irradiation-induced macrophage depletion completely prevented the glomerular macrophage recruitment and decreased α_1 -chain type IV collagen mRNA and mesangial area in diabetic rats, whereas ACE inhibitor treatment had an incomplete effect. It can be concluded that in the streptozotocin model, hyperglycemia is followed by an early macrophage recruitment that contributes to the

molecular and structural events that could lead to glomerulosclerosis. Therefore, besides direct stimulation of mesangial cells by hyperglycemia, macrophages recruited in the glomeruli during the early phase of hyperglycemia could secondarily act on mesangial cells. *Diabetes* 49:466–475, 2000

In occidental countries, diabetes is currently the major cause of chronic renal failure. Diabetic nephropathy is characterized mainly by glomerulosclerosis. Various pathological conditions that injure the glomerulus lead to glomerulosclerosis, which is a common lesion that can be considered a scar or an active progressive phenomenon that hampers glomerular filtration and results in proteinuria. Glomerulosclerosis is defined by an increase in extracellular matrix (ECM) (1). In diabetes, it is mainly composed of α_1 - and α_2 -chain type IV collagens, laminin, and fibronectin (2). ECM accumulation could result from nonmutually exclusive mechanisms: increased synthesis of ECM macromolecules (3) and/or decreased degradation because of nonenzymatic glycation (4) and reduced collagenase activity (5). Increased ECM synthesis depends mainly on mesangial cell activation (i.e., proliferation, matrix and cytokine-growth factor production) (6). Mesangial cells are directly activated in vitro by high levels of glucose, which stimulate matrix synthesis (7), protein kinase C (8), protooncogen c-fos and c-jun expression (9), and synthesis of transforming growth factor (TGF)- β and platelet-derived growth factor (PDGF) (10,11) with an autocrine action. Furthermore, in vivo, mesangial cells could be indirectly stimulated by other cell types present within the glomerulus in diabetes; a candidate cell is macrophage because of its ability to infiltrate tissues from the blood stream and to produce paracrine growth factors. The macrophage has been implicated in many pathological conditions in which kidney is injured (12), mainly focusing on acute glomerulonephritides where glomerular inflammatory infiltration by macrophages is major. However, macrophages are also observed at a lower level in chronic glomerular involvement that is induced by nonimmune mechanisms (13–16). In such circumstances, the role of macrophages and the mechanisms of their recruitment are not completely understood. In this study, we tested the hypotheses that in streptozotocin-induced diabetic rats, the macrophages could be recruited in the glomeruli and that their recruitment is associated with adhesion molecule synthesis, cytokine synthesis, and type IV collagen mRNA synthesis to lead to glomerular structural changes through a discrete inflammatory phenomenon.

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ANOVA, analysis of variance; BSA, bovine serum albumin; DTT, dithiothreitol; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GS, glomerulosclerosis; ICAM, intercellular adhesion molecule; IL, interleukin; MCP, monocyte chemoattractant protein; PCR, polymerase chain reaction; PDGF, platelet-derived growth factor; RT-PCR, reverse transcriptase-polymerase chain reaction; TGF, transforming growth factor; VCAM, vascular cell adhesion molecule.

RESEARCH DESIGN AND METHODS

Animal models

Study 1: dynamics of macrophage recruitment. Male Sprague-Dawley rats aged 10 weeks (Charles River, St. Aubin lès Elbeuf, France) were used in this protocol. Diabetic rats ($n = 36$) and controls ($n = 36$) were given free access to food and water. The experiments were conducted in accordance with institutional guidelines and the recommendations for the care and use of laboratory animals put forward by the French Ministry of Agriculture (authorization no. 004814). Diabetes was induced by a single intraperitoneal injection of 70 mg/kg body weight of streptozotocin (Sigma, St. Louis, MO) in 0.5 ml of citrate buffer 0.05 mmol/l, pH 4.6. Animals presenting glycosuria values >10 mmol/l the day after streptozotocin injection were enrolled in the diabetic group. Diabetic and control rats were killed 1, 2, 4, 8, 15, and 30 days after the assessment of glycosuria. Six rats from each group were used for RNA extraction, morphological studies, and immunohistochemical studies at each time. All rats were weighed, and levels of glycemia were determined by using a Glucometer II (Bayer Diagnostic, Puteaux, France) just before death. Under anesthesia with sodium pentobarbital, both kidneys were perfused with 20 ml of a saline solution (NaCl 135 mmol/l, Na_2PO_4 1 mmol/l, Na_2SO_4 1.2 mmol/l, MgSO_4 1.2 mmol/l, KCl 5 mmol/l, CaCl_2 2 mmol/l, glucose 5.5 mmol/l, and HEPES 5 mmol/l, pH 7.4) at 4°C, as derived from the technique described by Peten et al. (17). Left kidney was then perfused alone at 37°C with 3 ml of the saline solution containing 1 mg/ml type I collagenase (Sigma), 1 mg/ml bovine serum albumin (BSA) (Sigma), 1.2 U/ μl human placental RNase inhibitor (RNasin; Promega, Madison, WI), and 1 mmol/l dithiothreitol (DTT) (Sigma). Both kidneys were then carefully removed and weighed.

Study 2: pharmacological modulation. Male Sprague-Dawley rats aged 10 weeks were divided into four groups: 1) control rats ($n = 18$), 2) diabetic rats ($n = 18$), 3) insulin-treated diabetic rats ($n = 18$), and 4) ACE inhibitor-treated diabetic rats ($n = 18$). Diabetes was induced according to the above-described protocol. Treatment started on the day of the assessment of glycosuria. The insulin-treated diabetic rats received daily a subcutaneous injection of 6 IU of insulin (Ultralente; Novo Nordisk, Boulogne-Billancourt, France); the ACE inhibitor-treated diabetic rats received 10 mg \cdot kg $^{-1} \cdot$ day $^{-1}$ of Cilazapril (Hoffmann-LaRoche, Basel, which was obtained from Dr. Jean-Paul Clozel (Basel, Switzerland), as food admix. The systolic blood pressure at day 7 was measured by using the tail-cuff method in control, diabetic, and ACE inhibitor-treated diabetic rats. Nine rats from each group were killed after 8 and 30 days of experimentation. Levels of glycemia were measured at time of death by using a Synchron CX7 Beckman analyzer (Beckman, Fullerton, CA). Kidney perfusion and removal was performed as described above.

Study 3: macrophage depletion. Four groups of 10-week-old male Sprague-Dawley rats were used in this study: 1) control nondiabetic rats ($n = 18$), 2) X-irradiated nondiabetic rats ($n = 18$), 3) diabetic rats ($n = 18$), and 4) X-irradiated diabetic rats ($n = 18$). Diabetes was induced as described above. The irradiated rats had a whole-body 600-rad X-irradiation using a 200-kV X-ray generator with a dose rate of 39.3 cGy/min. For X-irradiation, the animals were anesthetized, and their kidneys were shielded with two lead pieces to avoid direct irradiation. Streptozotocin injection was performed on the day of irradiation. The X-irradiated rats were grown in protected cages with sterilized sawdust, food, and drinking water. Nine rats from each group were killed after 8 and 30 days of experimentation. The 30-day rats received two extra 500-rad doses of whole-body X-irradiation on days 10 and 20 to prevent leukocyte recovery. At death, glycemia was measured as

described above, and the blood cell count was assessed by using a STKS analyzer (Coultronics, Mergency, France). Kidney perfusion and removal were performed as described above.

Glomerular microdissection. Small superficial cortical fragments from the left kidney were transferred under a binocular microscope in a microdissecting dish that was cooled at 4°C and contained the saline solution with 1.2 U/ μl RNasin and 1 mmol/l DTT. Glomeruli were separated from tubules, afferent and efferent arterioles, and Bowman's capsule. In study 1, 30 isolated glomeruli per animal were washed free of tissue debris. The isolated glomeruli of each group (i.e., diabetic and control rats at each time) were pooled (one sample of 180 glomeruli per group) and transferred to a solution of Trizol (Gibco-BRL, Gaithersburg, MD). In studies 2 and 3, 40 glomeruli per animal were isolated. Then, 120 isolated glomeruli from three animals were pooled and transferred to a solution of Trizol; this resulted in three samples of 120 glomeruli per group.

RNA extraction and quantitative RT-PCR. Total RNA from the isolated glomeruli samples was extracted by a procedure derived from Chomczynski and Sacchi (18).

As previously described by Nicoletti and Sassy-Prigent (19), to achieve a relative quantitative measurement of RNA by RT-PCR, a range of seven progressive dilutions obtained by mixing pools of RNA from the two different comparison groups were prepared in a constant final volume. This procedure eliminated tube-to-tube efficiency variations. Indeed, after gel densitometric analysis, the alignment of the seven measurements along a regression line accounted for the stability of PCR efficiency in the seven tubes. If the data for one tube were off the line, the experiment was repeated once more. The alignment of the points along the regression line was assessed by a Student's *t* test. The comparison between the two samples was determined by the slope of the regression line.

An aliquot of each dilution mix was incubated at 65°C for 10 min with 1 μg oligo-dT $_{12-18}$ (Pharmacia Biotech, Uppsala, Sweden) and then at 37°C for 1 h with 200 U of Moloney Murine Leukemia Virus reverse transcriptase (Gibco-BRL) in a buffer containing Tris-HCl 50 mmol/l (pH 8.3), KCl 75 mmol/l, DTT 10 mmol/l, 19 U RNasin, and 0.5 mmol/l of each dNTP in a final volume of 20 μl . Of each cDNA, 2 μl was amplified in a total volume of 25 μl containing 50 pmol of oligonucleotide primers, 10 mmol/l of each dNTP, 1 \times polymerase chain reaction (PCR) buffer (Tris-HCl 10 mmol/l, pH 8.3, and KCl 50 mmol/l), 0 or 1% dimethyl sulfoxide, MgCl_2 0.75–1.5 mmol/l, and 1.25 U of AmpliTaq DNA Polymerase (Perkin-Elmer Cetus, Emeryville, CA). The sequences and characteristics of the primers used for the amplifications are described in Table 1. By use of primer analysis software (Oligo 4.04; National Biosciences, Plymouth, MN), the primers were designed from rat sequences (except for monocyte chemoattractant protein [MCP]-1, which was designed from guinea pig sequences) obtained from GenBank (the National Institutes of Health database). The specificity of each PCR product was assessed using a unique restriction site in the amplified sequence. PCR was carried out in a Robocycler (Stratagene, La Jolla, CA). The appropriate number of cycles to be included within the exponential phase of amplification was determined for samples of isolated glomeruli for every set of primers. Of each PCR product, 10 μl was then run on an ethidium bromide-stained agarose gel. Polaroid photographs of gels were digitalized into gray-scale images. The amounts of nucleic acids were determined by densitometry, and each amount was proportional to the logarithm of the optic density. The sum of the logarithms of the pixel values was used to estimate the amount of nucleic acid in a band, which was expressed as arbi-

TABLE 1
Primers for PCR amplifications

	Sequence	Length (bp)	MgCl_2 (mmol/l)	Annealing temperature (°C)	Cycles (n)
3' GAPDH	3'-GTGAAGGTCGGAGTCAACG-5'	299	1.0	55	30
5' GAPDH	3'-GGTGAAGACGCCAGTGGACTC-5'				
3' type IV collagen	3'-TCGGCTATTCCTTCGTGATG-5'	185	1.5	55	35
5' type IV collagen	3'-TCTCGCTTCTCTCTATGGT-5'				
3' CD14	3'-ATTGTTTGGGGCGGCAGATG-5'	445	0.75	60	35
5' CD14	3'-GTGGAGAGGGCAGGGAAGACG-5'				
3' VCAM-1	3'-CACCTCCCCAAGAATACAGA-5'	476	0.75	56	40
5' VCAM-1	3'-GCTCATCCTCAACACCCACAG-5'				
3' ICAM-1	3'-GGCGTCCATTTACACCTATTA-5'	413	1.0	56	30
5' ICAM-1	3'-TTCCTTTTCTTCTCTTGGCTT-5'				
3' MCP-1	3'-TCCTTCTGTGCTGTGGTC-5'	336	1.0	56	40
5' MCP-1	3'-TCTTGGGGTCTTGGGTTGTG-5'				
3' IL-1	3'-CAGAAACACAAAACATCA-5'	209	1.0	52	35
5' IL-1	3'-GAATAGACTCCCGAAATAAGG-5'				

rary units. Analysis was performed using the public domain NIH Image 1.62b program (National Institutes of Health, Bethesda, MD). The amount of amplified cDNAs was normalized by the amount of amplified glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to correct for variations in the initial RNA content. Each RNA aliquot was amplified in triplicate.

Immunohistochemical study. Of liquid nitrogen snap-frozen right kidney, 5- μ m-thick sections were air-dried and acetone-fixed. The tissue sections were then incubated for 60 min with the primary antibodies ED-1, a murine macrophage marker, and anti-ICAM-1 (CD54) from Serotec (Oxford, U.K.). After phosphate buffer saline (0.01 mol/l, pH 7.4) rinses, the sections were incubated with biotinylated rabbit anti-IgG mouse antibody (Amersham, Les Ulis, France) and then alkaline phosphatase-anti-alkaline phosphatase complexes (Dako, Carpinteria, CA) with freshly prepared Fast Red Substrate System (Dako) and 0.33 mg/ml of levamisole (Sigma). Sections were counterstained with hematoxylin. The number of macrophages (ED1-positive cells) per glomerulus was determined in 100 glomeruli for all animals of the three studies, and a density of positive cells per glomerulus was assessed.

Glomerular type IV collagen was assessed in study 1 at days 8 and 30 by incubating frozen sections from diabetic and nondiabetic rat kidneys for 60 min at room temperature with an IgG fraction of a polyclonal rabbit antibody against mouse type IV collagen (Institut Pasteur, Lyon, France) diluted at 1/20 in Tris-HCl buffer (pH 7.2) with 0.1% BSA. The sections were then incubated with a fluorescein-conjugated anti-rabbit swine antibody (Dako) and observed with a Leitz ultraviolet microscope (Leica Microsystems, Rueil-Malmaison, France).

Morphometric analysis. As previously described (20,21), a transversal section at the level of the hilus of the right kidney was fixed in alcoholic Bouin's solution and embedded in paraffin. Of all of the kidneys, 5- μ m-thick sections were stained with silver staining for morphometric analysis. Briefly, the image analysis processor digitalized microscopic images into 512 \times 512 pixel images within 256 gray levels. Digitalized images were submitted to predefined transformations allowing the automatic measurement of selected glomerular morphometric parameters. The glomeruli were measured with a \times 25 objective yielding a final calibration of 0.4219 μ m/pixel. To correct a potential variability in the staining intensity of the different sections, the program permits the observer to calibrate the image according to the defined gray levels. The measured glomerular parameters were as follows: 1) total glomerular surface area delimited by the internal edge of the Bowman's capsule; and 2) mesangial surface area defined by the glomerular tuft surface area minus the areas of the capillary lumens, the areas of the urinary lumens, and the glomerular capillary free walls. In a preliminary study, the total number of glomeruli to be measured to yield convergent data was established at 30. In each kidney section, the observer blindly measured 30 consecutive unselected glomeruli randomly distributed over the depth of the cortex. Polar glomerular sections were not measured.

Statistical analysis. The results were expressed as means \pm SE. Statistical analysis was carried out using a two-way analysis of variance (ANOVA) with time and diabetes as factors for study 1 and two-way ANOVA with time and diabetes as factors in the nontreated groups or time and treatments as factors in the diabetic groups for studies 2 and 3.

The ANOVA test was followed by a Scheffe's test for comparisons among groups. Statistical significance was achieved if P was <0.05 . Data were analyzed using the Statview 4.5 software (Abacus Concepts, Berkeley, CA).

RESULTS

Study 1. All of the diabetic rats had levels of glycemia >22 mmol/l. Body weight was significantly lower in diabetic rats compared with control rats from day 4 (211.9 ± 4.7 vs. 250.3 ± 1.3 g, $P = 0.0001$). Renal hypertrophy, as assessed by the increase in the ratio of 2 kidney weight to body weight, was present as early as day 1 in the diabetic rats (1.23 ± 0.02 vs. 0.8 ± 0.01 , $P = 0.0001$).

Values of total glomerular and mesangial surface areas measured at days 1, 2, 4, 8, 15, and 30 in the nondiabetic and diabetic rats are reported in Fig. 1A and B. Glomerular hypertrophy, as assessed by the total glomerular surface area (Fig. 1A), was observed as early as day 1 in diabetic rats ($11,705 \pm 301$ vs. $9,404 \pm 160 \mu\text{m}^2$, $P = 0.0001$). Mesangial surface area (Fig. 1B) was significantly increased in diabetic rats from day 8 (691.3 ± 46.5 vs. $301.3 \pm 35.7 \mu\text{m}^2$, $P < 0.001$). Type IV collagen mRNA was increased in the glomeruli of diabetic rats from day 8 (281.8 ± 1.4 vs. 99.6 ± 1.1 , $P = 0.0001$) (Fig. 2A). Immunofluorescence with an anti-collagen IV anti-

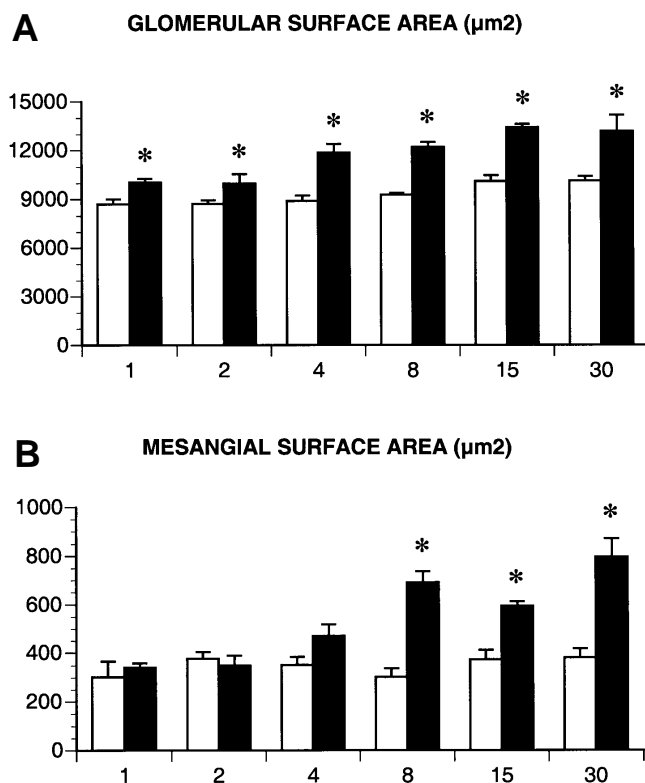


FIG. 1. Study 1: morphometric parameters (means \pm SE) in control (\square) and diabetic (\blacksquare) rats at days 1, 2, 4, 8, 15, and 30. A: Diabetes induces a significant glomerular hypertrophy as early as day 1. B: Mesangial surface area is increased in diabetic rats from day 8. *Diabetic versus control rats.

body (Fig. 3A) showed no clear-cut difference in the labeling in diabetic rats compared with control rats at days 8 and 30. For CD14, a macrophage marker, mRNA was increased as early as day 2 (717.3 ± 73.9 vs. 100.2 ± 0.1 , $P < 0.001$) (Fig. 2B), whereas ED1 immunohistochemical labeling (Fig. 3C and D) showed a significant increase in the density of macrophages within the diabetic glomeruli from only day 8 (1.43 ± 0.09 vs. 0.88 ± 0.08 , $P < 0.001$) (Fig. 4A). Intercellular adhesion molecule-1 (ICAM-1) mRNA was increased as early as day 1 (Fig. 2C) in diabetic rats (239.2 ± 26.5 vs. 100.4 ± 0.3 , $P < 0.01$). This increase had a tendency to decline with time. ICAM-1 immunohistochemical labeling was diffuse along the glomerular capillary walls (Fig. 3B) without any obvious difference between diabetic and non-diabetic rats. Vascular cell adhesion molecule 1 (VCAM-1) RT-PCR showed a dramatic increase in VCAM-1 mRNA in the diabetic rats (Fig. 2D) as early as day 1 ($1,444 \pm 140$ vs. 98.6 ± 0.2 , $P < 0.001$). MCP-1 mRNA had an increased expression in diabetic rat glomeruli (Fig. 2E) from day 4 ($11,385 \pm 4,805$ vs. 93 ± 7.5 , $P < 0.05$). Interleukin (IL)- 1β mRNA expression showed a slight increase in diabetic rats (Fig. 2F) as early as day 1 (134.9 ± 4.7 vs. 100.7 ± 0.4 , $P < 0.01$).

Study 2. Levels of blood glucose were significantly higher in diabetic rats compared with those in control rats (33.9 ± 3.61 vs. 7.6 ± 0.18 mmol/l, $P = 0.0001$). Insulin treatment significantly decreased this hyperglycemia (12.7 ± 1.8 vs. 33.9 ± 3.6 mmol/l, $P = 0.0001$). ACE inhibitor treatment with Cilazapril did not influence glycemia as compared with untreated diabetic rats (43.3 ± 0.9 vs. 33.9 ± 3.6 mmol/l, NS). Diabetic rats

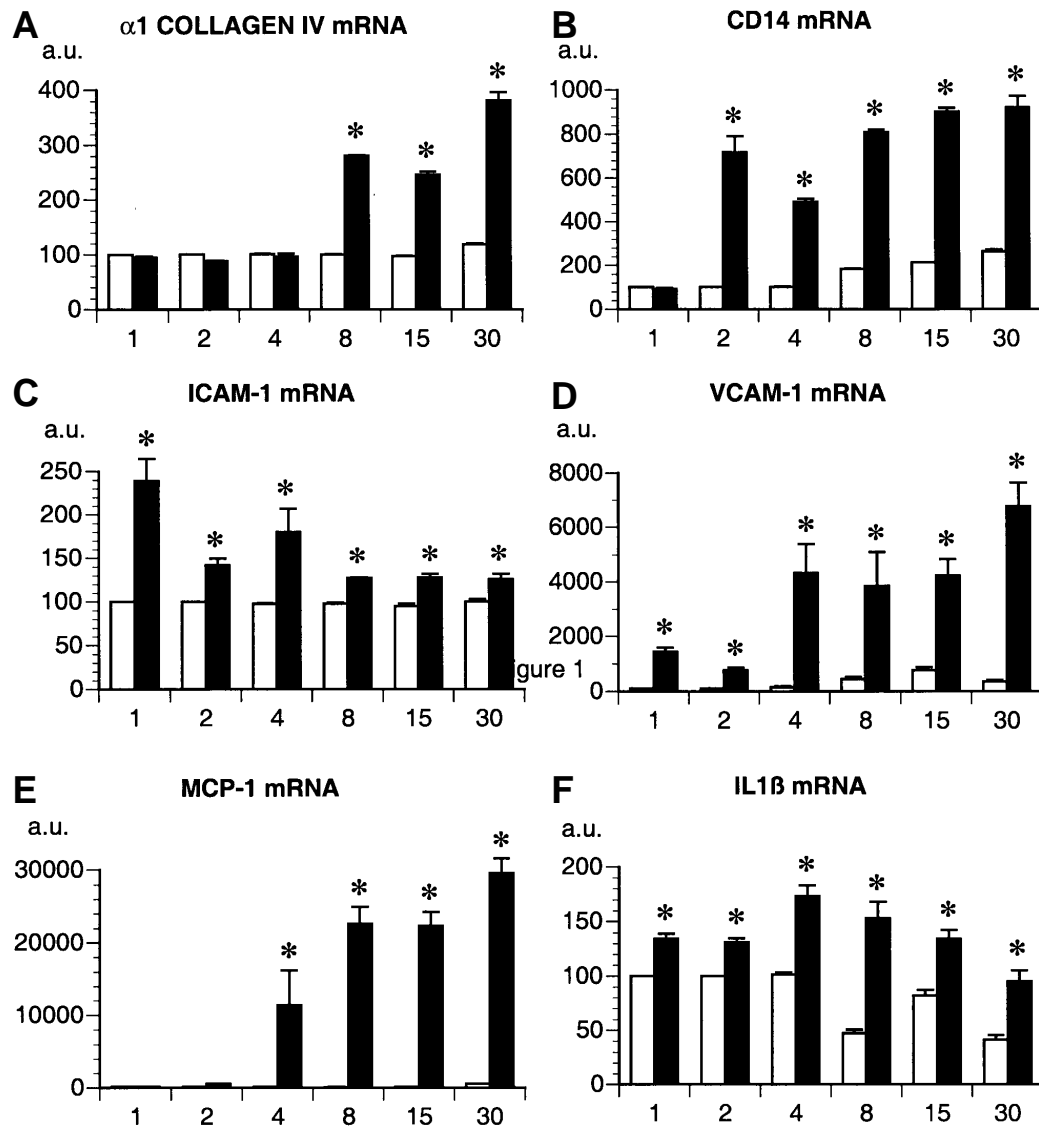


FIG. 2. Study 1: RT-PCR quantitative analysis of mRNA extracted from control (\square) and diabetic (\blacksquare) rats at days 1, 2, 4, 8, 15, and 30. A: Diabetes increases α_1 -chain type IV collagen mRNA expression from day 8. B: CD14 mRNA expression from day 2. C: ICAM-1 mRNA expression as early as day 1. D: VCAM-1 mRNA expression as early as day 1. E: MCP-1 mRNA expression from day 4. F: IL-1 β mRNA expression as early as day 1. Data are arbitrary units (mean \pm SE). *Diabetic versus control rats.

had no higher systolic levels of blood pressure compared with those of control rats (158 ± 10.3 vs. 149 ± 5.6 mmHg, NS). ACE inhibitor treatment significantly decreased the systolic levels of blood pressure of diabetic rats as compared with those in nontreated diabetic rats (116 ± 4.8 vs. 158 ± 10.3 mmHg, $P < 0.01$).

The loss in body weight observed in diabetic rats as compared with that in control rats (288.7 ± 8.9 vs. 364.1 ± 12.2 g, $P = 0.0001$) was abolished by insulin treatment (340.6 ± 17.9 vs. 288.7 ± 8.9 g, $P = 0.0001$). Treatment with ACE inhibitor increased the loss in body weight in diabetic rats (247.8 ± 8.2 vs. 288.7 ± 8.9 g, $P = 0.0001$). Renal hypertrophy observed in diabetic rats compared with that in control rats (1.24 ± 0.05 vs. 0.83 ± 0.02 , $P = 0.0001$) was reverted by insulin treatment (0.99 ± 0.03 vs. 1.24 ± 0.05 , $P < 0.01$) but not by ACE inhibitor treatment (1.18 ± 0.09 vs. 1.24 ± 0.05 , NS).

Diabetic glomerular hypertrophy ($11,674 \pm 642$ vs. $7,791 \pm 82.6 \mu\text{m}^2$, $P = 0.0001$) was significantly decreased by insulin

($7,823 \pm 258.7$ vs. $11,674 \pm 642 \mu\text{m}^2$, $P = 0.0001$) and ACE inhibitor ($8,683 \pm 156.6$ vs. $11,674 \pm 642 \mu\text{m}^2$, $P = 0.0001$) treatments (Fig. 5A). Increased mesangial surface area (Fig. 5B) in diabetic rats (601 ± 74.6 vs. $262 \pm 8.4 \mu\text{m}^2$, $P = 0.0001$) was significantly reduced by insulin treatment as early as day 8 (224.6 ± 17.2 vs. $386.6 \pm 20.4 \mu\text{m}^2$, $P < 0.001$) and by ACE inhibitor treatment at only day 30 (392 ± 12 vs. $816.2 \pm 75 \mu\text{m}^2$, $P = 0.0001$).

Increased α_1 -chain type IV collagen mRNA synthesis in diabetic rats compared with that in control rats (760.2 ± 240.7 vs. 134.2 ± 15.8 , $P < 0.01$) was reverted by insulin treatment (141.3 ± 20.5 vs. 760.2 ± 240.7 , $P < 0.01$) from day 8 but not by ACE inhibitor treatment (Fig. 6A).

The same pattern of mRNA expression was observed with CD14: the increased synthesis in diabetic rats compared with that in control rats (470.6 ± 41.9 vs. 120.6 ± 9.3 , $P = 0.0001$) was significantly diminished only by insulin treatment (191.6 ± 17.7 vs. 470.6 ± 41.9 , $P = 0.0001$) (Fig. 6B). In parallel, diabetes

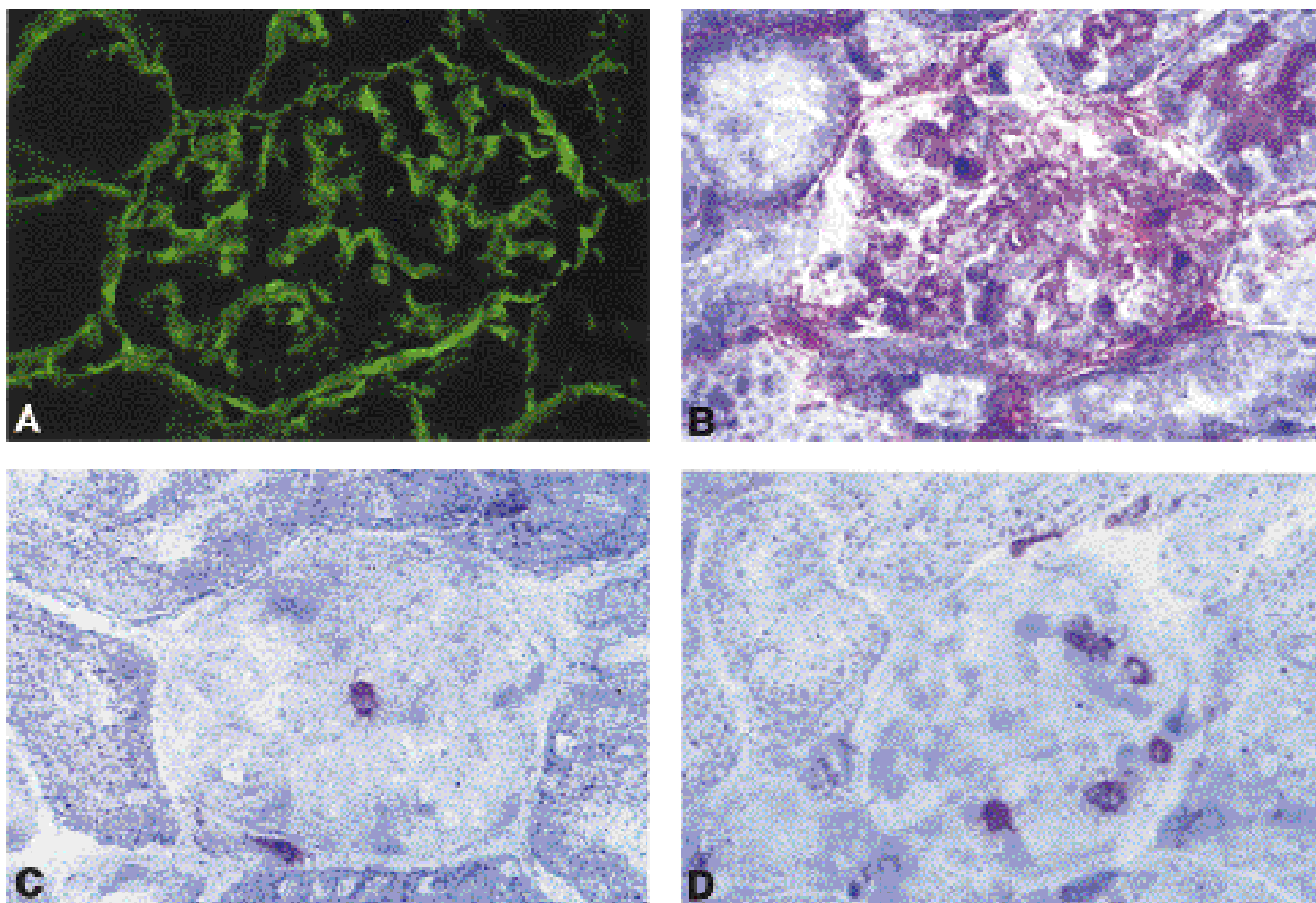


FIG. 3. A: Type IV collagen immunofluorescence in a control rat: labeling of the glomerular tuft, the Bowman's capsule, and the tubular base-membranes. B: ICAM immunohistochemistry in a 30-day diabetic rat: diffuse labeling in the glomerular tuft including capillary lumens. Note the labeling of peritubular capillaries. C: ED1 immunohistochemistry in control rats. D: ED1 immunohistochemistry in 30-day diabetic rats. C and D show one positive intraglomerular cell in the control rat and five positive intraglomerular cells in the diabetic rat. $\times = 350$.

induced an increase in the density of ED1-positive cells in the glomeruli compared with that in control rats (1.66 ± 0.2 vs. 1.14 ± 0.1 , $P < 0.01$) (Fig. 4B). This effect was decreased by insulin (0.91 ± 0.05 vs. 1.66 ± 0.2 , $P = 0.0001$) and by ACE inhibitor (1.2 ± 0.1 vs. 1.66 ± 0.2 , $P < 0.01$) treatments.

The increased VCAM-1 mRNA synthesis in diabetic rats compared with that in control rats ($1,980.9 \pm 435$ vs. 188.8 ± 60.5 , $P < 0.001$) was significantly attenuated by insulin treatment (718 ± 151 vs. $1,980.9 \pm 435$, $P < 0.01$) (Fig. 6C).

The dramatic MCP-1 mRNA increase in diabetic rats compared with that in control rats ($1,466.4 \pm 247.1$ vs. 171.6 ± 47.9 , $P < 0.001$) was abolished by insulin treatment (165.9 ± 41.2 vs. $1,466.4 \pm 247.1$, $P = 0.0001$) at both times. ACE inhibitor treatment was also efficient to normalize this overexpression at only day 30 (100.8 ± 19.2 vs. $1,850.1 \pm 394.1$, $P < 0.01$) (Fig. 6D).

The increased IL-1 β mRNA expression in diabetic versus control rats (631.5 ± 209.3 vs. 124.1 ± 9.1 , $P < 0.05$) was reverted by insulin treatment only (154.4 ± 8.4 vs. 631.5 ± 209.3 , $P < 0.05$) from day 8 (Fig. 6E).

Study 3. Levels of glycemia were significantly higher in diabetic rats than in nondiabetic rats (41.4 ± 1.2 vs. 7.6 ± 0.2 mmol/l, $P = 0.0001$). X-irradiation had no influence on glycemia.

The X-irradiated rats experienced a significant decrease in the peripheral leukocyte count as compared with that of the

nondiabetic or diabetic non-X-irradiated rats at day 8 ($1.1 \pm 0.1 \cdot 10^8$ vs. $9.4 \pm 0.7 \cdot 10^8$ cells/dl, $P = 0.0001$) and day 30 ($0.6 \pm 0.1 \cdot 10^8$ vs. $8.0 \pm 1.0 \cdot 10^8$ cells/dl, $P = 0.0001$), which indicated that leukocyte depletion was sustained up to day 30.

The nonirradiated diabetic rats had a low body weight compared with that of nonirradiated nondiabetic rats (225.3 ± 7.1 vs. 364.1 ± 12.2 g, $P = 0.0001$). X-irradiation increased the body weight loss in diabetic rats (180.6 ± 8.5 vs. 225.3 ± 7.1 g, $P = 0.0001$). X-irradiation had no effect on the diabetic renal hypertrophy.

X-irradiation had no effect on the diabetic glomerular hypertrophy (Fig. 7A). Mesangial surface area expansion in diabetic rats compared with that in control rats (609.9 ± 66.9 vs. $262.2 \pm 8.4 \mu\text{m}^2$, $P = 0.0001$) was decreased by X-irradiation at day 30 (557.4 ± 57.8 vs. $876.1 \pm 73.9 \mu\text{m}^2$, $P < 0.01$) (Fig. 7B).

In diabetic rats, X-irradiation significantly decreased the overexpression of the mRNA of α_1 -chain type IV collagen (104.7 ± 2.3 vs. 134.3 ± 2.7 , $P = 0.0001$) (Fig. 8A), CD14 (97.8 ± 1.2 vs. 285.9 ± 30.4 , $P = 0.0001$) (Fig. 8B), MCP-1 (126.7 ± 8.5 vs. 169.5 ± 6.5 , $P < 0.001$) (Fig. 8D), and IL-1 β (106.7 ± 2.9 vs. 325.4 ± 87.6 , $P < 0.05$) (Fig. 8E) as early as day 8 (irradiated diabetic versus nonirradiated diabetic rats respectively). X-irradiation had no effect on the overexpression of VCAM 1 mRNA in diabetic rats (Fig. 8C). In parallel, it was efficient to

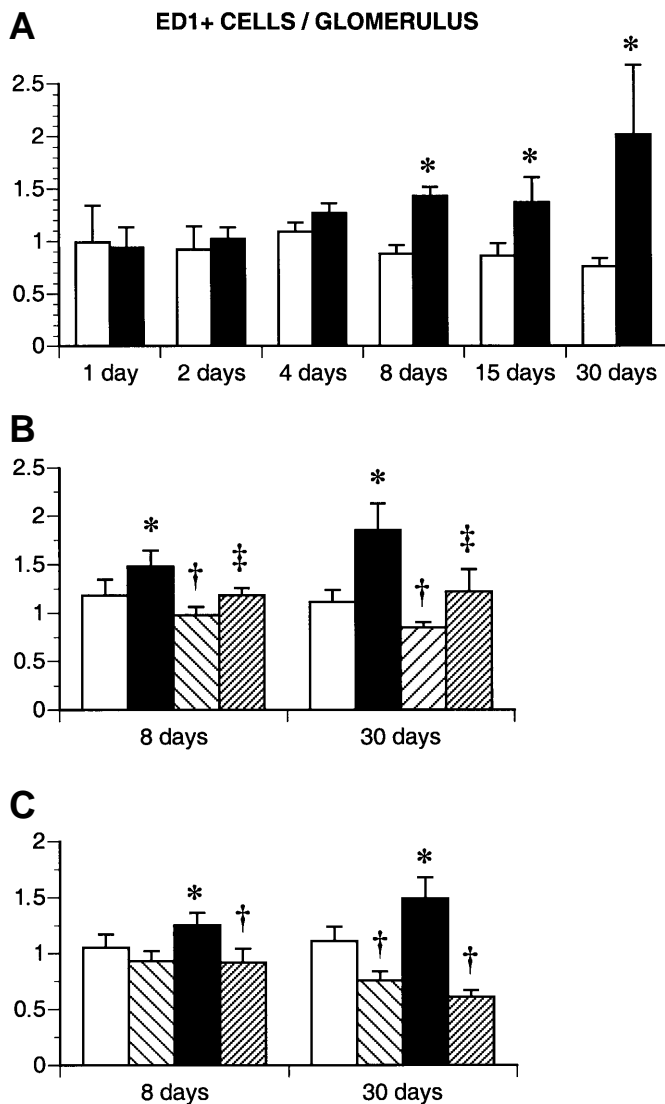


FIG. 4. Immunohistochemical-labeling density of ED1-positive cells per glomerulus (means \pm SE). A: Study 1: control (□) and diabetic (■) rats at days 1, 2, 4, 8, 15, and 30. Labeling density is increased with diabetes from day 8. *Diabetic versus control rats. B: Study 2: control (□), diabetic (■), insulin-treated diabetic (▨), and ACE inhibitor-treated diabetic (▩) rats at days 8 and 30. The increase in the density of ED1-positive cells is reduced by insulin and ACE inhibitor treatments from day 8 in diabetic rats. *Untreated diabetic rats versus control rats; †insulin-treated diabetic rats versus untreated diabetic rats; ‡ACE inhibitor-treated diabetic rats versus untreated diabetic rats. C: Study 3: control (□), X-irradiated nondiabetic (▨), non-X-irradiated diabetic (■), and X-irradiated diabetic (▩) rats at days 8 and 30. X-irradiation is efficient to reduce the macrophage density in the diabetic glomeruli from day 8. *Diabetic rats versus nondiabetic non-X-irradiated rats; †X-irradiated rats versus either nondiabetic or diabetic non-X-irradiated rats.

reduce the macrophage density in the diabetic glomeruli (0.75 ± 0.07 vs. 1.35 ± 0.1 , $P = 0.0001$) (Fig. 4C) from day 8.

DISCUSSION

In this study, macrophage recruitment has been demonstrated within rat glomeruli early after the onset of hyperglycemia in the streptozotocin model. It is associated with an increase in α_1 -chain type IV collagen mRNA synthesis in the glomeruli and with glomerular structural changes. The

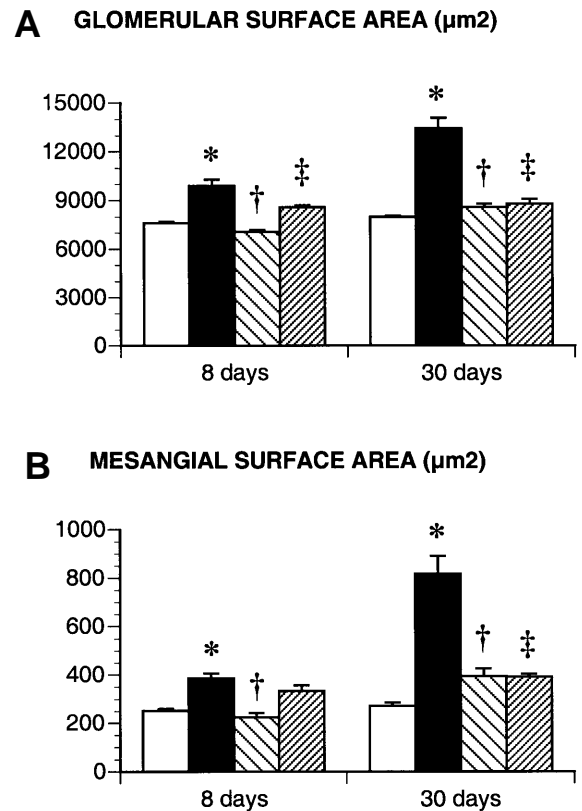


FIG. 5. Study 2: morphometric parameters (means \pm SE) in control (□), diabetic (■), insulin-treated diabetic (▨), and ACE inhibitor-treated diabetic (▩) rats at days 8 and 30. A: Insulin and ACE inhibitor treatments are efficient to reduce diabetic glomerular hypertrophy as early as day 8. B: Increased mesangial surface area in diabetic rats is reduced by insulin treatment as early as day 8 and by ACE inhibitor treatment at day 30. *Untreated diabetic rats versus control rats; †insulin-treated diabetic rats versus untreated diabetic rats; ‡ACE inhibitor-treated diabetic rats versus untreated diabetic rats.

macrophage infiltration in the diabetic glomeruli is mediated by adhesion molecule and chemotactic factor expression. Furthermore, IL-1 β is involved and could contribute to type IV collagen and adhesion molecule synthesis. Insulin treatment reduced all of these changes in diabetic rats, whereas ACE inhibitor had an incomplete effect. Macrophage depletion decreased hyperglycemia-induced mesangial expansion and α_1 -chain type IV collagen mRNA.

In this model, a significant mesangial expansion occurs as early as day 8 and is therefore associated with glomerular hypertrophy. Glomerular hypertrophy occurs within the hours after the onset of hyperglycemia, whereas mesangial expansion is delayed after day 8 of the onset of hyperglycemia. In a previous study (21), morphometric detection of mesangial domain expansion in the glomeruli of diabetic rats without overt glomerulosclerosis (GS) at 3 and 6 months has been interpreted as an early marker of incipient GS. However, at day 8, mesangial domain expansion cannot be considered as incipient GS. Thus, the increase in type IV collagen mRNA, a primary event for ECM type IV collagen accumulation, cannot be sufficient at this very early time of hyperglycemia to contribute significantly to the mesangial expansion, especially because type IV collagen immunofluorescence is unchanged. Besides ECM accumulation, the increase in

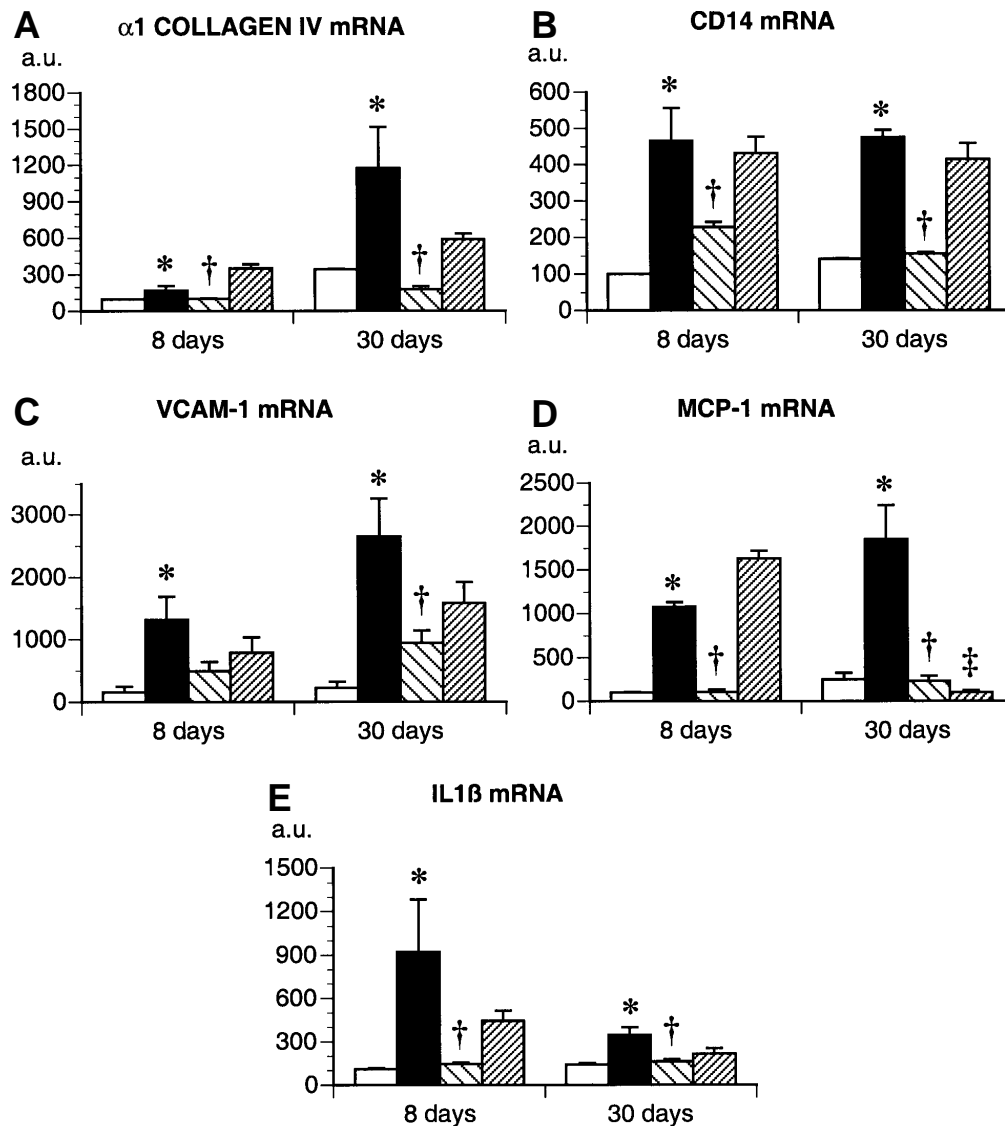


FIG. 6. Study 2: RT-PCR quantitative analysis of mRNA extracted from control (□), diabetic (■), insulin-treated diabetic (▨), and ACE inhibitor-treated diabetic (▩) rats at days 8 and 30. Insulin treatment is efficient to reduce the mRNA overexpression in diabetic glomeruli for (A) α_1 -chain type IV collagen mRNA, (B) CD14, (C) VCAM-1, (D) MCP-1, and (E) IL-1 β . ACE inhibitor treatment is efficient to normalize only the overexpression of MCP-1 mRNA (D). Data are arbitrary units (means \pm SE). *Untreated diabetic rats versus control rats; †insulin-treated diabetic rats versus untreated diabetic rats; ‡ACE inhibitor-treated diabetic rats versus untreated diabetic rats.

mesangial area could result from an expansion of the cellular component of the mesangium (i.e., hypertrophy and/or hyperplasia of mesangial cells) (13) and/or macrophage influx. Such mesangial cell changes may reflect mesangial cell activation. With glomerular hypertrophy (22), therefore, mesangial expansion and increased α_1 -chain type IV collagen mRNA synthesis could be considered as conditions for the development of GS.

Macrophage recruitment in glomeruli was assessed using both ED1 immunohistochemistry and RT-PCR of CD14, the lipopolysaccharide receptor, which is considered as a marker of macrophages (23,24). In addition to the increase in glomerular macrophages during hyperglycemia, both techniques showed that resident macrophages are present in normal rat glomeruli. In this respect, except for macrophage recruitment from blood monocytes, this study does

not rule out any proliferation of these resident macrophages that could contribute to the increase in glomerular macrophage density.

The macrophages assessed either by RT-PCR or immunohistochemistry are recruited early (i.e., within a few days after the induction of hyperglycemia). Concomitantly, the α_1 -chain type IV collagen mRNA synthesis is activated, and the mesangial domain is enlarged in the glomeruli of diabetic rats. This *in vivo* dynamic study demonstrates that the phenomena that could lead later to GS are engaged in the very early phase of diabetes. This early engagement was probable given that the data from cell culture studies in high-glucose conditions were obtained within hours or a few days (7–10). Macrophage depletion was used to assess the role of macrophage recruitment in diabetic glomeruli and especially its involvement in the concomitant α_1 -chain type IV collagen

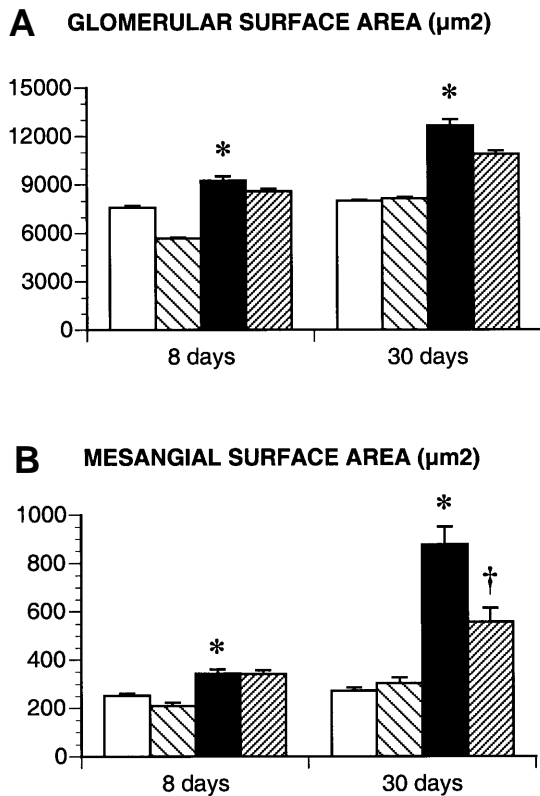


FIG. 7. Study 3: morphometric parameters (means \pm SE) in control (□), X-irradiated nondiabetic (▨), non-X-irradiated diabetic (■), and X-irradiated diabetic (▩) rats at days 8 and 30. A: X-irradiation has no effect on the diabetic glomerular hypertrophy. B: Diabetic mesangial expansion is decreased by X-irradiation at day 30. *Diabetic rats versus nondiabetic non-X-irradiated rats; †X-irradiated rats versus non-X-irradiated diabetic rats.

mRNA overexpression and the mesangium expansion. In our X-irradiation experiment, we induced a significant decrease in the glomerular macrophage recruitment either assessed by ED1 immunohistochemistry or CD14 RT-PCR. This decrease resulted in the reduction of α_1 -chain type IV collagen mRNA synthesis and of mesangial area. These data strongly suggest that, in experimental diabetes, glomerular macrophages contribute, at least in part, to the phenomena that could lead to GS. It is supported by an *in vitro* study where a macrophage-conditioned medium stimulated fibronectin synthesis by mesangial cells (25). Although diabetes is a metabolic disorder and not an immune or overtly inflammatory injury of the renal tissue, it can involve cells and mediators usually observed in inflammation. Therefore, a discrete inflammatory phenomenon could contribute to diabetic glomerulopathy. Furthermore, macrophages that exhibit proliferation at a lower level than mesangial cells have been shown by using immunochemistry to be present very early in the glomeruli of diabetic rats (13). They were associated with growth factor synthesis and type IV collagen accumulation but, surprisingly, not with type IV collagen mRNA overexpression. No macrophage depletion was tested. In a human study based on renal biopsies from diabetic patients at different stages of diabetic nephropathy, EBM11 immunohistochemistry detected macrophages in glomeruli, in which glomerulosclerosis was patent, by using standard semiquantitative scoring analysis (26). In this study, the macrophage density was higher in

mild and moderate glomerulosclerosis than it was in severe glomerulosclerosis. This finding suggests that fibrosis per se is not a factor for macrophage recruitment and that, on the contrary, macrophages that are more prominent in early stages of glomerulosclerosis could contribute to the progression of fibrosis. Thus, in experimental diabetes, macrophages are involved in glomerulosclerosis pathogenesis, just as they are in other models of chronic glomerular injury that lead to glomerulosclerosis, puromycin (14), high lipoprotein (15), and renal mass ablation (16,27).

Our study also assessed the mechanisms involved in glomerular macrophage recruitment and action. VCAM-1 and the chemotactic factor MCP-1 were early and intensely overexpressed in the diabetic glomeruli that facilitate glomerular recruitment of macrophages from blood monocytes. ICAM-1 is clearly present in glomeruli in steady-state conditions, whereas its expression is only slightly increased in diabetic glomeruli. We did not evaluate whether the overexpression of adhesion molecules and chemotactic factor is mediated directly by hyperglycemia or indirectly through increased IL-1 β or another mediator not tested in this study. However, a high-glucose condition can increase ICAM-1 in human umbilical vein endothelial cell (28). IL-1 β was increased early in diabetic glomeruli and could contribute to the overexpression of adhesion molecules and chemotactic factors (29). IL-1 β could also act as a growth factor on mesangial cells to increase expression of type IV collagen (30), TGF- β , and PDGF in diabetes (6,31). In this study, the cellular origin of IL-1 β was not assessed; it could be either recruited macrophages (32,33) or stimulated mesangial cells (34). The efficiency of insulin to prevent macrophage recruitment as previously observed by Young et al. (13) suggests that hyperglycemia directly plays a major role. Insulin effect is mediated by the decrease in levels of adhesion molecules, chemotactic factors, and cytokine overexpression. Furthermore, insulin treatment strongly reduces the molecular (α_1 -chain type IV collagen mRNA) and structural (mesangial expansion and glomerular hypertrophy) events that could lead to GS. ACE inhibitor is partly efficient and less potent than insulin when reducing the cellular and molecular changes induced by hyperglycemia. However, ACE inhibitor is as powerful as insulin when reducing the glomerular structural changes observed in diabetes. The partial effect of ACE inhibitor on macrophage recruitment is not mediated by VCAM, which was unchanged. It could be related to the effect of angiotensin II on mesangial cells and on macrophages (35) via nuclear activation and MCP-1 synthesis.

In conclusion, this study shows that pure preparations of glomeruli obtained by microdissection allow RT-PCR and are very sensitive to assess and quantify gene expression within the glomeruli (36). Contrary to total renal cortex or to glomeruli isolated by sieving, which yields an impure preparation of glomeruli, microdissection allows clear distinctions to be made between glomerular and tubular, interstitial, or vascular phenomena. When possible, immunohistochemistry on tissue sections, when driven in parallel with RT-PCR, confirms protein expression in the precise structures of the kidney. Our study demonstrates that macrophages are recruited in glomeruli at the very early phase of streptozotocin-induced diabetes. The glomerular macrophages play an active role in diabetes, as demonstrated by insulin-treatment and X-irradiation macrophage depletion. The glomerular macrophages contribute to glomerular changes that could lead to GS.

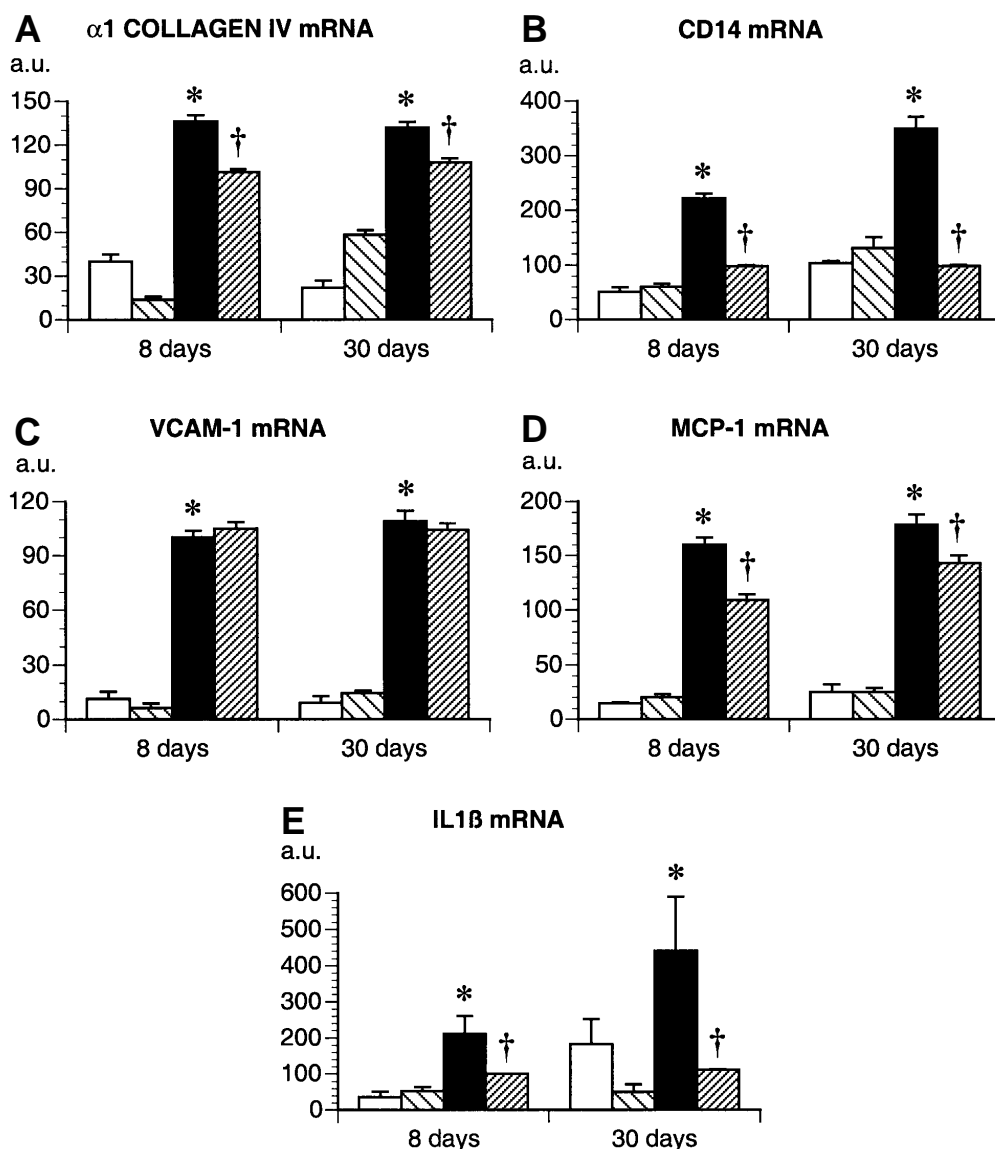


FIG. 8. Study 3: RT-PCR quantitative analysis of mRNA extracted from control (□), X-irradiated nondiabetic (▨), non-X-irradiated diabetic (■), and X-irradiated diabetic (▩) rats at days 8 and 30. X-irradiation is efficient to reduce the diabetic overexpression of the mRNA of (A) α_1 -chain type IV collagen, (B) CD14, (D) MCP-1, and (E) IL-1 β . C: RT-PCR quantitative analysis has no effect on the overexpression of VCAM-1 mRNA in the diabetic glomeruli. Data are arbitrary units (means \pm SE). *Diabetic rats versus nondiabetic non-X-irradiated rats; †X-irradiated rats versus non-X-irradiated diabetic rats.

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