Early Influx of Glomerular Macrophages Precedes Glomerulosclerosis in the Obese Zucker Rat Model

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ABSTRACT

Because hyperlipidemia and macrophage influx appear to play a key role in the genesis of renal glomerulosclerosis, this study examined the temporal relationship between hyperlipidemia (triglycerides and cholesterol), mononuclear cell influx, changes in glomerular structure, and expansion of the extracellular matrices in obese Zucker rats, which rapidly develop hyperlipidemia and spontaneous glomerulosclerosis. Lean and obese Zucker rats were fed a standard diet, and were euthanized at 14 days, 1, 3, 6, 9, and 12 months. Plasma lipid, insulin, and creatinine levels were measured, and the presence of inflammatory cells in the glomerulus was assessed by Immunohistochemistry on kidney sections. Plasma lipids and insulin and macrophage density were significantly greater in obese than in lean rats as early as 1 month. Computer-assisted image analysis was used to evaluate the glomerular domain surface areas. The morphometric measurements showed that glomeruli of obese rats rapidly became hypertrophied after 3 months, as a result of a very large increase in the mesangial domain. The expression of genes for extracellular matrix components and inhibitors of extracellular matrix proteinases (TIMP-1 and TIMP-2) was monitored in microdissected glomeruli. Reverse transcription-polymerase chain reaction showed increases in mRNA for Type IV collagen and fibronectin and for the two metalloproteinase inhibitors, each of which might participate in this matrix expansion. Thus, the development of hyperlipidemia plus macrophage influx at a very early age may initiate a sequence of events leading to glomerulosclerosis later on.

Key Words: Inflammation, hyperlipidemia, extracellular matrix, morphometry, RT-PCR

Diffuse glomerulosclerosis and focal and segmental glomerular hyalinosis (FGSH) (1) (often called focal and segmental glomerulosclerosis [2]) occur in diverse circumstances including aging; hypertension; metabolic disorders such as diabetes, obesity or hyperlipidemia; partial nephrectomy; and intoxication by puromycin aminonucleoside (reviewed in References 1 and 2). Among the different factors implicated in the pathogenesis of these lesions, the accumulation of circulating monocytes/macrophages within the glomerular tuft has been described in almost all circumstances of FSGH (3), suggesting that several mechanisms involved in glomerulosclerosis are analogous to those observed in atherosclerosis (4) In this respect, disturbances of lipoprotein metabolism may accelerate the onset of glomerulosclerosis (5).

The obese Zucker rat is a model of spontaneous glomerulosclerosis (6–8). Obesity, an autosomal recessive trait accompanied by hyperlipidemia, develops at an early age (8, reviewed in Reference 9). Obese rats also suffer from mild glucose intolerance and peripheral insulin resistance similar to that found in humans with Type II diabetes (10). These metabolic abnormalities precede the development of proteinuria and glomerular injury in obese rats (8, 11). Lean littermates have normal serum lipids (11, 12) and normal renal structure and function (6). The glomerular hemodynamic function of obese rats and their lean littermates is not significantly different and is probably not involved in the pathogenesis of glomerulosclerosis in this strain (13).

Several studies have been published on the putative mechanisms involved in the pathogenesis of diffuse glomerulosclerosis and FSGH in Zucker rats, but little attention has been paid to the early events occurring in the glomeruli of weaning animals when metabolic disorders begin to worsen. The importance of a monocyte/macrophage influx during and after the weaning period is not known, although hypertriglyceridemia, hypercholesterolemia, and hyperinsulinemia develop markedly. We have, therefore, attempted to examine the contribution of inflammation to the initiation and development of diffuse glomerulosclerosis and FSGH lesions in obese rats, by analyzing the temporal relationship between hyperlipidemia and hyperinsulinemia, the mononuclear cell influx, changes in glomer-
ular structure, and the expansion of the glomerular tuft. Little is known about the composition of the expanded matrices observed in the sclerosed glomeruli of Zucker rats, or about the change in the balance between the synthesis and degradation of extracellular matrix components (ECM) that occurs with age. Accordingly, to consider only the matrix expansion within the glomerulus, we used microdissected glomeruli from lean and obese rat kidneys to follow the kinetics of messenger RNA expression of some major ECM components (Types I, III, and IV collagens, and fibronectin) and of the specific proteinase inhibitors TIMP-1 and TIMP-2.

METHODS

Animals

Male lean (Fa/fa) and obese (fa/fa) Zucker rats were identified and selected at 4 wk of age by visual examination of inguinal fat deposit. They were raised under standard husbandry conditions, fed regular laboratory chow ad libitum (A04; Villemoisson, Épinay sur Orge, France) and had free access to water until euthanization at 5 wk (1 month), 3, 6, 9, and 12 months of age. A second group of 14-day-old suckling Zucker pups (Fa/fa and fa/fa) were euthanized, and the obese genotype was determined by plotting inguinal fat pad weight against body weight (14). Animal care compiled with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication 86–23, revised 1989; authorization 00577, 1989, Paris, France).

Blood Pressure, Serum Creatinine, Insulin, Lipids, and Urine Proteins

Conscious systemic blood pressure (BP) was measured by a tail-cuff system (Ugo Basile Apelx, Varese, Italy). Blood was collected from animals at the time of euthanization into tubes containing heparin as anticoagulant for serum lipid and creatinine assays. These samples were centrifuged and aliquots of plasma were frozen and stored at −20°C. Twenty-four-hour urine samples were collected from fasting animals housed individually in metabolic cages with free access to water. Triglycerides and total cholesterol were determined by the enzymatic and colorimetric GPO-PAP and CHOD-PAP methods respectively (Boehringer, Mannheim, Germany). Serum and urine creatinine concentrations were measured on a Synchron CX7 Beckman analyser (Beckman, Fullerton, CA). Proteinuria was determined enzymatically using the Coomassie Protein Assay Reagent with BSA as standard (Pierce, Rockford, IL). Serum insulin levels were measured by RIA (CIS, GIF sur Yvette, France) with a rat insulin standard (Novo, Copenhagen, Denmark).

Kidney Structure

Lean and obese rats aged 1, 3, 6, 9, and 12 months, were anesthetized with pentobarbital (ip, 0.1 mL/100 g body wt) and the kidneys were removed and weighed. For light microscopy, transverse sections at the hilus were directly frozen in liquid nitrogen, others were fixed in alcoholic Bouin’s solution, embedded in paraffin, sectioned (4-µm thick), and stained with Masson’s trichrome or silver methenamine, according to routine histological staining. For electron microscopy, small samples of the kidney cortex were fixed in 2% glutaraldehyde/0.2 M cacodylate buffer, dehydrated in acetone, and embedded in Glucidether 100 (Merck, Darmstadt, Germany).

Masson’s trichrome stained sections of at least 100 glomeruli were evaluated for the presence of FSGH lesions. The percentage of glomeruli having FSGH lesions was determined for each tissue specimen, and a severity index was calculated for each glomerulus by using the equation:

\[
(S_1 \times 1/4) + (S_2 \times 1/2) + (S_3 \times 3/4) + (S_4 \times 1)
\]

total number of glomeruli

where S is the number of glomeruli with FSGH involving 1/4, 1/2, 3/4, and 4/4 of the glomerulus.

Morphometric Measurements

The thickness of the glomerular basement membrane (GBM) was evaluated from electron micrographs enlarged to a magnification of 42,000. Only capillaries in which the plasma membrane of the foot processes was clearly visible along the basement membrane were considered to guarantee that the section was perpendicular to the GBM. The width of the lamina densa was measured with a ruler oriented at a right angle to the GBM, in areas not directly contiguous to the mesangial matrix. At least three glomeruli were examined from each rat, with a minimum of five capillary sections surveyed in each glomerulus. The exact magnification of the image was calculated using diffraction grating replica standard (Polaron Equipment, Watford, U.K.).

Morphometric determination of the different glomerular domains and parameters was done using an automated image-analyzing system on transverse kidney sections processed with silver methenamine staining, which reveals the extracellular matrices. The image-analyzing system was composed of a light microscope (Nachet; Microvision, Evry, France), a black-and-white video camera (Cohu, San Diego, CA), an image-analysis processor (NS-15000, Nachet), and a microcomputer to store the data and pilot the processor using a personal program written in C language. The image-analysis processor, based on the principles of mathematical morphology, digitizes microscopic images into 512 × 512 pixel images within 256 gray levels. The digitized images were submitted to predefined transformation allowing automatic measurements of selected glomerular morphometric parameters (15). The glomeruli were measured with a ×25 objective, yielding a final calibration of 0.4219 µm/pixel. Any variability in the staining intensity of the sections could be corrected by calibrating the image according to the defined gray levels. The glomerular parameters measured were: (1) total glomerular surface area delimited by the internal edge of the Bowman’s capsule; (2) urinary space surface area; (3) cumulative surface area of the capillary lumen sections; (4) glomerular tuft surface area, defined by the total glomerular measurement minus the urinary space; (5) mesangial surface area, defined by the glomerular tuft measurement minus areas of the capillary lumens and the glomerular capillary free walls (see Figure 1). Although the individual capillary lumen section surface area was not obtained by the program used in the automated image analysis, the mean capillary section surface area was estimated by dividing the cumulative surface area of the capillary lumen by the number of capillary sections. The total number of glomeruli necessary to yield convergent data was established at 30 glomeruli per rat (15). The observer, unaware of the code of the sections, measured 30 glomeruli randomly over the depth of the cortex on each kidney section. Glomeruli show-
Using clear glomerular tuft retraction or extensive FSGH were not measured because, in these cases, differences between matrices, remnant capillary lumen sections, and urinary spaces were indistinguishable.

**Immunohistochemical Studies**

The differentiation and activation of intraglomerular mononuclear cells was assessed by incubating frozen sections from rats aged 14 days, 1, 3, 6, or 9 months for 60 min at room temperature (RT) with a panel of mouse monoclonal antibodies diluted in Tris-buffered saline pH 7.4, containing 0.1% BSA (Sigma Chemical, St Louis, MO): (1) ED1, diluted 1:1000 (Serotec, Oxford, UK), specific for a monocyte/macrophage cytoplasmic antigen (16); (2) ED2, diluted 1:1000 (Serotec, Oxford, UK), considered to be a marker of tissue-resident macrophages (16); (3) OX-4, diluted 1:50 (Sera-Lab; Crawley Down, Sussex, UK), which detects the MHC Class II la antigen; and (4) W3/25 diluted 1:50 (Serotec, Oxford, UK), which reacts with a CD4 epitope and binds to T lymphocyte helper cells and to a monocyte/macrophage subpopulation (17, 18). FSGH lesions were particularly well developed in the glomeruli of the 12-month-old obese rats, making the estimation of mononuclear cell densities difficult and imprecise. This age group was therefore not included in the immunohistochemical study. The sections were washed in Tris-buffered saline and incubated with rabbit anti-mouse immunoglobulin antibody (Dako Corporation, Carpinteria, CA) and alkaline-anti-phosphatase alkaline complexes (diluted 1:75) (Dako). The enzyme was revealed with freshly prepared Fast Red Substrate System (Dako) containing 0.33 mg/mL le-vanisol (Sigma) to reduce the staining background. Sections were counterstained with hematoxylin. The number of positive cells in each glomerulus was counted, with a minimum of 50 glomeruli surveyed per kidney section.

The presence of matrix protein was assessed by incubating frozen sections from rats aged 1, 3, 6, or 9 months for 60 min at RT with antibodies (Institut Pasteur Lyon, France) diluted in Tris/0.1% BSA buffer (pH 7.2), such as: (1) an IgG fraction of polyclonal rabbit anti-mouse Type IV collagen (1:20); (2) anti-rat Type III collagen rabbit immune serum (1:5); and (3) anti-rat Type I collagen rabbit immune serum (1:5). The sections were then incubated with a fluorescein-conjugated anti-rabbit swine antibody (Dako).

**Preparation of Isolated Microdissected Glomeruli**

The expression of genes for ECM components and inhibitors of ECM proteinases was monitored in glomeruli microdissected from a second series of lean and obese rats (1, 3, 6, 9, and 14 months old), after modifications of the technique described by Peten et al. (19). Rats were anesthetized with pentobarbital and the left kidney was perfused at 4°C, first with solution I (135 mM NaCl, 1 mM Na2HPO4, 1.2 mM Na2SO4, 1.2 mM MgSO4, 5 mM KCl, 2 mM CaCl2, 5.5 mM glucose, and 5 mM N-hydroxyethylpiperazin-N’-2-ethanesulfonic acid, pH 7.4) and then with 3 mL of the solution I containing I mg/mL Type I collagenase (300 U/mg) (Sigma) and 1 mg/mL BSA (Sigma). Small, superficial fragments of the kidney cortex were rapidly excised and transferred to a microdissecting dish, placed on ice, containing solution I.
supplemented with 1.2 U/μL RNasin, a RNase inhibitor (Promega, Madison, WI) and 1 mM dithiothreitol (Sigma). Glomeruli were separated from tubules and from afferent and efferent arterioles and removed from their Bowman's capsules under a binocular microscope. A total of 20 glomeruli were taken from each rat, and placed in acid guanidium thiocyanate solution for RNA extraction by the phenol/chloroform method.

**Reverse Transcription and Polymerase Chain Reaction Analysis**

The mRNA were extracted from glomeruli from three rats in each group and pooled to ensure sufficient material for the subsequent polymerase chain reaction (PCR) analysis. They were reverse transcribed into cDNA with oligo(dT) and MMLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). The reverse transcription products were amplified with primers for mouse fibronectin (upper primer: 5’-TTA TGA CGA TGG GAA GAC CTA-3’; lower primer: 5’-GTC GGG CTT GAA TTA CTC C-3’; product length, 295 base pairs [bp], for mouse α1 (IV) collagen (upper primer: 5’-TGC GCT ATT CCT TCG TGA TG-3‘; lower primer: 5’-TCT CGC TTC TCT CTA TGG TG-3‘; product length, 185 bp), mouse α2 (I) collagen (upper primer: 5’-TGC CCA CAG CCT TCT ACA CTT-3‘; lower primer: 5’-CAG CCA TTC CTC CCA CTC CAG-3‘; product length, 244 bp), mouse α2 (I) collagen (upper primer: 5’-TGC CCA CAG CCT TCT ACA CTT-3‘; lower primer: 5’-TTC TGG TAG CAG GGT TTC TTT-3‘; product length, 254 bp), mouse TIMP-1 (upper primer: 5’-ACC CAG AAA TCA AGG AGA CCA-3‘; lower primer: 5’-ACA CCC CAC AGC CAG CAC TAT-3‘; product length, 303 bp), and GAPDH (upper primer: 5’-GCA GAA GAT GCC AAG ATG-3‘; lower primer: 5’-GGG GGA GAT GAT GCA AGG-3‘; product length, 162 bp) and for GAPDH (upper primer: 5’-GTT AAG GTC GGA GTC AAC G-3‘; lower primer: 5’-GCT GAA GCC AGT GGA GTC C-3‘; product length, 299 bp). All PCR were carried out in 25 μL of a mixture containing 10 mM dNTP, 1× PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 40% dimethyl sulfoxide, 0.001% gelatin, MgCl2 1 mM for Type IV collagen, TIMP-1, TIMP-2, and GAPDH primers; 1.5 mM for fibronectin primers and Type I collagen primers; 0.75 mM for Type III collagen primers) and 2.5 U amplitaq polymerase (Perkin-Elmer Cetus, Norwalk, CT)]. Each sample was incubated in a DNA thermal cycler (Perkin-Elmer Cetus) at 55 to 58°C for 25 to 30 cycles, depending on the primer. The PCR fragments were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. Polaroid photographs of ethidium bromide-stained gels were digitized into 512x512 pixel gray-scale images. The amount of nucleic acid was determined by densitometric analysis of the dots. The intensities of the cDNA bands for each protein were normalized to the GAPDH band intensities. All experiments were performed in duplicate.

**Statistical Analysis**

Results are expressed as means ± SE. Statistical analysis was carried out using two-way analysis of variance with age and genotype as factors. Statistical significance was achieved if P < 0.05. In cases of interaction between the factors, one-factor analysis of variance was used at one level of the other factor. Data were analyzed using Statview 4.0 software (Abacus Concept Inc., Berkeley, CA).

**RESULTS**

Both lean and obese Zucker rats increased in weight with age, almost two times faster between 1 and 3 months than in the following period (Table 1). The obese rats were significantly heavier than age-matched lean littermates at and after 3 months of age, with body weights 40 to 50% greater. The cumulative weights of the two kidneys also increased with age in both groups of animals, more rapidly in the obese than in lean rats, the difference being significant at 9 and 12 months. However, the growth of the kidney with respect to the body weight remained roughly similar in the two groups of animals (Table 1). Mean systolic tail-cuff blood pressure increased slightly and similarly in both lean and obese groups between 3 and 12 months (Table 1).

Serum levels of cholesterol and triglycerides were higher in obese than in lean Zucker rats as early as 1 month (Table 1). Cholesterol levels increased twofold in obese rats between 1 and 12 months and triglyceride levels increased sevenfold. Plasma insulin levels, higher in the obese than in the lean group on and after 1 month, plateaued at 3 to 6 months and declined thereafter. No differences existed between the two groups by 12 months (Table 1). Serum creatinine concentration increased with age in the same manner in both groups of animals up to 9 months, but rose dramatically thereafter in obese rats (Table 1).

The creatinine clearance rate decreased with age in the obese group, dropping from 2.0 ± 0.8 mL/min at 3 months to 0.5 ± 0.1 mL/min at 12 months, but remained close to 1.3 mL/min in lean rats of any age (1.2 ± 0.3 mL/min at 3 months and 1.4 ± 0.1 mL/min at 12 months). Proteinuria developed markedly in obese rats between 3 and 9 months of age and stabilized thereafter. At 9 and 12 months, proteinuria was nearly 20 times greater in the obese rats than in the lean littermates (302.8 ± 50.7 and 16.5 ± 11.8 mg/24 h, respectively, at 12 months).

There was no sign of FSGH in lean-rat kidneys until 12 months. At 12 months, a faint FSGH, with a low severity index of 2, was detectable and affected 2% of the glomeruli. On the contrary, the incidence of FSGH in obese rats was 2% (severity index: 2) at 6 months and rapidly increased in quantity and severity to affect 26% of the glomeruli at 9 months with a severity index of 15, with no further development of the lesion thereafter. Histopathological examination of the kidney revealed a moderate tubulopathy in the obese group at 6 months, which worsened with age, affecting all of the obese animals after 9 months with the presence of tubular casts and cystic formations. Cystic formations were generally close to the most damaged glomeruli of the examined kidney section, and were often associated with areas of interstitial fibrosis.

The automated morphometric analysis revealed that glomeruli and glomerular tufts increased in size, in both obese and lean rat kidneys. They were larger in the obese group than in the lean group at all ages but
Early Macrophages and Glomerulosclerosis

### TABLE 1. Evolution of weights, blood pressure, and blood chemistries with age in obese rats and lean littermates

<table>
<thead>
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<th>Parameter</th>
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<tr>
<td>Lean</td>
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<td>398 ± 10</td>
<td>440 ± 1</td>
<td>471 ± 10^b</td>
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<td>128 ± 7</td>
<td>411 ± 14^c</td>
<td>594 ± 11^a</td>
<td>656 ± 12^a</td>
<td>654 ± 34^b,c</td>
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<td>Two-Kidney Weight (g)</td>
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<td>Lean</td>
<td>1.18 ± 0.05</td>
<td>2.38 ± 0.06</td>
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<tr>
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<td>36 ± 6</td>
<td>57 ± 3</td>
<td>73 ± 26</td>
<td>176 ± 52^b,c</td>
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^a N = six rats per group except in 12-month obese group where N = five rats.
^b Age effect: P < 0.001.
^c Lean versus obese: P < 0.05.

12 months (Figure 2A). The whole glomerular surface area began to decline in the obese group between 9 and 12 months. The urinary space surface area remained constant between 3 and 9 months, and tended to decline thereafter (Figure 2A). The cumulative surface area of the capillary lumen sections and the number of cut sections of the capillary lumen are shown in Figure 2B. These two parameters indicated significant differences in age in the two groups of rats. However, the curves had to be divided into two parts. The first part, from 1 to 3 months, corresponded to the growing period of the kidney (Table 1, Figure 2A) whereas the second period covered 3 to 12 months. When only the second period was considered, on and after 3 months of age, neither the cumulative surface area nor the number of capillary lumen sections changed significantly in the obese or lean group of animals (age effect between 3–12 months, P = 0.16 for both groups). With age, the mesangial domain increased in size in the two groups of animals, with a sharp expansion in obese rat kidneys between 6 and 12 months (Figures 1, 2C). At 12 months, the mesangial domain was three times larger in obese than in lean animals. Whereas in the 12-month-old obese rat kidneys, the glomerulus, urinary, and the capillary lumen surface areas tended to decline (Figure 2, A and B), the mesangial domain continued to expand in old obese rats (Figure 2C). The Bowman’s capsule basement membrane became thicker with age in both groups of animals (lean, 1.9 ± 0.3 μm versus 2.4 ± 0.1; obese, 1.8 ± 0.3 versus 2.9 ± 0.1 at 3 months and 12 months, respectively).

Electron microscopy showed that the GBM regularly thickened between 3 months and 12 months by a factor of 1.8 in lean rats (0.119 μm ± 0.003 versus 0.217 μm ± 0.018, N = three rats) and by a factor of 2.4 in obese rats (0.123 μm ± 0.003 versus 0.287 μm ± 0.018, N = three rats) (Figure 3). The difference between lean and obese rats became statistically significant at 12 months (P < 0.05).

Macrophage-ED1^+ cell density increased 3.5-fold in obese rat glomeruli between 15 days and 1 month and remained high for the following periods (Figure 4A). In lean rats, the density of such ED1-positive cells increased moderately (twofold) in early life and remained stable after 1 month, at a much lower level (almost twofold less) than in obese rats. La^+ cell density (Ox6-detected cell) (Figure 4B) increased in lean and obese rat glomeruli between 14 days and 1 month. Therefore, this density remained roughly constant in the lean group (if the 14-day data are excluded, the statistical analysis did not show any age effect: P = 0.1). On the
contrary, Ia<sup>+</sup> cell density continuously increased with age in the obese rat glomeruli. At 9 months, there were significantly more Ia<sup>+</sup> cells in obese than in lean rat glomeruli. The number of CD4<sup>+</sup> cells per glomerulus (recognized by W3/25: T cells and monocyte/macrophage subpopulation) was always low compared with the number of other cell types (Figure 4C). Again, at 9 months, obese rats had statistically more cells per glomerulus than did lean animals. There was no ED<sub>2</sub> antibody labeling in any case.

The change in mRNA synthesis for extracellular matrix proteins is shown in Figure 5. Alpha(I) collagen (Figure 5B) and fibronectin (Figure 5C), which are among the major components of the mesangial matrix, were actively synthesized in younger animals. Thereafter, the mRNA synthesis in the two groups of rats varied. In lean rats, alpha(I) collagen and fibronectin mRNA decreased with age, whereas in obese rats, after a decrease at 3 months, they regularly increased until 14 months. Messenger RNA for alpha(I) collagen (Figure 5D) was barely detected after 1 month of age and did not change, no matter what the experimental group and the age of the animals were, except for a sharp increase in 9-month-old obese rats. No mRNA for Type III collagen was detected in any rats of either group, at any age. These observations were paralleled by the immunodetection of the proteins in the glomeruli in kidney sections (Figure 6). Levels of mRNA for TIMP-1 and TIMP-2 slowly decreased in lean rats to reach minima in 9- and 14-month-old animals but remained constant in obese rats, with a peak at 9 months (Figure 5, E and F). They then increased 5.2-fold (TIMP-1) and 4.5-fold (TIMP-2) in obese rats as compared with lean rats.

DISCUSSION

With age, FSGH developed dramatically in obese Zucker rats between 6 and 9 months, affecting 25% of the glomeruli. In comparison, only 2% of the glomeruli show FSGH at 36 months in a model of true kidney aging. The Wistar (WAG)/Rij rat raised in specific pathogen-free conditions (15). Automated image analysis showed that all glomerular domains markedly increased in size during the first 3 months of life in obese and lean animals. This corresponds to the developing phase of young animals and kidneys. Thereafter, the glomeruli rapidly became hydropnephrotic in obese rat kidneys, as in aging (15), because of enlargement of the glomerular tuft after the age of 3 months if we ignore the growing period (1-3 months). There were more capillary sections in obese rat kidneys than in lean rat kidneys (P < 0.05; N = three rats/group). C: The mesangial domain enlarged in both groups of rats between 1 and 12 months (age effect: P < 0.01), with a marked increase at 6 to 9 months in obese rat glomeruli. The mesangial surface area in 12-month-old obese animals was three times greater than in lean rats (P < 0.001; N = three rats/group).
months. This was caused by a specific, large increase in argyrophilic extracellular matrices (GBM and mesangial matrix) with no statistical significant change in the number of capillary lumen sections or their surface area, or in the urinary space, between 3 and 12 months, except for a tendency to decline in the oldest animals. Contrary to human diabetic nephropathy, in which mesangial expansion could lead to glomerular functional deterioration in insulin-dependent diabetes mellitus by restricting the glomerular vasculature (20), the expansion of the glomerular tuft did not modify the capillaries in these obese Zucker rats, at least until 9 months, as was observed in true aging (15). Thereafter, although the mesangial domain was still expanding, a tendency toward a decline in glomerulus, urinary space, and capillary surface areas was observed at 12 months, a result that was significantly confirmed at 15 months in another series of animals (Michel et al., manuscript in preparation). This decline is not an artifact of measurements, as glomeruli showing manifest glomerular tuft retraction or extensive FSGH were not taken into account because glomerular structures were almost indistinguishable in these cases. It is probably a result of an impairment in the GFR (as shown by the decrease in creatinine clearance and a rise in creatininemia), indirectly provoked by a general alteration of the tubulointerstitial area, a phenomenon observed in progressive renal diseases (reviewed in Reference 21). This hypertrophy of the glomerular tuft might be associated with the genetic character of the Zucker rats or a consequence of hyperlipidemia and the associated events (cytokine and eicosanoid production) (22) rather than being directly induced by a glomerular hyperfunction, as micropuncture experiments indicate that glomerular hemodynamic alterations play little role in the initiation of glomerular injury in this strain of rats (13,23).

Because the interaction between serum lipids, macrophages, and mesangial cell activation must play a key role in the pathogenesis of glomerulosclerosis, we investigated the correlation between hyperlipidemia and trapping of monocytes/macrophages in the mesangial matrix in young Zucker pups. The number of ED1+ macrophages markedly increased in obese pups
cells were not glomerular-resident macrophages, as they were not labeled by the ED1 antibody (24), but our study does not discriminate between the local proliferation of recently recruited monocytes and a continuous influx of circulating cells with age. Although obese rats always had significantly more ED1$^+$ cells than lean rats, this number (close to two cells per glomerulus) remained lower than the densities generally seen in other experimental models of glomerulosclerosis, such as nephrotoxic serum nephritis (25), partial nephrectomy (26), or aminonucleoside nephrosis (27), but are in agreement with those seen by Magil and Frohlich (28) in obese Zucker rats and Mai et al. (29) in hypertension-induced renal injury. It is interesting to note that the use of an antibody which reveals all of the MHC Class II Ia$^+$ antigens instead of specific ED1$^+$ monocytes/macrophages did not seem adequate to detect the early inflammatory cells involved in the genesis of glomerulosclerosis in this strain of rat: In younger obese rats, the density of Ia$^+$ cells (which was low and similar to the ED1$^+$ cell density) did not differ from that seen in lean littermates (Figure 4B) or in the Wistar/Rij model (Lavaud, unpublished observations). This indicates that other cells (for example, a subset of mesangial cells) probably intervene in the Ia$^+$ labeling. The number of Ia$^+$ cells was significantly increased in 9-month-old obese rats, as were CD4$^+$ T helper lymphocytes and/or a subpopulation of monocytes/macrophages recognized by W3/25 antibody. This shows that inflammation involving major inflammatory cell types was taking place in the glomeruli in parallel with the development of tubular lesions and interstitial fibrosis seen in aged obese rat kidney.

Lipoproteins and oxidized lipoproteins play a major role in the recruitment and/or activation of macrophages (4). In obese Zucker rats, hyperlipidemia was associated with significant increases in serum very-low-density lipoprotein triglycerides and very-low-density lipoprotein cholesterol, which occurred very early and massively accumulated thereafter (9, 11). This study shows that hyperlipidemia (triglyceridemia and cholesterolemia) and glomerular macrophage density are already dramatically greater in 1-month-old obese rats than in their lean littermates. This close relationship between hyperlipidemia and macrophages is in agreement with the observation that lipid-supplemented diets, which induced hyperlipidemia and glomerulosclerosis in some animal models, increase the number of macrophage cells in the early stages of glomerular injury (22). Conversely, diets reducing hyperlipidemia should decrease the number of glomerular macrophages. Kasiske et al. (31) succeeded in reducing glomerular injury by feeding rats polyunsaturated diets, but found no decrease in the number of Ia$^+$ cells trapped into the mesangium of 9-month-old obese Zucker rats. This could be because of the fact that they did not use the specific ED1 antibody but a broader-range antibody to detect macrophages.

Figure 4. Immunohistochemical detection of ED1$^+$ (monocytes/macrophages), OX$^+$ (MHC Class II Ia$^+$ cells) and W3/25$^+$ (T helper CD$^+$ cells) cells (hatched bars, obese rats; open bars, lean rats). Macrophages (A) invaded the glomeruli of very young obese rats (14 days to 1 month) and their number per glomerulus remained significantly higher in obese than in lean rats (lean versus obese group: $P < 0.001$). In contrast, Ia$^+$ cells (B) and CD$^+$ cells (C) differed significantly in the two experimental groups only at 9 months ($P < 0.05$; $N = six$ rats per group).

In the very first weeks after weaning (Figure 4). This macrophage density remained low in lean rats from 1 to 12 months, and was similar to the value found during true kidney aging in the Wistar (WAG/Rij) model (Lavaud, unpublished observations). These
Figure 5. Synthesis of messenger RNA for α1(IV), α2(I) collagens, fibronectin, TIMP-1, and TIMP-2 in isolated, microdissected glomeruli from lean and obese rat kidneys. After an early phase of maturation of the glomeruli, as evidenced by the 1-month values, α1(IV) collagen (B) and fibronectin (C) mRNA decreased in lean rats, but increased between 3 and 14 months in obese animals. However, α2(I) collagen mRNA were barely detected after 1 month of age and did not change in either the experimental group regardless of the age of the animals, except for a sharp increase in 9-month-old obese rats. TIMP-1 (E) and TIMP-2 (F) mRNA decreased in lean rats to reach low values in 9- and 14-month-old animals, but were still synthesized in obese rats with a peak at 9 months. Glomerular mRNA isolated from three rats were pooled in each age group. Experiments were run in duplicates. The intensities of the cDNA bands for each protein were normalized to the GAPDH band intensities (A).
Figure 6. Immunofluorescence detection of Types I, III, and IV collagens in obese (A, C, E, F) and lean (B, D) rat kidneys. With age, Type IV collagen accumulated in obese and lean rat glomeruli, but more in obese than in lean animals (A, B: 3-month-old rats; C, D: 9-month-old rats). Type I collagen was barely seen in 9-month-old obese-rat glomeruli, associated with foci of FSGH lesions (E, arrow). Type III collagen was not detected in any group (F: 9-month-old obese-rat glomerulus). (Original magnification: A through D, ×500; E, ×300; F, ×600).

Activated macrophages can stimulate both mesangial cell proliferation and their synthesis activity via several cytokines and growth factors, as shown by studies on animal and human mesangial cells in culture (32). Lipoproteins also directly stimulate mesangial cells (33). Although this study provides no
precise information on mesangial cell hyperplasia, it shows that extracellular matrices (GBM and mesangial matrix) largely expand with age in the glomeruli of obese rats. This expansion is associated with a net increase in Type IV collagen and fibronectin (mRNA and proteins) and occurs late after a significant increase in plasma lipids, in parallel with the development of proteinuria and the beginning of kidney-function impairment. This influence of hyperlipidemia and macrophages on the upregulation of fibronectin mRNA was also found in experimental nephrotic syndrome induced by puromycin aminonucleoside (34). The synthesis of fibronectin and \( \alpha_1(IV) \) collagen mRNA continued to increase until the rats were 12 months old, whereas the macrophage density did not vary after 3 months. This could indicate that there is an autocrine production of cytokines by mesangial cells in the cascade of events initiated by the lipoproteins and the activation of monocytes/macrophages. This increase in fibronectin and \( \alpha_1(IV) \) collagen mRNA could also be the result of hyperinsulinemia, which reached a peak at 3 to 6 months, as insulin seems to be a potent factor in mesangial matrix accumulation \textit{in vivo} (35). We also detected a band corresponding to \( \alpha_2(II) \) collagen mRNA and the protein itself in 9-month-old obese rats when FSGH lesions were well established. This is in agreement with the results of Floege \textit{et al.} (36), who found Type I collagen in the remnant kidney model. Type III collagen mRNA and protein remained undetectable, whereas it has been found in a diabetic model (37). As the expansion of ECM components is the result of the balance between synthesis and degradation of the components, we looked for a possible reduction in protein degradation by checking the mRNA for TIMP-1 and TIMP-2, enzymes that specifically inhibit 72-kd metalloproteinase (TIMP-1) and 92-kd gelatinase (TIMP-2). These metalloproteinases are involved in the degradation of the major matrix components, Types IV and V collagens, fibronectin, proteoglycans, elastin, and laminin (38). The synthesis of both TIMP-1 and TIMP-2 mRNA increased in obese animals in parallel. Our results are in agreement with those of Guitjarro \textit{et al.} (22), who found increased glomerular TIMP-2 mRNA in a rat model of dietary-induced hypercholesterolemia. This synthesis of TIMP-1 and TIMP-2 mRNA in microdissected glomeruli is probably a result of the glomerular constitutive cells, primarily mesangial cells (39). However, the inhibition of the ECM degradation could also be partly mediated by infiltrating macrophages, as they produce collagenase inhibitors (40).

In summary, we have shown that the glomerular macrophage density is markedly greater in 1-month-old obese Zucker rats than in their lean littermates. This increase parallels the worsening of hyperlipidemia in the obese animals in the very first few days after weaning. Hyperlipidemia associated with hyperinsulinemia develops in preobese pups from 10 days onward (9). Low- and very-low-density lipoproteins are known to activate mesangial cells and to stimulate the production of chemokines (reviewed in Reference 5). Mesangial cells and endothelial cells also alter low-density lipoproteins to form minimally modified and oxidized low-density lipoproteins, which are cytotoxic for endothelial cells, aggravating the recruitment of macrophages by newly synthesized adhesion molecules and via the production of chemokines (reviewed in Reference 41). The invasion of glomeruli by monocytes/macrophages, which occurs on and after weaning, is probably the result of the increases in lipoproteins at very early ages in these obese Zucker pups. Thus, this study underscores the importance of the Zucker fa/fa rats as an appropriate model for examining the relationship between obesity, early changes in plasma lipids, plasma insulin, glucose intolerance, and the cellular and molecular events involved in the genesis of glomerulosclerosis.

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