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Research Article

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Leukotriene D₄ Is a Mediator of Proteinuria and Glomerular Hemodynamic Abnormalities in Passive Heymann Nephritis

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Abstract

We assessed the role of leukotrienes (LTs) in Munich-Wistar rats with passive Heymann nephritis (PHN), an animal model of human membranous nephropathy. 10 d after injection of anti-Fx1A antibody, urinary protein excretion rate (Upr) in PHN was significantly higher than that of control. Micropuncture studies demonstrated reduced single nephron plasma flow and glomerular filtration rates, increased transcapillary hydraulic pressure difference, pre- and postglomerular resistances, and decreased ultrafiltration coefficient in PHN rats. Glomerular LTB₄ generation from PHN rats was increased. Administration of the 5-LO activating protein inhibitor MK886 for 10 d markedly blunted proteinuria and normalized glomerular hemodynamic abnormalities in PHN rats. An LTD₄ receptor antagonist SK&F 104353 led to an immediate reduction in Upr and to reversal of glomerular hemodynamic impairment. Ia(+) cells/glomerulus were increased in PHN rats. In x-irradiated PHN rats, which developed glomerular macrophage depletion, augmented glomerular LT synthesis was abolished. Thus, in the autologous phase of PHN, LTD₄ mediates glomerular hemodynamic abnormalities and a hemodynamic component of the accompanying proteinuria. The synthesis of LTD₄ likely occurs directly from macrophages or from macrophage-derived LTA₄, through LTC₄ synthase in glomerular cells. (*J. Clin. Invest.* 1993. 91:1507-1515.) Key words: leukotriene D₄ • passive Heymann nephritis • proteinuria • glomerular pressure • lipoxygenase

Introduction

Passive Heymann nephritis (PHN)¹ is a complement-dependent animal model of human membranous nephropathy that

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1. Abbreviations used in this paper: π , colloid osmotic pressure; AP, arterial pressure; C, protein concentration; C_A, femoral arterial blood plasma C; C_E, surface efferent arteriolar C; FLAP, 5-LO activating protein; GEC, glomerular epithelial cell; GFR, glomerular filtration rate; Hct, hematocrit; K_f, glomerular capillary ultrafiltration coefficient; LC, leukocyte common antigen; LO, lipoxygenase; OD⁴⁹⁰, optical density at 490 nm of wavelength; ΔP , transcapillary hydraulic pressure difference; P_E, hydraulic pressure in surface efferent arterioles;

is induced by the administration of antibody against the brush border membrane antigen (FX1A) present on proximal tubule cells in rats (1). The antibody binds to a surface glycoprotein, gp-330, on glomerular epithelial cells (GEC) and activates the complement system leading to the assembly of the membrane attack complex of complement, C5b-9, and GEC injury (2). The morphological and pathological features of PHN, such as subepithelial cell deposits of antigen-antibody complex and effacement of GEC are shared with human membranous nephropathy. Proteinuria develops within 3-5 d after the injection of anti-Fx1A antibody and accompanies decreased renal plasma flow (RPF) and glomerular filtration rate (GFR) (3-5). Glomerular micropuncture studies reveal increased transcapillary hydraulic pressure difference (ΔP) and reduced glomerular capillary ultrafiltration coefficient (K_f) (3-5) as underlying hemodynamic changes in PHN. These microcirculatory characteristics were also shown to participate in the generation of proteinuria in PHN (4). Yoshioka et al. (4) demonstrated that, after administration of acetylcholine, a nonspecific vasodilator, proteinuria decreased to ~60% of preacetylcholine values, coincident with normalization of glomerular hemodynamic parameters in the autologous phase of PHN. This observation suggested the existence of vasoactive agent(s) that mediate perturbations in renal hemodynamics and the associated enhancement of glomerular capillary permeability to protein observed in PHN. There have been several reports describing roles for cyclooxygenase products or reactive oxygen species (6-11) in the development of proteinuria and hemodynamic abnormalities in PHN. Emerging evidence also suggests a role for lipoxygenase (LO) pathway products of arachidonic acid metabolism in the pathogenesis of PHN (10, 12). Lianos et al. (12) demonstrated enhanced production of leukotriene (LT) B₄, a 5-LO metabolite of AA, in the isolated glomeruli of PHN rats. Interestingly, the profile of changes in the glomerular microcirculatory parameters in PHN described above (i.e., decreased single nephron [SN] plasma flow and SNGFR, increased ΔP , and decreased K_f) is strikingly similar to that caused by the administration of LTD₄, a potent vasoconstrictive product of 5-LO pathway (13). To gain insight into the potential role of 5-LO products in the development of proteinuria and hemodynamic changes in PHN, we undertook a series of experiments using MK886, an inhibitor of 5-LO

P_{GC}, hydraulic pressure in surface glomerular capillaries; PHN, passive Heymann nephritis; P_T, hydraulic pressure in proximal tubules; Q_A, initial glomerular capillary plasma flow; R_A and R_E, resistance of single afferent and efferent arterioles, respectively; RPF, renal plasma flow; SN, single nephron; SNFF, SN filtration fraction; Upr, urinary protein excretion rate.

activation, and SK&F 104353, a specific LTD₄ receptor antagonist, coupled with glomerular micropuncture analysis, histologic evaluation, and measurement of glomerular LTB₄ generation in the absence or presence of macrophage depletion.

Methods

Induction of PHN and histologic examination

Sheep immune serum was raised against rat tubular brush border antigen Fx1A isolated according to the method described by Edginton et al. (14). Before use, sera were heat inactivated at 56°C for 30 min and adsorbed with rat peripheral blood cells and liver powder. Alternatively, the IgG fraction of immune sera was isolated by anion exchange column chromatography using DE-52 (Bio-Rad Laboratories, Richmond, CA) as the cellulose ion exchanger. A dose of 150 µg/g body weight IgG was used to induce the disease. Glomerular anti-Fx1A and complement deposition was assessed by cryostat immunofluorescence microscopy performed in 7–8-µm sections and used FITC-labeled immunoglobulin against sheep IgG and rat complement component C3. The presence of monocytes/macrophages and of leukocytes in glomeruli was also assessed by immunofluorescence using FITC-labeled mouse MAb against the rat monocyte/macrophage Ia determinant and the rat leukocyte common antigen (LC) (Sera-Lab, Sussex, UK). The presence of Ia(+) or LC(+) cells was expressed as number of cells per glomerulus (mean±SEM of the mean, $n = 20$ glomeruli).

Measurement of urinary protein excretion rate

All experiments were performed on adult male Munich–Wistar rats (200–220 g), which were maintained in metabolic cages on a standard rat diet and allowed free access to water. After a 24-h urine collection for the measurement of baseline protein excretion, anti-Fx1A antibody or nonimmune serum was administered intravenously and 24-h urine protein excretion rate (Upr) was monitored daily until 10–12 d after anti-Fx1A antibody injection, when micropuncture and morphological studies were performed. Animals were randomly divided into three age-matched experimental groups as follows.

Group 1. ($n = 8$). These animals were control rats to which 0.1 ml of nonimmune serum, as the vehicle of anti-Fx1A antibody, was administered intravenously.

Group 2. ($n = 9$). These animals received 0.1 ml of anti-Fx1A antibody.

Group 3. ($n = 8$). These animals received 0.1 ml of anti-Fx1A antibody, as in group 2, and were also given MK886 orally at a dose of 10 mg/kg every 12 h starting 1 d before the antibody injection and continuing daily until the day of micropuncture experiments.

Micropuncture studies

Micropuncture studies were performed on rats of each group described above 10–12 d after the injection of anti-Fx1A antibody or nonimmune serum according to protocols described previously (15). In brief, after Inactin anesthesia (100 mg/kg i.p.), the left femoral artery was catheterized with PE-50 tubing, which was used to monitor mean systemic arterial pressure (AP) by means of a pressure transducer (P23Db; Statham Instruments, Oxnard, CA) connected to a direct writing recorder (Gould Inc. Instruments Div., Santa Clara, CA) and to perform blood sampling. After a tracheostomy, PE-50 catheters were inserted into both jugular veins for infusion of plasma, and [³H]inulin (New England Nuclear) (300 µCi/experimental period in 0.9% NaCl at 1.2 ml/h). Another PE-50 catheter was inserted into the right femoral vein for infusion of SK&F 104353 or vehicle (0.9% NaCl at 1.2 ml/h). The left kidney was exposed by a left subcostal incision, separated from the surrounding fat, and suspended on a Lucite holder. The kidney surface was bathed with warm isotonic NaCl. Homologous rat plasma was administered intravenously at a rate of 10 ml/kg per h for

45 min followed by a reduction in infusion rate to 1.5 ml/kg per h for the remainder of the experiment. This protocol has been shown previously to adequately replace surgically induced plasma losses, thus maintaining euolemia (16).

In all experiments, measurements were started 60 min after the onset of plasma infusion and carried out as follows: two or three samples of urine from the experimental kidney were collected, each over 15 min, for the determination of flow rate (V) and [³H]inulin and protein concentrations and for the calculation of whole-kidney GFR, RPF, and urinary protein excretion rate (Upr). For these urine collections, indwelling ureteral catheters (PE-10) were used. Coincident with these urine collections, two or three blood samples of femoral artery and left renal vein were obtained in each period for determination of hematocrit (Hct) and plasma concentration of [³H]inulin for the calculation of GFR and RPF. The inulin concentration difference between renal arterial and venous blood was employed to calculate filtration fraction and RPF. Blood samples from renal vein were obtained from a 30-gauge needle placed in the left renal vein distal to the adrenal vein. Whole-kidney inulin clearance studies were performed during baseline conditions and repeated during administration of SK&F 104353 and other experimental conditions to be described.

Time-averaged hydraulic pressures were measured in surface glomerular capillaries (P_{GC}), proximal tubules (P_T), and surface efferent arterioles (P_E) by use of a continuous recording, servo-null micropipette transducer system (model 5; Instrumentation for Physiology and Medicine, Inc., San Diego, CA). Micropipettes with outer tip diameters of 2–4 µm and containing 2.0 M NaCl were used. Hydraulic output from the servo-nulling system was coupled electronically to a second channel of the Gould recorder by means of a pressure transducer.

Experiments were performed on seven groups of rats as follows.

Group 1. PHN control ($n = 8$). These animals were control rats that were treated as described above.

Group 2. PHN ($n = 9$). This group of animals received 0.1 ml of anti-Fx1A antibody.

Group 2-A. PHN and SK&F 104353 ($n = 6$). After the first set of micropuncture measurements was obtained from group 2 rats, six of the rats received SK&F 104353 (Smith Kline Beecham Pharmaceuticals, King of Prussia, PA) (10 mg/kg i.v. bolus injection followed by a continuous infusion at a rate of 1 mg/h) infused intravenously. A second set of micropuncture measurements was then performed.

Group 3. PHN treated with the 5-LO activating protein (FLAP) antagonist MK886 ($n = 8$). These animals received 0.1 ml of anti-Fx1A antibody, as in group 2, and were given MK886 (Merck Frosst Canada, Montreal, Quebec, Canada) orally at a dose of 10 mg/kg every 12 h starting 1 d before the injection of anti-Fx1A antibody and continuing until the day of micropuncture studies. The dose of MK886 employed in this study has been demonstrated to suppress the activity of 5-LO for ≥ 10 h (17).

Group 3-A. PHN and MK886 and SK&F 104353 ($n = 4$). After the first set of measurements was obtained from group 3 rats, SK&F 104353 was given to four of the rats as an initial bolus of 10 mg/kg and was followed by a continuous infusion of 1 mg/kg per h for the remainder of the experiment. A second set of micropuncture measurements was subsequently performed.

Group 4. Effects of MK886 or SK&F 104353 on normal untreated rats.

Group 4-A. MK886 control ($n = 5$). To test the renal hemodynamic effects of MK886 on normal animals, rats of this group were given MK886 orally at a dose of 10 mg/kg every 12 h for 1–2 d before the hemodynamic measurements.

Group 4-B. SK&F 104353 control ($n = 4$). To test the effect of SK&F 104353 on the renal hemodynamics of normal rats, two-period micropuncture studies were performed on this group of untreated rats before and after the administration of SK&F 104353 as described above.

All these measurements were completed within 60–90 min after the SK&F 104353 administration.

Enzyme-linked immunosorbent assay for anti-sheep IgG in PHN rat sera

To examine the possible effect of inhibition of 5-LO by the administration of MK886 on the immune system of PHN rats, we studied serum concentration of anti-sheep IgG in the autologous phase of PHN rats using ELISA. ELISA for anti-sheep IgG in PHN rat sera was performed as previously described with slight modification (18). In brief, 100 μ l of 200 time-diluted rat test sera was incubated in sheep IgG (Organon Teknika-Cappel, Malvern, PA)-coated 96-well microtiter plates (Becton Dickinson Labware, Lincoln Park, NJ) for 1 h at room temperature. After discarding sera and washing wells, 1,000 time-diluted biotylated goat anti-rat IgG (Organon Teknika-Cappel) was added and incubated for 1 h at room temperature. After washing wells, 2.5 μ g/ml of avidin-conjugated peroxidase (Organon Teknika-Cappel) was added and incubated for 30 min at room temperature. After washing wells, 100 μ l of developing buffer containing 50 mM phosphate-citrate (pH 5.0), 0.04% α -phenylenediamine, and 0.014% H₂O₂ was added and incubated for 10 min at room temperature. Reaction was terminated by addition of 100 μ l of 2.5 M H₂SO₄ and optical density at 490 nm of wavelength (OD⁴⁹⁰) was measured by a microplate reader (Titertek, Elfab Oy, Finland). OD⁴⁹⁰ of nonspecific binding was estimated by addition of PBS instead of test serum and corrected OD⁴⁹⁰ was obtained by subtracting that of nonspecific binding from each OD⁴⁹⁰. Data are expressed as means \pm SE of three or four determinations of ELISA units (corrected OD⁴⁹⁰ times primary serum dilution factor [=200]) as described elsewhere (18). Sera from PHN rats (PHN; $n = 4$) and PHN rats treated with daily oral MK886 administration (PHN + MK; $n = 3$) 10 d after the injection of anti-Fx1A antibody as described above were compared with those from normal control rats (Control; $n = 4$).

Glomerular macrophage depletion

This was accomplished by the use of whole-body x-irradiation. Rats received 250 KVp orthovoltage x-rays with a half value of 1 mmCi at a dose of 133 rad/min for a total dose of 900 rad, using parallel opposed fields. Kidneys were shielded with 6-mm-thick lead blocks, which covered the kidneys within 5-mm margins. Positioning of the block was verified with diagnostic x-rays done simultaneously with treatment (Port Fields). Dosimeter was done in a Plexiglas phantom using a Farmer-type ionization chamber. The effect of x-irradiation was assessed 4 d later on peripheral leukocyte counts determined by an automated hematology analyzer and on glomerular Ia(+) cell counts. These cells were identified by immunofluorescence microscopy (direct) performed in 7- μ m cortical sections using an FITC-labeled MAb against the rat monocyte/macrophage Ia determinant (Sera-Lab) as described above. The presence of Ia(+) cells was expressed as number of cells per glomerulus (mean \pm SEM of the mean, $n = 20$ glomeruli). X-irradiated animals ($n = 8$) received anti-Fx1A IgG doses identical to those given to nonirradiated rats.

Glomerular synthesis of LTB₄

Glomerular LTB₄ synthesis was assessed 24–48 h after the intravenous administration of sheep IgG ($n = 6$) or anti-Fx1A antibody ($n = 14$) and in animals subjected to whole-body x-irradiation followed by administration of anti-Fx1A antibody ($n = 8$). In the latter group, anti-Fx1A antibody was given 4 d after total body x-irradiation and glomerular LTB₄ synthesis was assessed 24–48 h later. We have previously shown that 24–48 h after anti-Fx1A administration the synthesis of LTB₄ in isolated glomeruli is enhanced (12). Moreover, in animals undergoing total-body x-irradiation, a high mortality rate occurs (primarily due to gastrointestinal hemorrhage) early (at time point past day 5) after administration of x-irradiation. Glomerular LT synthesis was, therefore, also assessed 24–48 h after anti-Fx1A administration (day 7 after x-irradiation). Glomeruli were isolated by differential sieving, suspended in 1 ml RPMI 1640 and incubated at 37°C for 45 min in the presence of the phospholipase A₂ activator, A23187 (2 mmol).

Glomerular suspensions were subsequently extracted with 3 vol of ice-cold ethanol and acidified with formic acid to pH 3.0–3.5. After a 30-min vigorous agitation at 4°C to precipitate tissue protein, glomerular suspensions were centrifuged to separate tissue from the solvent/ aqueous phase mixture. The tissue pellet was dissolved in 1 ml sodium hydroxide, 0.5 N, to denature protein, and this solution was used for glomerular protein determination by a colorimetric method. Supernates were dried, reconstituted in 1 ml of HPLC solvent (methanol/water/acetic acid, 65:35:0.02, pH 5.7) and injected into a high pressure liquid chromatography. Eluted LTB₄ was subsequently quantified by radioimmunoassay using a specific antibody provided by Dr. A. Ford-Hutchinson (Merck-Frosst Laboratories, Pointe-Claire, Quebec, Canada).

Analytical

Colloid osmotic pressures (π) of plasma entering and leaving glomerular capillaries were estimated from values of protein concentration (C) in femoral arterial (C_A) and surface efferent arteriolar (C_E) blood plasmas. π was calculated according to previously derived equations (19). Values for C_A, and thus π _A, for femoral arterial plasma were taken as representative for values for C and π for the afferent end of the glomerular capillary network. These estimates of pre- and postglomerular protein concentration permit calculation of single-nephron filtration fraction (SNFF) and K_f , as well as the resistance of single afferent (R_A) and efferent (R_E) arterioles and the initial glomerular capillary plasma flow (Q_A), using equations described in detail elsewhere (20).

The volume of fluid collected from individual proximal tubules was estimated from the length of the fluid column in a constant-bore capillary tube of known internal diameter. The concentrations of inulin in tubule fluid, plasma, and urine were determined by measuring the radioactivity of [³H]inulin in a scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Calculation of values for GFR and SNGFR was performed using conventional formulas. C_A and C_E were determined using a fluorometric method developed by Viets et al. (19). Urine protein concentration was measured using Coomassie brilliant blue method (21).

Statistical

Within-group comparisons were carried out using the *t* test, while inter-group multiple comparisons were made with ANOVA followed by the Newman-Keuls test. A *P* value < 0.05 was required for statistical significance. All values are reported as means \pm SEM.

Results

Progression of proteinuria. In group 1 control rats, Upr measurements were constant and less than 10 mg/d throughout the experimental period (Fig. 1). In group 2, Upr was not different from the control values from day 0 (1 d before the injection of anti-Fx1A antibody or control serum) until day 4, at which time Upr in this group increased significantly compared with that of group 1 and increased progressively thereafter (Fig. 1). On day 10, Upr in group 2 was 172 \pm 45 mg/d, a value significantly different from that of control animals (10 \pm 3 mg/d; *P* < 0.005). In group 3 PHN rats treated with MK886, Upr was not different from the control values from day 0 until day 4, at which time Upr of group 3 was significantly greater than that of group 1 and increased thereafter. Upr in group 3, however, was significantly less than that of group 2 on days 8–10 (Fig. 1). On day 10, Upr in group 3 was 75 \pm 21 mg/d, a value statistically different from that of control group 1 (*P* < 0.01) and PHN group 2 (*P* < 0.05) (Fig. 1).

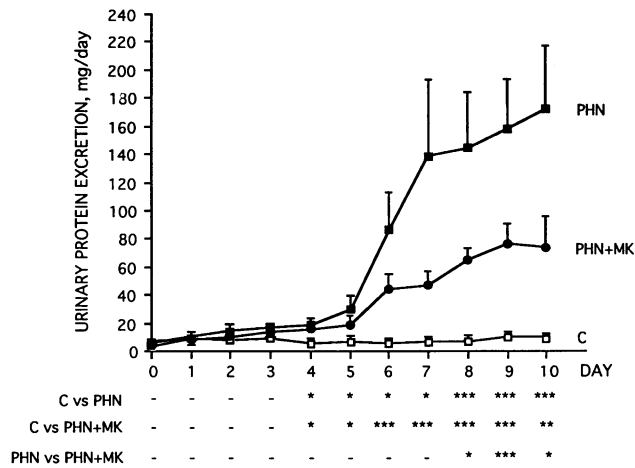


Figure 1. Urinary protein excretion levels in control (C; n = 8), passive Heymann nephritis (PHN; n = 9), and PHN treated with the oral 5-lipoxygenase activating protein inhibitor MK886 (PHN + MK; n = 8) groups. Symbols denote P values between two groups indicated at left (-, not significant; *P < 0.05, **P < 0.01, ***P < 0.005).

Micropuncture studies. Values for systemic and whole-kidney functions of rats 10–12 d after the injection of anti-Fx1A or control serum are summarized in Table I. Examination of parameters at the SN level (summarized in Fig. 2) revealed that Q_A and SNGFR of group 2 PHN rats (141.8 ± 11.9 and 47.7 ± 3.0 nl/min, respectively) were lower than those of group 1 control animals (219.8 ± 17.5 nl/min, $P < 0.005$; and 57.2 ± 1.3 nl/min, $P < 0.05$, respectively) and group 3 PHN rats (216.0 ± 16.9 nl/min, $P < 0.005$; and 58.9 ± 3.7 nl/min, $P < 0.05$, respectively) treated with the FLAP antagonist MK886. There were no significant differences in QA and SNGFR between groups 1 and 3. SNFF in group 2 (0.34 ± 0.01) was statistically different from that of groups 1 (0.27 ± 0.02 ; $P < 0.005$) and 3 (0.28 ± 0.02 ; $P < 0.01$). There was no significant difference in SNFF between groups 1 and 3. R_A of group 2

($2.42 \pm 0.18 \cdot 10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$) was statistically higher than that of groups 1 ($1.26 \pm 0.13 \cdot 10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$; $P < 0.0001$) and 3 ($1.53 \pm 0.18 \cdot 10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$; $P < 0.005$). No differences in R_A were found between groups 1 and 3. R_E of group 2 animals ($1.65 \pm 0.14 \cdot 10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$) was also higher than that in groups 1 ($0.80 \pm 0.09 \cdot 10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$; $P < 0.0001$) and 3 ($0.99 \pm 0.08 \cdot 10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$; $P < 0.005$). R_E in group 3 was not different from that in group 1. An increased P_{GC} in group 2 (53.6 ± 1.7 mmHg; $P < 0.05$ vs. group 1 [48.9 ± 0.9 mmHg] and not significant [NS] vs. group 3 [50.0 ± 0.6 mmHg]) with equivalent proximal tubule pressure (P_T) values among three groups (11.5 ± 1.3 , 8.4 ± 0.7 , and 10.6 ± 1.2 mmHg in groups 1, 2, and 3, respectively) resulted in a significantly higher value for ΔP in group 2 (45.1 ± 1.3 mmHg; $P < 0.005$ vs. group 1 [37.4 ± 1.0 mmHg], $P < 0.005$ vs. group 3 [39.4 ± 1.2 mmHg]). ΔP in group 3 was not different from that in group 1. C_E , which was 6.8 ± 0.2 g/dl in group 1, 6.8 ± 0.1 g/dl in group 2, and 6.3 ± 0.2 in group 3, translated to values for postglomerular (π_E) equal to 25.6 ± 0.7 , 24.4 ± 0.8 , and 21.9 ± 1.2 mmHg, respectively. The presence of filtration pressure disequilibrium in all three experimental groups allowed for the calculation of unique values for K_f . K_f in group 2 ($0.030 \pm 0.003 \text{ nl} \cdot \text{s}^{-1} \cdot \text{mmHg}^{-1}$) was significantly lower than the values in groups 1 (0.054 ± 0.005 ; $P < 0.005$) and 3 (0.045 ± 0.004 ; $P < 0.005$). There was no difference in K_f between groups 1 and 3. In normal untreated rats, MK886 had no effects on systemic (see Table I) as well as glomerular hemodynamic parameters in group 4-A rats (Q_A , 210.6 ± 21.4 nl/min; SNGFR, 54.1 ± 2.6 nl/min; SNFF, 0.26 ± 0.02 ; ΔP , 36.8 ± 2.1 mmHg; R_A , $1.26 \pm 0.21 \cdot 10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$; R_E , $0.81 \pm 0.05 \cdot 10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$; K_f , $0.052 \pm 0.008 \text{ nl} \cdot \text{s}^{-1} \cdot \text{mmHg}^{-1}$) compared with the numbers in the control periods of group 4-B untreated rats described below.

Effects of SK&F 104353 on glomerular circulation and protein excretion. The changes in the systemic and glomerular microcirculation before and after the administration of SK&F 104353 in groups 3-A and 4-B are summarized in Table I and Figure 3. In group 4-B (untreated [normal] rats), SK&F

Table I. Summary of Parameters of Systemic Hemodynamics and Whole-Kidney Function

Group	n	Body wt g	Hct		AP		CA		RPF		GFR		FF	
			C	E	C	E	C	E	C	E	C	E	C	E
			vol%		mmHg		g/dl		ml/min					
1 Control	8	232±5	47±2		113±5		5.0±0.1		5.32±0.40		1.32±0.0		0.25±0.01	
			* vs. 3				** vs. 2		** vs. 2		* vs. 2		*** vs. 2	
2 PHN	9	230±7	43±1		126±3		4.5±0.1		3.49±0.50		1.05±0.11		0.31±0.01	
							** vs. 1		** vs. 1		* vs. 1		*** vs. 1	
3 PHN + MK	8	214±8	41±2		115±6		4.6±0.2		5.18±0.51		1.35±0.08		0.27±0.02	
			* vs. 1						* vs. 2		* vs. 2			
2-A PHN + SK&F	6	236±15	42±5	41±1	124±4	122±4	4.5±0.3	4.5±0.1	3.40±0.64	4.65±0.64	1.05±0.15	1.26±0.13	0.32±0.02	0.28±0.01
				+		+				+++		++		++
3-A PHN + MK + SK&F	4	226±9	39±2	40±2	122±9	119±10	4.9±0.3	4.9±0.2	5.70±0.70	5.82±0.40	1.41±0.13	1.55±0.09	0.25±0.01	0.27±0.01
4-A con + MK	5	236±2	45±3		107±5		4.8±0.2		5.26±0.6		1.34±0.07		0.26±0.02	
4-B con + SK&F	4	235±3	44±4	43±4	102±5	96±4	4.8±0.2	4.9±0.2	5.46±0.09	6.04±0.69	1.31±0.03	1.36±0.09	0.25±0.03	0.23±0.02
						+								

In groups 2-A, 3-A, and 4-B, values before (C) and after (E) the administration of SK&F 104353 are demonstrated. Asterisks (*, **, and ***) indicate significant difference from the values of other groups ($P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively). Crosses (+, ++, and +++) indicate significant difference from the values of control groups ($P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively). n, numbers of animals studied.

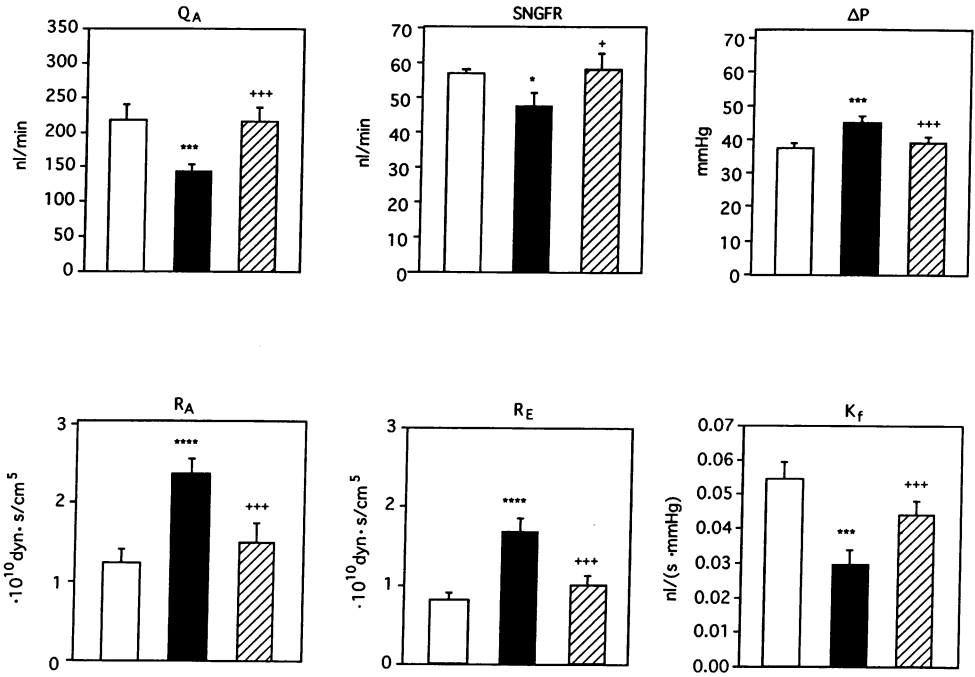


Figure 2. Summary of micropuncture studies in rats 10–12 d after the injection of either control serum (control group, open columns; $n = 8$), anti-Fx1A antibody (PHN group, closed columns; $n = 9$), or anti-Fx1A antibody with oral treatment with 5-lipoxygenase activating protein inhibitor MK886 (PHN + MK group, hatched columns; $n = 8$). Asterisk indicates significant difference from the value of control group (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, **** $P < 0.0001$). Cross on the value of PHN + MK group indicates significant difference from the value of PHN group (+ $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$).

104353, which was infused continuously at a rate of 1 mg/kg per h after a bolus injection of 10 mg/kg, caused no changes in the glomerular hemodynamic parameters measured. Q_A , from 205.3 ± 27.2 to 206.4 ± 39.0 nl/min; SNGFR from 56.4 ± 1.6 to 55.3 ± 4.3 nl/min; SNFF, from 0.28 ± 0.04 to 0.28 ± 0.04 ; ΔP , from 38.8 ± 1.0 to 36.8 ± 2.2 mmHg; R_A , from 1.25 ± 0.26 to 1.15 ± 0.30 $10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$; R_E , from 0.93 ± 0.14 to 0.93 ± 0.17 $10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$; and K_f , from 0.044 ± 0.003 to 0.054 ± 0.015 $\text{nl} \cdot \text{s}^{-1} \cdot \text{mmHg}^{-1}$. In group 3-A rats, which were PHN animals already treated with MK886, the LTD4 antago-

nist SK&F 104353 caused no statistical changes in glomerular hemodynamic parameters measured, including Q_A , from 226.0 ± 27.8 to 208.2 ± 32.3 nl/min; SNGFR, from 58.0 ± 7.2 to 62.1 ± 7.8 nl/min; SNFF, from 0.26 ± 0.03 to 0.30 ± 0.02 ; ΔP , from 41.0 ± 1.4 to 36.0 ± 2.7 mmHg; R_A , from 1.62 ± 0.37 to 1.86 ± 0.17 $10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$; R_E , from 0.92 ± 0.09 to 0.88 ± 0.13 $10^{10} \text{ dyn} \cdot \text{s} \cdot \text{cm}^{-5}$; and K_f , from 0.043 ± 0.006 to 0.060 ± 0.014 $\text{nl} \cdot \text{s}^{-1} \cdot \text{mmHg}^{-1}$.

In group 2-A PHN rats, SK&F 104353 infusion altered Hct and AP (values for systemic parameters are summarized in

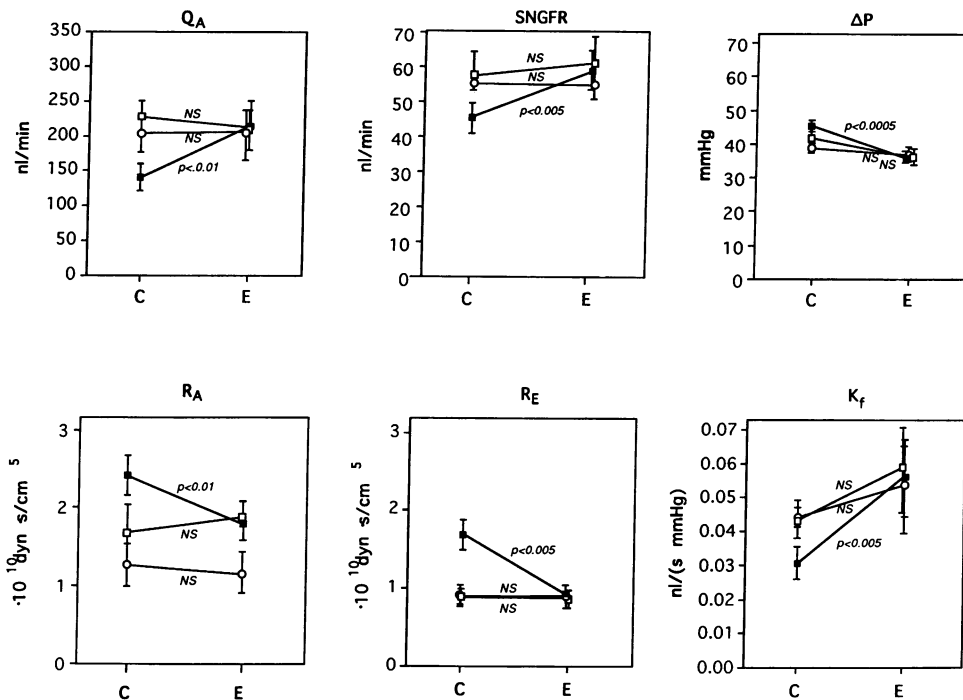


Figure 3. Effects of peptidoleukotriene receptor antagonist SK&F 104353 on glomerular hemodynamics of normal (open circle; $n = 4$), PHN (closed square; $n = 6$), and PHN rats pretreated with the 5-lipoxygenase activating protein inhibitor MK886 (open square; $n = 4$). Note that SK&F 104353 changed micropuncture parameters only in PHN rats. P value of each change of PHN group is indicated on the figure.

Table I). Despite the fall in AP, RPF increased from 3.40 ± 0.64 to 4.65 ± 0.64 ml/min ($P < 0.005$), and GFR also increased significantly from 1.05 ± 0.15 to 1.26 ± 0.13 ml/min ($P < 0.01$). Q_A increased from 142.4 ± 18.6 to 216.3 ± 37.9 nl/min ($P < 0.01$) and SNGFR from 46.8 ± 4.7 to 59.6 ± 5.8 nl/min ($P < 0.01$). SNFF changed from 0.34 ± 0.02 to 0.29 ± 0.03 ($P < 0.005$). These hemodynamic changes were accompanied by a significant reduction of R_A (from 2.44 ± 0.29 to 1.78 ± 0.20 $10^{10} \text{dyn} \cdot \text{s} \cdot \text{cm}^{-5}$ [$P < 0.01$]) and R_E (from 1.69 ± 0.21 to 0.94 ± 0.13 $10^{10} \text{dyn} \cdot \text{s} \cdot \text{cm}^{-5}$ [$P < 0.005$]). This reduction in R_E led to a decrease in P_{GC} from 53.3 ± 1.9 to 46.2 ± 1.9 mmHg ($P < 0.005$). P_T changed from 8.7 ± 0.5 to 10.5 ± 1.0 mmHg ($P < 0.05$), and ΔP decreased significantly from 44.7 ± 1.5 to 35.7 ± 1.3 mmHg ($P < 0.0005$). C_E and π_E changed from 6.6 ± 0.5 to 6.0 ± 0.3 g/dl ($P < 0.05$) and from 23.5 ± 1.5 to 20.3 ± 0.8 mmHg ($P < 0.05$), respectively. Calculated K_f increased from 0.030 ± 0.005 to 0.056 ± 0.011 $\text{nl} \cdot \text{s}^{-1} \cdot \text{mmHg}$ ($P < 0.005$). The effects of SK&F 104353 on the glomerular microcirculation are shown in Fig. 3.

LTD4 antagonist SK&F 104353 acutely reduced the Upr in group 2-A PHN rats from 0.23 ± 0.06 to 0.14 ± 0.02 mg/min ($P < 0.05$) although it had no effect on proteinuria in group 3-A PHN rats treated with MK886 (from 0.04 ± 0.01 to 0.04 ± 0.01 mg/min; Fig. 4).

Anti-sheep IgG. Anti-sheep IgG titer in PHN rat sera was significantly higher than that of control rats (Control, 71.08 ± 14.04 ELISA units/well; PHN, 422.73 ± 3.95 , $P < 0.01$ vs. Control). Simultaneous administration of FLAP antagonist, MK886, did not affect the titer of anti-sheep IgG (PHN + MK, 431.53 ± 6.18 ; $P < 0.01$ vs. Control, not significant vs. PHN), (Fig. 5).

Glomerular immunohistology. As shown in Table II, the number of Ia(+) cells in the glomerulus was increased in PHN rats and in PHN rats treated with MK886 compared with control rats. The number of Ia(+) cells in the PHN group was greater than those of the control group and less than those of PHN treated with MK886. The number of LC-positive cells in the glomerulus was not statistically different among the three groups.

Glomerular macrophage depletion. Fig. 6 demonstrates the effect of x-irradiation on peripheral leukocyte counts and on glomerular macrophage counts. Whole-body x-irradiation resulted both in peripheral leukopenia and in depletion of glomerular macrophages. In Fig. 7, glomerular synthesis of LTB4

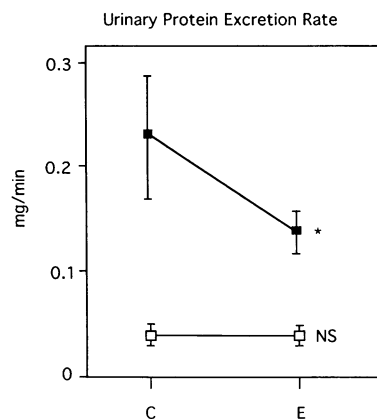


Figure 4. Changes in urinary protein excretion rate before (C) and after (E) the administration of the peptidoleukotriene receptor antagonist SK&F 104353 in PHN (closed square; $n = 6$) rats and in PHN rats treated with the oral 5-lipoxygenase activating protein inhibitor MK886 (open square; $n = 4$). Asterisk indicates significant difference from the control value ($P < 0.05$).

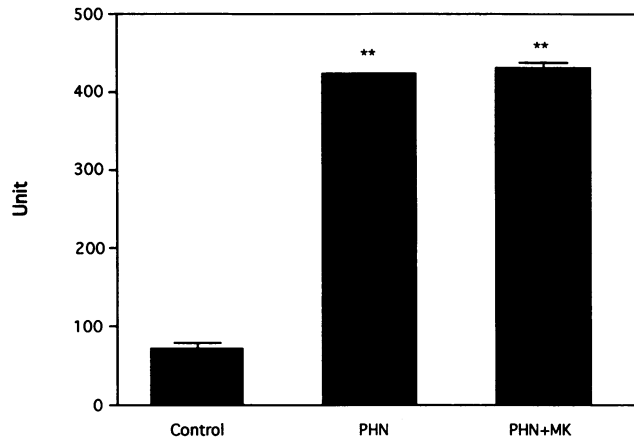


Figure 5. Anti-sheep IgG titer in sera of PHN rats and effect of MK886 on IgG titer. Data are expressed as means \pm SE of three or four determinations of ELISA units (corrected OD^{490} times primary serum dilution factor [=200]). Asterisks indicate significant difference from the control value ($P < 0.01$).

is shown in control animals (injected with sheep IgG), in animals that received anti-Fx1A IgG, and in animals with glomerular macrophage depletion induced before administration of anti-Fx1A antibody. Increased LTB4 synthesis was present in glomeruli isolated from anti-Fx1A-treated animals (4.9 ± 0.8 compared with 2.0 ± 0.3 ; $P < 0.05$). This increase was abolished in glomeruli isolated from animals with glomerular macrophage depletion.

Discussion

In this paper, we demonstrated that the enzyme activity of 5-LO was increased in PHN rats and that the inhibition of 5-LO by MK886 markedly attenuated the progression of both proteinuria and renal dysfunction in rats with PHN. LTB4 can be measured consistently and reliably in isolated glomeruli (11, 12) and its synthesis is augmented markedly in early PHN (Fig. 7) but suppressed to control levels at 12 d (12). This pattern of an early burst followed by apparent synthesis inhibition of LTB4 is not surprising and is also observed in nephrotoxic serum nephritis (11, 22). It suggests that the local biosynthesis of LTB4 is regulated by the activity of LTA4-hydrolase, a rate-limiting enzyme that converts LTA4 to LTB4 rather than by 5-LO. This is further supported by the fact that hemodynamic measurements on days 10–12 in the present studies assign a functional role for LTD4 in mediating glomerular hemodynamic changes in PHN, which indicates continued 5-LO activity.

Table II. LC(+) and Ia(+) Cells in Glomeruli of Animals 10 d after the Injection of PHN Antiserum

	Control	PHN	PHN + MK886
LC(+) cells	5.6 ± 0.7	5.9 ± 0.1	8.2 ± 1.3
Ia(+) cells	3.9 ± 0.2	$4.7 \pm 0.1^*$	$6.5 \pm 0.5^{**\dagger}$

$n = 4$ for each group. Values are cells per glomerulus. Asterisks (*) and (**) indicate significant difference from the values of control groups ($P < 0.05$ and $P < 0.01$, respectively). Cross (†) indicates significant difference ($P < 0.05$) from the value of PHN group.

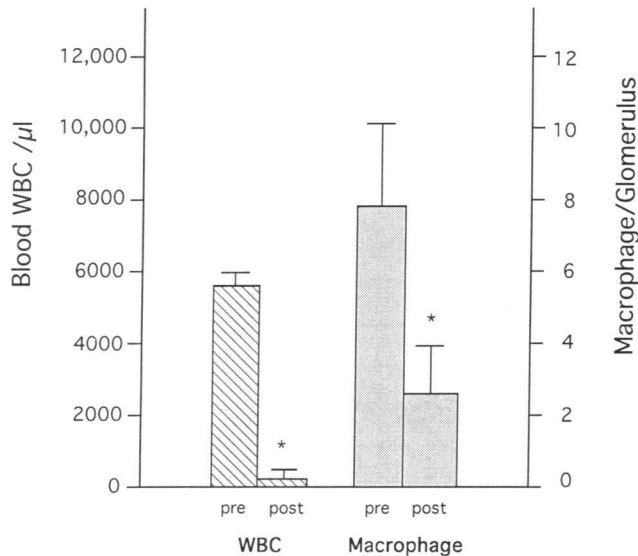


Figure 6. Effect of x-irradiation on peripheral leukocyte (WBC) counts and on glomerular macrophage counts. Whole-body x-irradiation resulted both in peripheral leukopenia and in depletion of glomerular macrophages. * $P < 0.05$.

MK886 is a specific FLAP antagonist that inhibits the association of cytosolic 5-LO to the membrane-bound protein, FLAP (23), a process essential to enzyme activity (24). MK886 was also shown to have no effects on cyclooxygenase and 12-LO activities (17). Hence, PHN rats treated with MK886 in this study lack all the products of 5-LO activity, including 5-HETE, LTB₄, and the peptidoleukotrienes LTC₄, LTD₄, and LTE₄, as well as the lipoxins throughout the observation period. Whereas MK886 treatment in itself had no effects on any glomerular hemodynamic parameters, as shown in

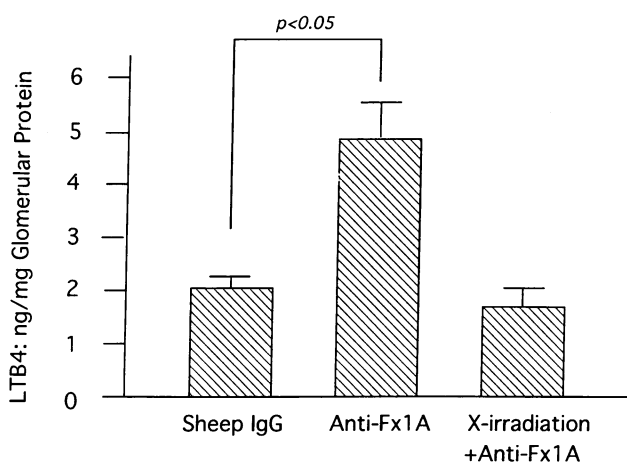


Figure 7. Glomerular synthesis of LTB₄ in control animals (injected with sheep IgG, $n = 6$), in animals that received anti-Fx1A immunoglobulin ($n = 14$), and in animals with glomerular macrophage depletion induced before administration of anti-Fx1A antibody ($n = 8$). Increased LTB₄ synthesis was present in glomeruli isolated from anti-Fx1A-treated animals ($P < 0.05$). This increase was abolished in glomeruli isolated from animals with glomerular macrophage depletion.

group 4, glomerular micropuncture studies revealed that, in rats with PHN, 5-LO inhibition reversed the increased R_A and R_E , increased ΔP , and decreased K_f , leading to normalization of Q_A and SNGFR (Fig. 2). MK886 did not affect the production of rat anti-sheep IgG 10 d after the administration of anti-Fx1A antibody (Fig. 7), suggesting that these effects of MK886 on renal hemodynamics and proteinuria in PHN rats were not through inhibition of the development of the autologous phase of PHN. Furthermore, the specific LTD₄ receptor antagonist SK&F 104353, which in itself had no effects on glomerular hemodynamics, as shown in group 5 (Fig. 3), completely normalized all the parameters of glomerular microcirculation and significantly reduced the urinary excretion of protein in PHN (group 2-A), confirming that the salutary effects of MK886 on proteinuria and renal dysfunction in these rats were through the inhibition of peptidoleukotriene production in the kidney (Figs. 3 and 4). MK886 might have additional actions, such as the decrease in Hct in group 3 rats, consistent with reversal of LTD₄/LTC₄-induced hemoconcentration (25). That the administration of SK&F 104353 did not affect glomerular hemodynamics and proteinuria in group 3-B PHN rats, which were already treated with MK886 (Figs. 3 and 4), presents additional evidence for the significance of LTD₄ production in the pathophysiology of PHN. Yoshioka et al. (4) have shown that exogenous administration of a nonspecific vasodilator, acetylcholine, decreased Upr in PHN rats in association with the normalization of glomerular hemodynamic parameters, indicating that the deterioration of renal hemodynamics and the elevated Upr observed in PHN rats were functional and reversible. The responsible factor(s) for these changes had remained unknown. Here, we demonstrate that endogenous LTD₄ is involved in the development of renal dysfunction and proteinuria observed in PHN.

Peptidoleukotrienes are a group of arachidonate 5-lipoxygenase metabolites that are derived by the action of putative LTC₄ synthase on LTA₄ and are widely known as mediators of a variety of allergic and inflammatory reactions (26). While the detection and measurement of endogenous renal production of LTD₄ is still fraught with major methodological difficulties (27), the functional significance of LTD₄ in the kidney has been well established mainly through the identification of receptors in cultured mesangial cells (28) and through the demonstration of the actions of exogenous LTD₄ on renal hemodynamics (13). In nephrotoxic serum nephritis in rats, we demonstrated that a specific LTD₄ receptor antagonist restored the decline in glomerular hemodynamics (27), which suggests that LTD₄ participates in the development of functional changes in nephrotoxic serum nephritis. In murine lupus nephritis, it has been shown that the renal production of LTB₄ and LTC₄ was increased (29) and that the administration of the LTD₄ receptor antagonist SK&F 104353 also reversed the renal dysfunction in this model (29). In addition to immunologic and hemodynamic actions of LTD₄, a number of papers have been published reporting that peptidoleukotrienes were involved in the activation of pertussis toxin-sensitive G protein gated muscarinic K⁺ channel in cardiac myocytes as an intracellular signal mediator (30, 31), which implies a wider scope of functions for this family of leukotrienes. Indeed, LTC₄ was demonstrated to bind to human GEC in culture promoting their proliferation (32), which suggests, together with our present results, a potential role for peptidoleukotrienes in the development of the "cel-

lular crescent," a histological finding observed in advanced membranous nephropathy (33). Cyclooxygenase products, including prostaglandin E₂ and thromboxane A₂, might also be involved in the pathogenesis of PHN (6–8). LTD₄ stimulates prostanoid synthesis in smooth muscle and endothelial cells in culture via activation of phospholipase A₂ (34), which in turn might result in a positive feedback loop for eicosanoid production.

The cellular origins of these leukotrienes are unknown, partly because of the technical difficulties in detecting LTD₄, and partly because LTC₄ synthase, which converts LTA₄ into LTC₄, has not been identified nor purified (26). As for 5-LO, which oxidizes arachidonic acid to produce LTA₄, initial attempts to detect this key enzyme in the rat kidney resulted in an apparent absence of mRNA for 5-LO by Northern hybridization analysis in rat renal mesangial, endothelial, and glomerular epithelial cells, as well as human renal microvascular endothelial cells in culture (35). Western blot analysis, using antibodies directed against rat basophilic leukemia 5-LO, also failed to reveal the presence of this enzyme in all four cell lines. These results are not surprising in light of the mode of production of leukotrienes. LTA₄, derived from cells that possess 5-LO activity, such as polymorphonuclear neutrophils and macrophages, can be utilized as substrate by other types of cells to be transformed into either leukotrienes or lipoxins (36).

Macrophages, which accumulate in the glomerulus of PHN rats (see Table II), are one possible source of LTs (37). The contribution of the macrophage to the evolution of the glomerular lesions has been demonstrated in immune (11) as well as nonimmune (38) models of progressive renal disease. Although PHN is widely recognized as a neutrophil-independent model of renal disease (1), several lines of evidence have been reported describing the accumulation of macrophages in the glomerulus of PHN. Increased numbers of macrophages have been identified in the glomerulus of membranous nephropathy (39). Hara et al. (40) demonstrated that monocyte infiltration was increased in the glomerulus of PHN rats and that monocyte depletion significantly delayed the onset of proteinuria. Because of their 5-LO activity (41), macrophages could supply LTA₄ to neighboring resident glomerular cells, which may in turn produce and secrete peptidoleukotrienes such as LTC₄, LTD₄, and LTE₄ through their LTC₄ synthase activity. In this study, we demonstrated that glomerular 5-LO activity, assessed by glomerular LTB₄ levels after administration of anti-Fx1A antibody, was significantly lower in x-irradiated rats, which developed depletion of glomerular macrophages, than in non-x-irradiated animals studied in parallel (Fig. 6). This implicates monocytes/macrophages as necessary cells for the biosynthesis of LTs in isolated glomeruli. The mechanism of accumulation of macrophages in the glomerulus of PHN rats remains unknown. One possible chemoattractant for macrophages is the anaphylatoxin C5a, which is cleaved from C5 in the process of assembly of the membrane attack complex C5b-9 or LTB₄ itself (12). The latter possibility seems unlikely because 5-LO inhibition by MK886 failed to inhibit macrophage accumulation in this study (Table II). Rather, PHN rats treated with MK886 demonstrated greater numbers of glomerular macrophages than PHN rats without MK886 treatment (Table II), suggesting that a 5-LO metabolite might act as an inhibitor of macrophage chemotaxis. In this regard, it is of interest that foam cells, tissue macrophages found in atherosclerotic lesions,

express 15-LO and its mRNA (42) and that lipoxin A₄, which is an inhibitor of leukocyte chemotaxis (41), might be produced through the interaction of 5-LO and 15-LO in this setting (41).

In summary, we demonstrated increased 5-LO enzyme activity in glomeruli of PHN rats and a role for endogenous LTD₄ as a mediator in the development of proteinuria and glomerular microcirculatory dysfunction in a rat model of membranous nephropathy in the autologous phase. The acute fall in urinary protein excretion rate with SK&F 104353 implicates an LTD₄-mediated hemodynamic component (elevated glomerular capillary pressure) for the proteinuria. The synthesis of LTD₄ likely occurs directly from infiltrating macrophages or from macrophage-derived LTA₄, through LTC₄ synthase activity within the glomerulus.

Acknowledgments

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